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Effects of oxidative stress induced by environmental relevant concentrations of fluoxetine on the embryonic development on *Danio rerio*

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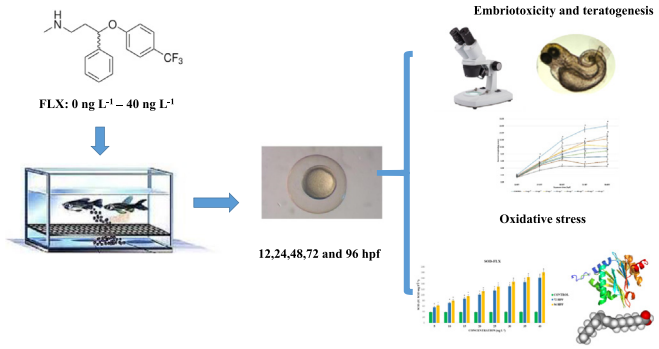
HIGHLIGHTS

- Developmental alterations and teratogenic effects by fluoxetine were evaluated.
- Fluoxetine proved to be teratogenic in embryos of *Danio rerio*.
- Fluoxetine promoted oxidative damage in zebrafish embryos.
- The main teratogenic alterations identified were pericardial edema, hatching retardation, spine alterations

GRAPHICAL ABSTRACT



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ABSTRACT

Fluoxetine (FLX) is a psychoactive drug that acts as an antidepressant. FLX is one of the world's best-selling prescription antidepressants. FLX is widely used for the treatment of various psychiatric disorders. For these reasons, this drug may eventually end up in the aquatic environment via municipal, industrial, and hospital discharges. Even though the occurrence of FLX in aquatic environments has been reported as ubiquitous, the toxic effects that this drug may induce, especially at environmentally relevant concentrations, on essential biological processes of aquatic organisms require more attention. In the light of this information, this work aimed to investigate the influence that fluoxetine oxidative stress-induced got over the embryonic development of *Danio rerio*. For this purpose, *D. rerio* embryos (4 h post fertilization) were exposed to environmentally relevant concentrations (5, 10, 15, 20, 25, 30, 35, and 40 ng L^{-1}) of fluoxetine, until 96 h post fecundation. Along the exposure, survival, alterations to embryonic development, and teratogenic effects were evaluated using a stereomicroscope. Furthermore, oxidative stress biomarkers (superoxide dismutase, catalase, glutathione peroxidase, lipid peroxidation, hydroperoxide, and carbonyl content) were evaluated at 72 and 96 h post fecundation. LC_{50}, EC_{50m}, and teratogenic index were 30 ng L^{-1}, 16 ng L^{-1}, and 1.9, respectively. The main teratogenic effects induced by fluoxetine were pericardial edema, hatching retardation, spine alterations and craniofacial malformations. Concerning oxidative stress, our integrated biomarkers (IBR) analysis demonstrated that as the concentration increased, oxidative damage biomarkers got more influence over the embryos than antioxidant enzymes. Thus,

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fluoxetine induces an important oxidative stress response on the embryos of *D. rerio*. Collectively, our results allow us to concluded that FLX is a dangerous drug in the early life stages of *D. rerio* due to its high teratogenic potential and that FLX-oxidative stress induced may be involved in this toxic process.

1. Introduction

Fluoxetine (FLX) is a selective serotonin reuptake inhibitor. It is the active pharmaceutical ingredient (API) of some psychotropic drugs used for the treatment of depression, obsessive-compulsive disorder/behaviors, anxiety, and bulimia nervosa (de Farias et al., 2019; Marvanova and Gramith, 2018). Its mechanism of action is related to the inhibition of the serotonin receptor carrier in the presynaptic terminal which leads to sustained concentrations of 5-hydroxytryptamine (5-HT) in certain areas of the brain. The ability of FLX to bind to 5-HT receptors (dopaminergic, adrenergic, muscarinic, and cholinergic) makes it more likely to cause adverse effects compared to other tricyclic antidepressant drugs (Chai et al., 2021; Saaristo et al., 2017). The mechanism of action of FLX in fish is still uncertain; however, Park et al. (2012), postulate that the effects of this antidepressant are related to the alteration of endogenous serotonin levels which intervenes directly in the reproductive, metabolic and physiological processes.

Regarding the global antidepressant drugs market, in 2019, its consumption was estimated at 14.3 billion dollars, and in 2020 approximately 28.6 billion dollars. This increase is mainly due to the effects of the COVID-19 pandemic. Furthermore, global consumption of this antidepressant in the U.S. was 379 million dollars in 2016; however, in 2020 it exceeded 400 million U.S. dollars (Research and Market, 2021).

FLX has a log Kow of 4.65 at pH 7 (Silva et al., 2012); thus, it can be inferred that the distribution of this drug in the aqueous phase is high, which is demonstrated by its ubiquitous presence in worldwide water bodies. For example, this drug has been previously reported in wastewater treatment plants (WWTPs) effluents of Canada, Germany, Italy, Mexico, UK, and the US at concentrations of 2 ng L^{-1} to 240 ng L^{-1} and in surface waters of Canada, China, Portugal, UK, and the US at concentrations of 0.28 ng L^{-1} to 141 ng L^{-1} (Metcalfe et al., 2003; Schultz et al., 2011; Verlicchi et al., 2012; Lajeunesse et al., 2012; Writer et al., 2013; Schlüsener et al., 2015; Evans et al., 2015; Petrie et al., 2015; Xiang et al., 2018; Fernandes et al., 2020).

In regard to the degradation data, Kwon and Armbrust (2006) pointed out that FLX is a highly recalcitrant and resistant compound, not susceptible to degradation by hydrolysis, photolysis, or microbial action. In addition, FLX is a secondary amine with a pKa value of 10.1, which favors that its toxicity and bioaccumulation in the aquatic life are high (De Assis et al., 2013; Schultz et al., 2011; Weinberger and Klaper, 2014). Several studies have pointed out that FLX induces alterations to different metabolic and physiological processes (reproduction, development, behavior), generates oxidative stress, induces neurotoxicity, and affects the immune system in non-target organisms (Park et al., 2012; Weinberger and Klaper, 2014; Franzellitti et al., 2014; Chen et al., 2018; Costa et al., 2021). Duarte et al. (2020) for instance, showed that FLX at concentrations of 0.3 and 3 μg L^{-1}, induces oxidative stress and DNA damage to the liver of *Argyrosomus regius*. In addition, acute exposure to FLX (2–20 μg L^{-1}), produced an anorexigenic effect in *Cichlasoma dimerus* fish, affecting their growth, development, and reproduction (Dorelle et al., 2020). Exposure to FLX has also been linked to alterations in behavior, as shown by de Abreu et al. (2020), who identified that at 5 μg g^{-1} of this antidepressant, generated a decrease in the predisposition to interact and increases in cortisol levels of *Labroides dimidiatus* fish. Finally, regarding teratogenicity studies, Nowakowska et al. (2020), demonstrated that 10 μg L^{-1} of FLX induced several malformations in the trunk and tail regions of *D. rerio*. Even though the above-mentioned study has shown that FLX is embryotoxic toxic in fish, the effects described by the authors were observed at concentrations of μg L^{-1}.

In this study, we selected the early stages of the zebrafish, since these stages are widely used in toxicity studies to evaluate the sublethal effects of contaminants. In addition, *D. rerio* has demonstrated great advantages as a bioindicator organism: for example, it has a perfectly sequenced genome, has an abundant spawning, a rapid embryonic, and a high sensitivity to environmental contaminants (Scholz et al., 2008; Tenorio-Chávez et al., 2020).

Since most of the toxic effects of FLX have been evaluated using high concentrations of this antidepressant (μg L^{-1} - mg L^{-1}). This work aimed to determine whether environmentally relevant concentrations of FLX may affect the embryonic development of *D. rerio*, as well as their redox system.

2. Material and methods

2.1. Fluoxetine determination

For sampling, we used the method reported by Elizalde-Velázquez et al. (2021). The method used to evaluate the FLX determination was as follows. Briefly, for ZFET, 140 μL of water from each of 60 wells were collected and pooled. This process was done for each of the FLX concentrations tested. Thus, 8.4 mL of water were gathered for each test solution of FLX. In regard to the oxidative stress experiment, 10 mL of water from each of the systems were collected. After their gathering, water samples were stored at −20 °C, until their quantification. Water samples, for both experiments, were gathered at 0, 12, 24, 48, 72 and 96 hpf. Hence, 6 water samples for each treatment group were quantified, per experiment. FLX determination was performed using an Agilent 1260 HPLC system coupled to an API 5500 Qtrap MS equipped with a Turbo V Ion spray source. Source parameters were: nebulizer gas 413 kPa; ion spay voltage 5 kV; ion source temperature 500 °C; and collision gas: medium. Nitrogen was used as collision activated dissociation gas and was set at 5 psi. Instrument control, data acquisition and data processing were performed with Analyst 1.6 software. Separation was performed using a Thermo Hypersil Gold C18 (50 mm × 4.6 mm, particle size 5 μm). The mobile phase consisted of a mixture of water with 2 mM ammonium acetate as eluent A and MeOH 100% as eluent B. The flow rate was kept at 1.0 mL min^{-1} and the injection volume was of 20 μL. The standard stock solution of FLX was prepared by dissolving its accurately weighted compound in water to give a final concentration of 1 mg L^{-1}. The calibration standards and quality control samples were prepared by spiking ultrapure water with FLX at a concentration ranging from 0 to 40 ng L^{-1}. Accuracy and precision of analytical method were <12% of the nominal concentration. Accuracy of the proposed method was confirmed by control spiking method, which was carried out by spiking ultrapure water with FLX at three different levels 80%, 100% and 120%. Triplicate determination of these 3 levels have been recorded to obtain the % RSD.

2.2. Test drug

Fluoxetine [(±)-N-Methyl-γ-[4-(trifluoromethyl) phenoxy] benzenepropanamine hydrochloride, CAS number: 56296-78-7] with empirical formula $C_{17}H_{18}F_3NO{\cdot}HCl$ was purchased from Sigma-Aldrich (Mexico), molecular weight 345.79. All other compounds used were purchased with the same supplier.

2.3. Animals

Adult zebrafish of either sex were obtained from a commercial supplier (MX Aquanimals, Toluca, Mexico). For their housing, zebrafish were separated by gender and placed in 40 L tanks. The organisms were kept in water at 28 ± 1 °C, pH 7–8, with 12-12 h light-dark cycles. The organisms were fed with dry flakes (TetraMin PRO) three times a day. Once a day they were also fed with *Artemia salina* larvae to promote oviposition (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013). The water in the tanks was maintained with continuous aeration and described conditions (Table 1S). The organisms were kept in these conditions during all experiment.

2.4. Egg production

The embryos used were obtained by natural mating. 6 males and 3 females of size 4–6 cm were selected and placed in a breeding tank equipped with a spawning tray. The breeding tank contained 15 L of water previously aerated (conditions similar to those referred to in Section 2.3), dechlorinated, and reconstituted with 1 mL per liter of water, of a stock solution 9 mg L^{-1} of commercial Instant Ocean® salts. Next day, eggs were collected and placed in Petri dishes filled with egg water (60μg ocean salt mL^{-1}). After spawning, eggs were washed with saline solution and observed using a stereomicroscope (Zeiss Stemi 305 at 10× of magnification). The fertilized eggs selected for all experiments were in the middle blastula stage (Beekhuijzen et al., 2015).

2.5. Zebrafish embryo acute toxicity (ZFET) test for fluoxetine

Exposure of zebrafish embryos to environmentally relevant concentrations of FLX was carried out in 24-well plates according to the method described in Test No. 236: Fish Embryo Acute Toxicity (FET) Test (2013). At 4-hour post fertilization (4 hpf), selected healthy embryos were washed and examined under the stereomicroscope (Zeiss Stemi 305 at 10× of magnification) and fertilized embryos were selected for the test. The concentrations of FLX used (5, 10, 15, 20, 25, 30, 35, and 40 ng L^{-1}) were environmentally relevant (Table 1). 60 fertilized eggs (4 hpf, n = 60) for each FLX concentration were used and experiment was performed in 3 independent replicates. The control (untreated group) was exposed to 2.5 mL of water without FLX. The development endpoints that were evaluated on embryos upon four-day exposure were mortality and survival of embryos. A maximum likelihood linear regression analysis was performed to calculate lethal concentration 50 (LC_{50}) and effective concentration of malformation (EC_{50m}) with their 95% confidence intervals ($p < 0.05$). Spearman-Karber method trimmed was used (US-EPA software ver. 1.5). The teratogenic index (TI) was also calculated using the ratio LC_{50}/EC_{50m}. If the TI was greater than 1, FLX was considered as teratogenic and if it was less than 1 as embryo lethal, according to criteria of Weigt et al. (2011). To guarantee the traceability of the results, two main validating criteria for the test were established; the first one was to ensure a fertilization percentage ≥90% and the second one, for the test to be considered valid, an analysis of the control group at the 96 hpf mark was conducted in order to review that it did not present a lethal teratogenic effect index greater than 10%.

2.6. Evaluation of alterations to embryonic development and teratogenic effects

The systems cited in Section 2.4 were used for this purpose. The evaluation of *D. rerio* oocytes and embryos exposed to FLX was performed at 12, 24, 48, 72, and 96 hpf. Morphological alterations in embryos were identified using a stereomicroscope (Zeiss Stemi 305). Alterations to embryonic development and teratogenicity endpoints were evaluated considering: 1) tail development, 2) formation of somites, 3) eye development, 4) movement, 5) blood circulation, 6) heartbeat, 7) head-body pigmentation, 8) pigmentation of the tail, 9) appearance of the pectoral fin, 10) mouth protuberance and 11) hatching. This process was performed using the methodology established by Hermsen et al. (2011) and Kimmel et al. (1995). Total of malformations were expressed as the percentage of embryos with at least one malformation or the individual malformation, respectively, in comparison to the control. A concentration-response curve was constructed with the main malformations induced by FLX exposure on embryos of *D. rerio* using IBM SPSS Statistics 22 software. The malformed images of embryos were captured with Canon Digital camera (EOS 80D).

2.7. Evaluation of oxidative stress in zebrafish embryos

For this experiment, nine test systems were used. Each system consisted of one gram of zebrafish embryos (corresponding to 1600 embryos) in 4 L. Each system was spiked with the environmentally relevant concentrations of FLX, as well as their corresponding controls. The systems were kept at constant temperature (27 ± 1 °C). At 72 hpf and

Table 1
Measured concentrations of FLX during ZFET and oxidative stress experiment.

Nominal concentrations of FLX	Measured FLX concentrations at different exposure times (ng L^{-1})					
	0 hpf	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
Control	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
Zebrafish embryo toxicity test						
5 ng L^{-1}	5.03 ± 0.42	5.87 ± 0.39	5.03 ± 0.43	5.02 ± 0.43	5.36 ± 0.47	5.25 ± 0.49
10 ng L^{-1}	10.09 ± 0.62	10.03 ± 0.60	10.21 ± 0.57	10.15 ± 0.61	10.01 ± 0.67	10.07 ± 0.65
15 ng L^{-1}	15.01 ± 0.82	15.19 ± 0.74	15.43 ± 0.78	15.31 ± 0.83	15.27 ± 0.79	15.18 ± 0.85
20 ng L^{-1}	20.06 ± 0.92	20.45 ± 1.03	20.32 ± 0.99	20.74 ± 0.94	20.45 ± 1.01	20.05 ± 0.94
25 ng L^{-1}	25.04 ± 0.94	25.28 ± 0.98	25.04 ± 1.10	25.33 ± 0.99	25.83 ± 0.97	25.45 ± 1.08
30 ng L^{-1}	30.88 ± 1.52	30.90 ± 1.31	30.03 ± 1.48	30.31 ± 1.53	30.21 ± 1.87	30.88 ± 1.83
35 ng L^{-1}	35.53 ± 2.14	35.21 ± 2.49	35.73 ± 2.21	35.73 ± 2.54	35.54 ± 2.38	35.15 ± 2.27
40 ng L^{-1}	40.24 ± 3.47	40.19 ± 3.12	40.11 ± 3.56	40.21 ± 3.51	40.32 ± 3.23	40.85 ± 3.81
Oxidative stress test						
5 ng L^{-1}	4.98 ± 0.12	5.67 ± 0.15	5.03 ± 0.12	4.97 ± 0.11	4.59 ± 0.15	5.03 ± 0.16
10 ng L^{-1}	10.01 ± 0.17	10.28 ± 0.20	10.13 ± 0.19	10.34 ± 0.16	10.35 ± 0.18	10.05 ± 0.20
15 ng L^{-1}	15.95 ± 0.24	15.05 ± 0.22	15.15 ± 0.26	15.33 ± 0.29	15.40 ± 0.29	15.24 ± 0.31
20 ng L^{-1}	19.89 ± 0.38	20.09 ± 0.40	20.22 ± 0.47	20.42 ± 0.43	20.54 ± 0.39	20.43 ± 0.42
25 ng L^{-1}	24.94 ± 0.40	25.11 ± 0.43	24.23 ± 0.46	24.91 ± 0.40	25.47 ± 0.45	25.67 ± 0.46
30 ng L^{-1}	30.52 ± 0.91	30.32 ± 0.98	30.89 ± 0.95	30.89 ± 0.95	30.43 ± 0.94	30.87 ± 0.93
35 ng L^{-1}	35.32 ± 1.02	35.78 ± 1.11	35.56 ± 1.08	35.63 ± 1.03	35.74 ± 1.05	35.34 ± 1.10
40 ng L^{-1}	35.19 ± 1.43	35.35 ± 1.39	35.67 ± 1.47	35.19 ± 1.41	35.94 ± 1.38	35.73 ± 1.49

Values represent mean (n = 18) ± standard deviation (SD) of each concentration. Limit of Quantification: 0.5 ng L^{-1}.

96 hpf, 800 embryos, respectively, were randomly selected and homogenized using 1 mL of phosphate buffer solution (pH 7.4) at 12,500 rpm. The exposure times were selected considering that between 72 and 96 hpf, the larvae had already hatched, and their antioxidant enzyme system was already functioning appropriately. For the evaluation of oxidative stress, the following biomarkers of cellular oxidation were determined: lipoperoxidation level (Buege and Aust, 1978), hydroperoxide content (Jiang et al., 1992), and protein carbonylation (Levine et al., 1994). In addition to the antioxidant enzymes superoxide dismutase (Misra and Fridovich, 1972), catalase (Radi et al., 1991) and glutathione peroxidase (Günzler and Flohé, 1985, as modified by Stephenson, 2000). All results were harmonized considering the protein contents determined by the method of Bradford (1976). The experiment was performed in triplicate in three independent experiments. Reproducibility of results was assessed calculating the precision of them. Precision was calculated using the following formula: C.V. $= \left(\frac{s}{m}\right) \times 100\%$. Where: CV: coefficient of variation, s: standard deviation, m: mean of the concentrations.

2.8. Integrated biomarker response index (IBRv2)

The integrated biomarker response index was employed to analyze the influence of FLX oxidative stress-induced on the embryos of *D. rerio* (Sanchez et al., 2013). For IBR determination, the biomarkers of each tested drug (Xi) were divided against the biomarkers of the control group (Xo). The relation of Xi to Xo was log-transformed (Yi) to reduce variance. Next, Yi values were harmonized by applying the formula Zi = (Yi-m)/s and, using the mean (m) and standard deviation (s) of Yi. Subsequently, the biomarker deviation index (A) was estimated by the difference between Zi and Z0. Finally, the values of A were plotted in star plots representing the integrated responses of biomarkers. In addition, the absolute value of A of each biomarker was summed to obtain IBR.

2.9. Ethical approval

This research protocol was reviewed and approved by the Ethics and Research Committee of the Autonomous University of the State of Mexico (UAEM) to ensure that experiments are conducted in accordance with institutional standards for animal care (approval ID: RP. UAEM.ERC.119.2020). The provisions of the Mexican official standard on the breeding, care and use of laboratory animals (NOM-062-ZOO-1999) were also considered.

2.10. Statistical design and traceability criteria

LC_{50} and EC_{50} of malformations were calculated using US-EPA software ver 1.5, by means of a probit analysis with maximum likelihood linear regression. The teratogenic index (TI) was calculated using the ratio of LC_{50} and EC_{50} of malformations. For the evaluation of embryonic development (Hermsen score-scale), we performed a two-way ANOVA followed by Student-Newman-Keuls post hoc test. Oxidative stress biomarkers data was examined using a two-way analysis of variance (ANOVA), considering time as factor A and concentration as factor B. Variations between the means were examined with the Student-Newman-Keuls method, using SigmaPlot 12.3 software. All oxidative stress biomarkers passed the normality test.

3. Results

3.1. Fluoxetine determination

Concentrations of FLX in both experiments decreased in a time-dependent manner (Table 1). In addition, concentrations of this drug in the control group remained <LoQ. As concentrations of FLX were maintained above 80% for all samples, analyses of the results were based on nominal values.

3.2. Zebrafish embryo acute toxicity (FET) evaluation

According to our results, the number of dead and malformed embryos increased in a concentration- dependent manner. LC50 and EC50m values were calculated, reaching a value of 30 ng L^{-1} and 16 ng L^{-1}, respectively. Furthermore, FLX got a teratogenic index of 1.9, and consequently, this drug should be classified as teratogenic (Weigt et al., 2011). For the determination of LC_{50} and EC_{50}, a linear regression analysis was performed. Our correlation coefficient, standard error and degrees of freedom values were: 0.996, 1.71, and F (1,7) = 827.254, $p < 0.05$ respectively.

The percentage of dead embryos increases in a concentration-dependent manner, reaching its maximum value (70%) at the concentration of 40 ng L^{-1}. In agreement with the aforementioned, the percentage of normal embryos decreased in a concentration-dependent manner, reaching its minimum value (11%) at the concentration of 40 ng L^{-1}. The percentage of malformed embryos remained relatively constant from 20 ng L^{-1} to 40 ng $L^{-1}$of FLX. Nonetheless, it is noteworthy to say that as the concentration of this drug increased the sternness of malformations also increased.

3.3. Evaluation of embryonic development due to exposure to FLX

Concentration-response curves for each FLX concentration are shown in Fig. 1. Taking into account the criteria of Hermsen et al. (2011) and Kimmel et al. (1995), no alterations to embryonic development were observed in the control group. Unlike the control group, FLX significantly decreased the score of the embryos in all treatment groups ($p < 0.05$). This decline in the score of the embryos is associated with a greater prevalence of malformations in the embryos. The main malformations observed at the lower scored embryos were pericardial edema, skeletal alterations, and craniofacial malformations.

3.4. Alterations to embryonic development and teratogenic effects induced by FLX exposure

Fig. 2 shows the teratogenic effects presented at all FLX concentrations tested and at different exposure times. As can be seen at concentrations of 30 to 40 ng L^{-1}, the most severe and life-threatening malformations included body malformation, hatching retardation, pericardial edema, and yolk sac malformation. Although this information was not reported in this work, many of the embryos died at 100 hpf.

Fig. 3 shows the most frequent and severe malformations presented in embryos exposed to environmentally relevant concentrations of fluoxetine. As can be seen, many of these malformations put the life of the embryos at risk, leading to their death. All malformations observed in the embryos were concentration-dependent and are represented as a frequency histogram, where the incidence of malformations per concentration is reported. The control value is not represented in the graph since the incidence of malformations was zero. The images show that some of the embryos exposed to the highest concentrations (30–40 ng L^{-1}) of FLX presented bifurcation of the head.

3.5. Antioxidant activity induced by FLX

The antioxidant activity of SOD, CAT, and GPX, in *D. rerio* embryos exposed to FLX, is depicted in Fig. 4. As can be seen in this figure, the activity of all enzymes, in all exposure groups, showed a significant increase compared with the control group. Furthermore, as the concentration increased, the activity of the enzymes also increased, reaching the maximum peak at the concentration of 40 ng L^{-1}. Finally, at concentrations of 10,15, 20, 25, 30, 35, and 40 ng L^{-1}, significant differences in the enzymatic activity of CAT and GPX were observed between 72 hpf.

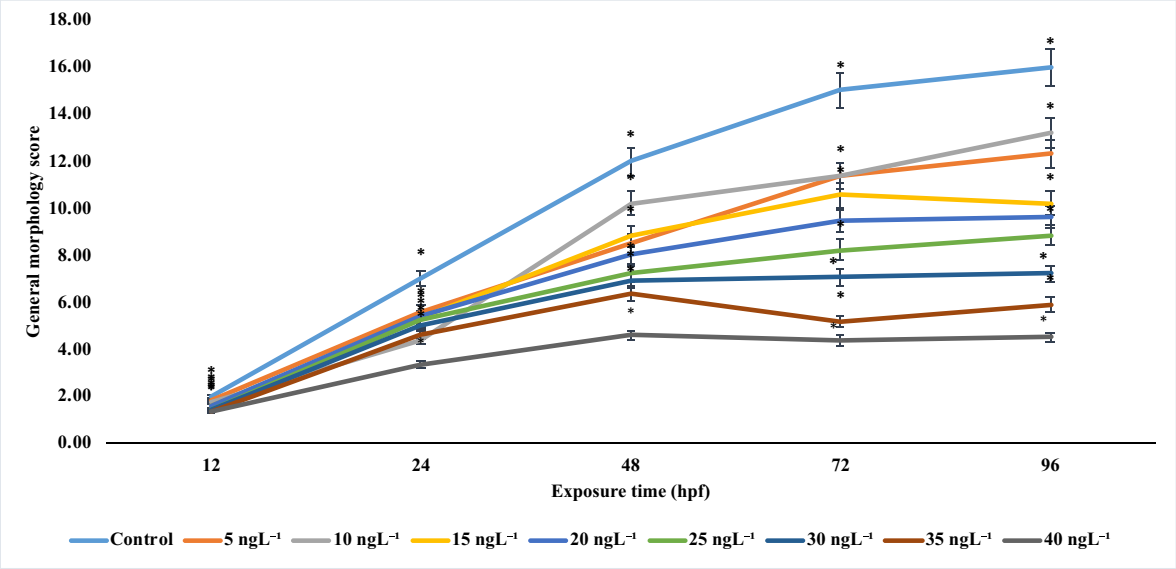


Fig. 1. Concentration-response curves of FLX in *D. rerio* embryos.
All FLX concentrations were significantly different from the control group. (ANOVA two way, test post-hoc Student Newman Keuls, $F(8,32) = 5193.482$; $p < 0.001$).

3.6. Oxidative damage induced by FLX

The levels of LPX, POX, and HPX in *D. rerio* embryos exposed to FLX are shown in Fig. 5. Levels of all oxidative damage biomarkers, in all treatment groups, exhibited a significant increase in relation to the control group. The levels of all oxidative damage biomarkers increased as the concentration did, reaching its maximum value at a dose of 40 ng L^{-1} of FLX. Concerning LPX, a significant difference was observed for all concentrations between times 72 and 96 hpf. Furthermore, for HPX, a significant difference was observed for concentrations 20, 25, 30, 35, and 40 ng L^{-1} at 96 hpf to 72 hpf. Since C.V. reached a value of <10%, we can say results show good reproducibility. This value was consistent in both experiments.

3.7. Integral biomarker response index (IBRv2)

IBR values increased as FLX concentrations increased. Thus, as concentrations of this drug increased more severe effects were observed on the embryos (Fig. 6: A) 72 hpf, B) 96 hpf). According to our IBRv2 analyzes, oxidative damage biomarkers got more influence on the embryos than antioxidant enzymes. The oxidative damage biomarker that more impacted the embryos was HPX. Regarding exposure time, the concentration of 15 ng L^{-1} and 35 ng L^{-1} showed a significant increase in the mean IBR values.

4. Discussion

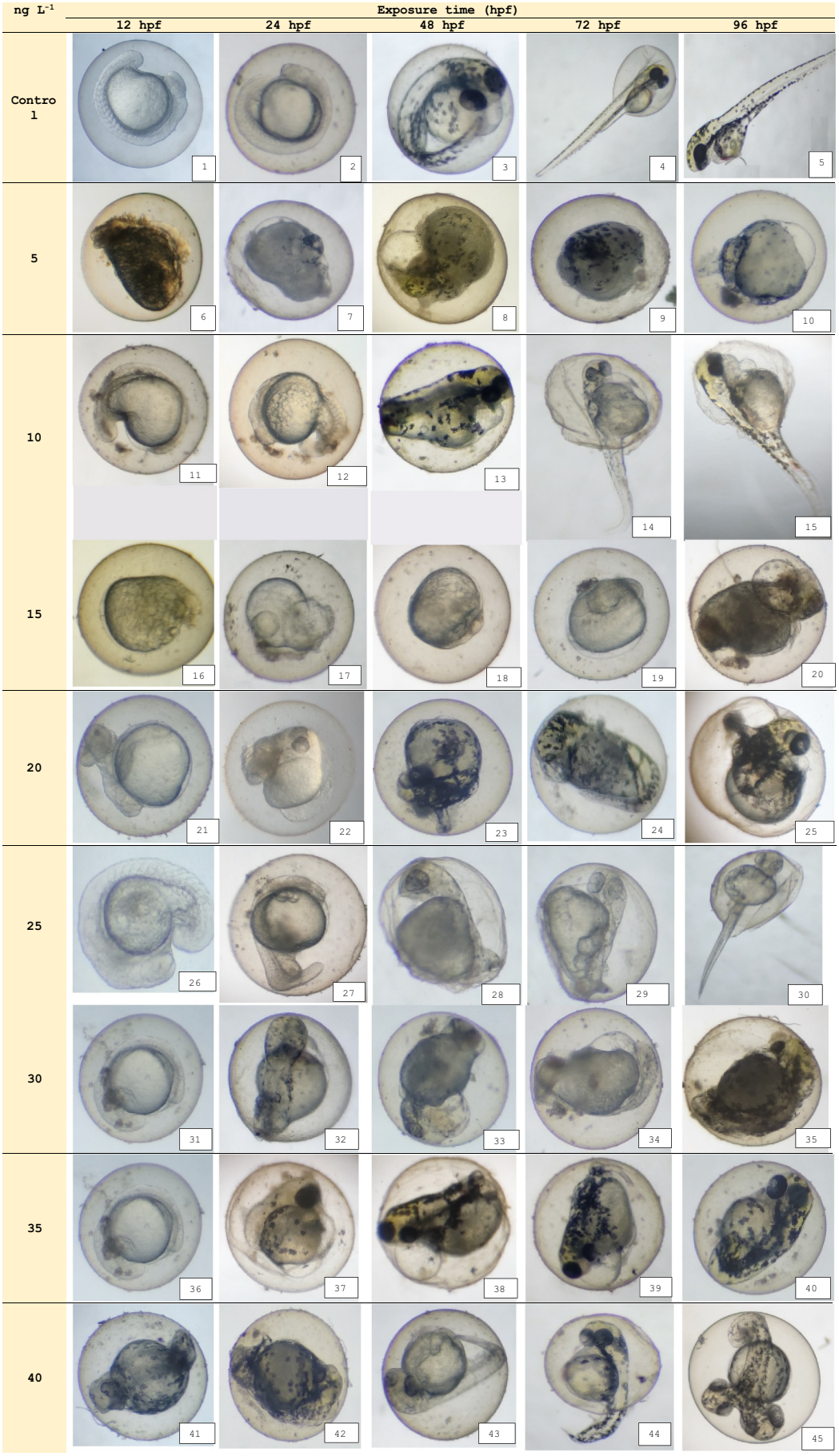
The findings identified in this work allow us to point out that FLX at environmentally relevant concentrations can alter embryonic development and generate teratogenic effects in the early life stages of zebrafish. Since FLX is a very highly recalcitrant and lipophilic compound, this drug can be widely distributed in aquatic environments, and consequently, bioaccumulate in organisms such as *Cyprinus carpio*, *Dorosoma cepedianum*, *Ameiurus nebulosus*, *Morone americana*, *Catostomus commersoni*, *Pimephales promelas*, *Carcharhinus leucas*, producing deleterious effects in them (Chu and Metcalfe, 2007; Dorelle et al., 2020; Duarte et al., 2020; Nowakowska et al., 2020; Ramirez et al., 2007; Schultz et al., 2010).

Our results in the ZFET test showed that the LC50 of FLX in zebrafish embryos was 30 ng L^{-1} with a 95% confidence interval of 26.3–35.5. The LC50 for FLX in embryos of other aquatic species were 705 μg L^{-1} at 48 hpf, for *Pimephales promelas* (Brooks et al., 2003), 7.5 mg L^{-1} at 96 hpf, for *Xenopus laevis* (Richards and Cole, 2006), 546 μg L^{-1} at 7 days, for *Gambusia affinis* (mosquito fish) (Henry and Black, 2008), and 5.5, 1.3, 0.2 mg L^{-1} at 96 h at pH 7, 8 and 9, respectively, for *Oryzias latipes* (Nakamura et al., 2008). Based on these data, we can conclude that *D. rerio* is more sensitive to the effects of FLX than the other organisms mentioned.

The effects of FLX on embryos are related to the ability of this drug to bind to vitellogenin, which is a precursor protein of egg yolk formation (Soares et al., 2009). Since FLX has the physicochemical properties to cross the zebrafish chorionic membrane, it can exert its effects on the oocytes. Pan et al. (2018), exposed *D. rerio* embryos to several concentrations (0.1, 1, 10, 100, and 1000 μg L^{-1}) of FLX. At 72 h, they determined the antidepressant uptake in the embryo's yolk obtaining values of 0.022, 0.044, 0.389, 1.88, and 29.7 μg L^{-1}, respectively, for each concentration tested. These findings show that FLX can cross the chorion and exert its toxic effects in zebrafish embryos.

When evaluating the alterations to embryonic development of zebrafish by exposure to FLX, we observed that yolk sac malformations and pericardial edema were presented in a high proportion and a concentration-dependent manner. Hollert et al. (2003), refer that this affectation can be explained by damage to the cell membrane which leads to an osmotic alteration with the consequent hyperaccumulation of liquids. Pericardial edema is a malformation that compromises the integrity of the embryos, because of the accumulation of fluid in the interstitium of the pericardium, which increases the size of the heart and the cardiac contraction (Zodrow et al., 2004). Both pericardial edema and yolk sac malformations had been evidenced in other studies conducted on zebrafish exposed to FLX (3, 5, 6, 12, 24, and 48 mg L^{-1}) (Ducharme et al., 2013; Zindler et al., 2019). In this study, we observed these malformations at much lower concentrations (5–40 ng L^{-1}), and we documented a new alteration (bubble-like formation in the yolk sac), which had not been evident in other studies. The bubble-like formation in the yolk sac is related to the hyperaccumulation of water in the embryo (Pereira et al., 2019).

Scoliosis was another important skeletal malformation observed in the embryos exposed to FLX. This is characterized by lateral curvature of the spine and usually occurs when there are defects in the spinal formation during the embryogenesis stage. Teleost like *D. rerio* have a natural susceptibility to develop spinal deformity, this situation is associated with genetic and environmental factors (Grimes et al.,


ng L-1
Exposure time (hpf)
12 hpf
24 hpf
48 hpf
72 hpf
96 hpf
Control
5
10
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1
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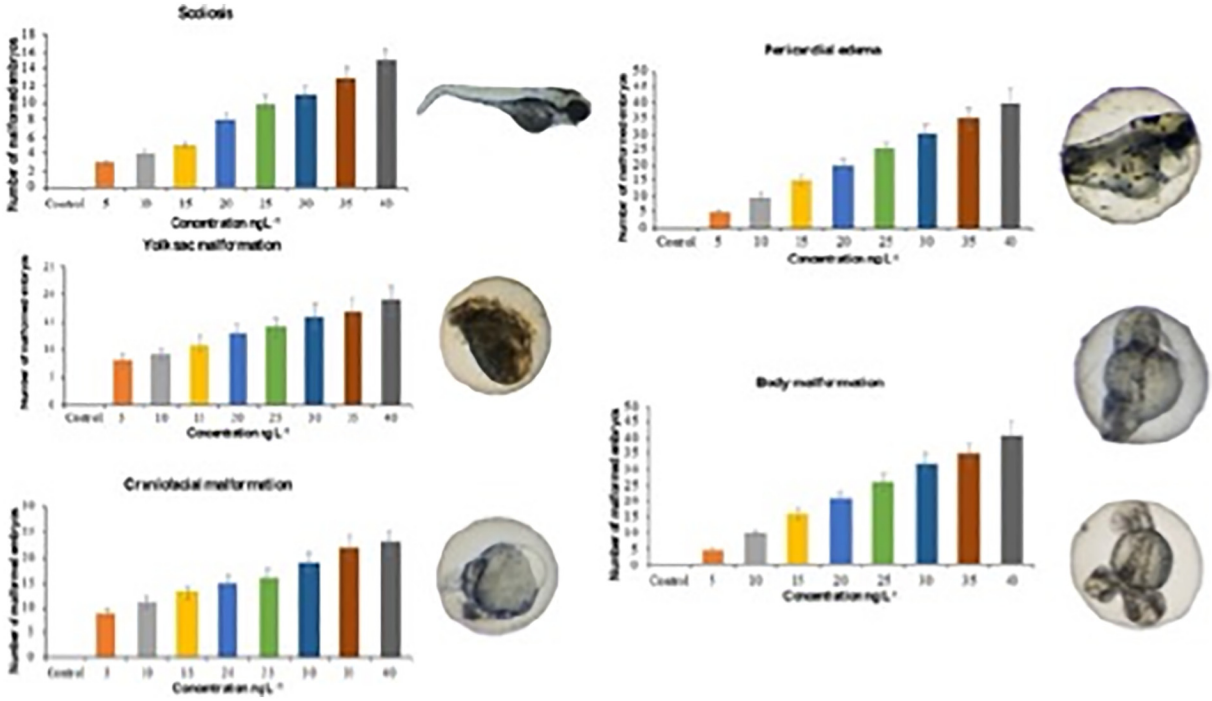

Fig. 3. More severe and frequent malformations induced by exposure to FLX concentrations in *D. rerio* embryos.

2016). In the control group of this study, we did not observe skeletal alterations in the embryos; however, in embryos exposed to FLX, scoliosis occurred. The occurrence of scoliosis on embryos may be due to the fact that FLX (5–40 ng L^{-1}) generates a deterioration of neurogenesis and abnormalities of the locomotor system, as well as deformities in the spine without them returning to their normal position (Stewart et al., 2013; Zindler et al., 2019).

Another alteration induced by the exposure of the embryos to FLX was hatching retardation. This malformation also affected the development and survival of the embryos. For example, the embryos that hatch early are immature and unable to perform their functions in the aquatic environment, on the other hand, those that hatch late are more susceptible to be damaged by contaminants found in the environment because their defense responses are not fully developed (Hallare et al., 2005). When the hatching process occurs in the zebrafish, movements of the embryo within the chorion are indispensable for embryonic development. In this process, the participation of the chorionic enzyme and osmotic rupture is fundamental for the larvae to hatch.

The hatching retardation induced by FLX in this study, could be explained by the absence of movements of the embryo that do not favor the exit of the larva. These findings are related to established by Tierney (2011), who reported that FLX inhibits the activity of acetylcholinesterase, generating progressive myopathy of skeletal muscles and consequently loss of mobility of the embryo.

Hypopigmentation was also one of the alterations with high prevalence on the embryos exposed to FLX. This finding could be explained by the fact that FLX produces the dysfunction of two serotonin receptor transcriptors (SERT-serotonin) and 5-HT1 (5-hydroxyindoacetic acid 1), present in the spinal cord. The dysfunction of the latter and its metabolite melatonin in the central nervous system, generates mental disorders and physiological diseases of the skin such as hypopigmentation (Arck et al., 2006; Slominski et al., 2003) It is well known that 5HT1 promotes melanogenesis in zebrafish. These findings are consistent with the study conducted by Zhou et al. (2018), who identified that at the concentration of 1 μg L^{-1}, hypopigmentation was observed in embryos *D. rerio*.

Margolis et al. (2016), refer that the FLX can alter the functions of SERT-serotonin and 5-HT1, generating an alteration in the organogenesis and morphogenesis of organisms, which is manifested in malformations of head, body, and tail in zebrafish embryos. These findings are consistent with those observed in our study since FLX exposures of 5 to 40 ng L-1 produce tail malformations and craniofacial malformation.

The presence of SSRIs such as FLX in the environment can lead to effects on the central nervous system and generate alterations in the signaling of growth factors. These alterations are a consequence of the reduced secretion of antagonists of two growth factors: 1) bone morphogenetic protein (BMP or Nodal) and 2) Wnt, present during embryonic development. Overexpression of Wnt or decreased expression of BMP inhibits head development. When there is high over-expression of BPM, there is interference in the formation of the fish embryo stem or body. The combined reduction in BPM and Wnt signaling induces additional heads (Foley et al., 2000; Niehrs, 2004). Over-expression or inhibition in the expression of these growth factor antagonists could explain the body and head bifurcation malformations identified in this study.

This study showed that fluoxetine at environmentally relevant concentrations was able to alter the early morphogenesis of zebrafish, leading to the appearance of malformations in the embryo such as: bubble-like formation in yolk sac, body malformation, craniofacial malformation, hatching retardation, head bifurcation, hypopigmentation, pericardial edema, scoliosis, tail malformation and yolk sac malformation.

Fig. 2. Effects of FLX exposure on morphological features in *D. rerio* embryos.
Embryos malformation presented: 1–5 normal development, 6 = BM, 7 = YSM, 8 = PE, 9 = DD, 10 = CM, 11 = TM, 12 = BLF, 13 = YSM, 14 = S, 15 = PE, HR and S, 16 = BM, 17 = BLF and PE, 18 = BM and BLF, 19 = YSM, BM, HR, 20 = PE, YSM, BM, 21 = CM and TM, 22 = CM, TM, PE and H, 23 = CM and YSM, 24 = CM, PE and BM, 25 = BM, PE, YSM, HR and TM, 26 = YSM and BLF, 27 = CM, BLF and TM, 28 = CM, YSM and TM, 29 = YSM, BLF and TM, 30 = BLF, HR and H, 31 = CM and BM, 32 = CM, PE and BM, 33 = CM, PE, TM and BM, 34 = BM, CM, PE, HR, H and YSM, 35 = BM, CM, PE, HR, H and YSM, 36 = CM and BM, 37 = CM,YSM, PE, TM and BM, 38 = CM,YSM, PE, TM and BM, 39 = CM,YSM, PE, TM, S and BM, 40 = CM,YSM, PE, TM, S and BM, 41 = CM, BM and YSM, 42 = CM, BM, S and YSM, 43 = YSM, BLF, S and H, 44 = YSM, S and BM, 45 = BH; TM and HR.
Meaning of the malformations: **BH** = Head bifurcation; **BLF** = Bubble-like formation in yolk sac; **BM** = Body malformation; **CM** = Craniofacial malformation; **HR** = Hatching retardation; **H** = Hypopigmentation; **PE** = Pericardial edema; **S** = Scoliosis; **TM** = Tail malformation, and **YSM** = Yolk sac malformation.

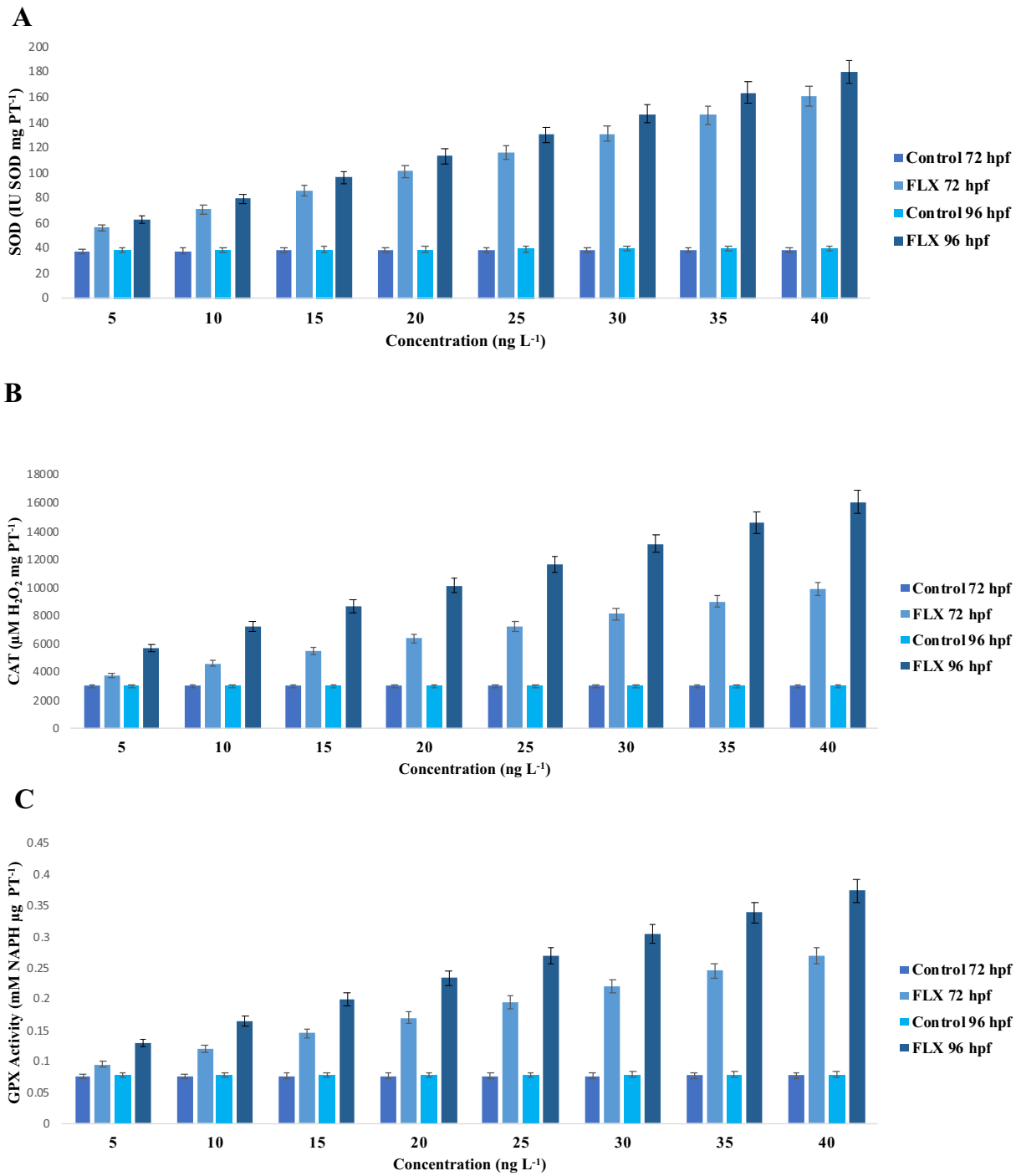

Fig. 4. A) Superoxide dismutase (SOD) ($F(8.244) = 643.922$; $n = 9$; $p < 0.001$), B) Catalase (CAT) ($F(8.244) = 3.145$; $n = 9$; $p < 0.042$) and C) Glutathione peroxidase (GPX) ($F(8.244) = 92.324$; $n = 9$; $p < 0.001$) in embryos of zebrafish *D. rerio* exposed to eight different concentrations of FLX at 72 and 96 hpf. Values are the mean of three replicates ± SE.

Some of these alterations will have repercussions during the alevin stage, or will lead to the death of *D. rerio*. As FLX consumption has increased significantly in the last year, its presence in water bodies leads to risks for the populations of aquatic organisms that are present in these environments.

One possible mechanism by which FLX may induce its embryotoxic in fish is oxidative stress. In the literature, it has been reported that reactive oxygen species act as messengers and as a signaling pathway to induce cell growth or cell death. Therefore, oxidative stress can disrupt embryo proliferation, development and growth. In our study, we demonstrated that exposure to FLX at environmentally relevant concentrations can alter the redox balance during early stages of zebrafish. These findings are congruent with (Duarte et al., 2020), who reported that FLX at concentrations of 0.3 to 3 µg L^{-1} induced an increase in the antioxidant response by inhibiting antioxidant enzymes (SOD, CAT and GST) causing lipid peroxidation and DNA damage in juvenile *Argyrosomus regius*. In the present study, we found that FLX at concentrations of 15 to 40 ng L^{-1} presented an increase in SOD, CAT and GPX activity, as well as an increase in LPX, POX and HPX. Carlos Fernández et al., 2013 and Pan et al., 2018 indicated that FLX toxicity has been related to increased ROS production, and an inhibitory effect on biotransformation enzymes of the cytochrome P450 family. However, Chen et al. (2018) reported that acute exposure (4 h) and chronic exposure (42d) of juvenile Chinese fish (*Pseudorasbora parva*) to 0, 50, and 200 µg L^{-1} of

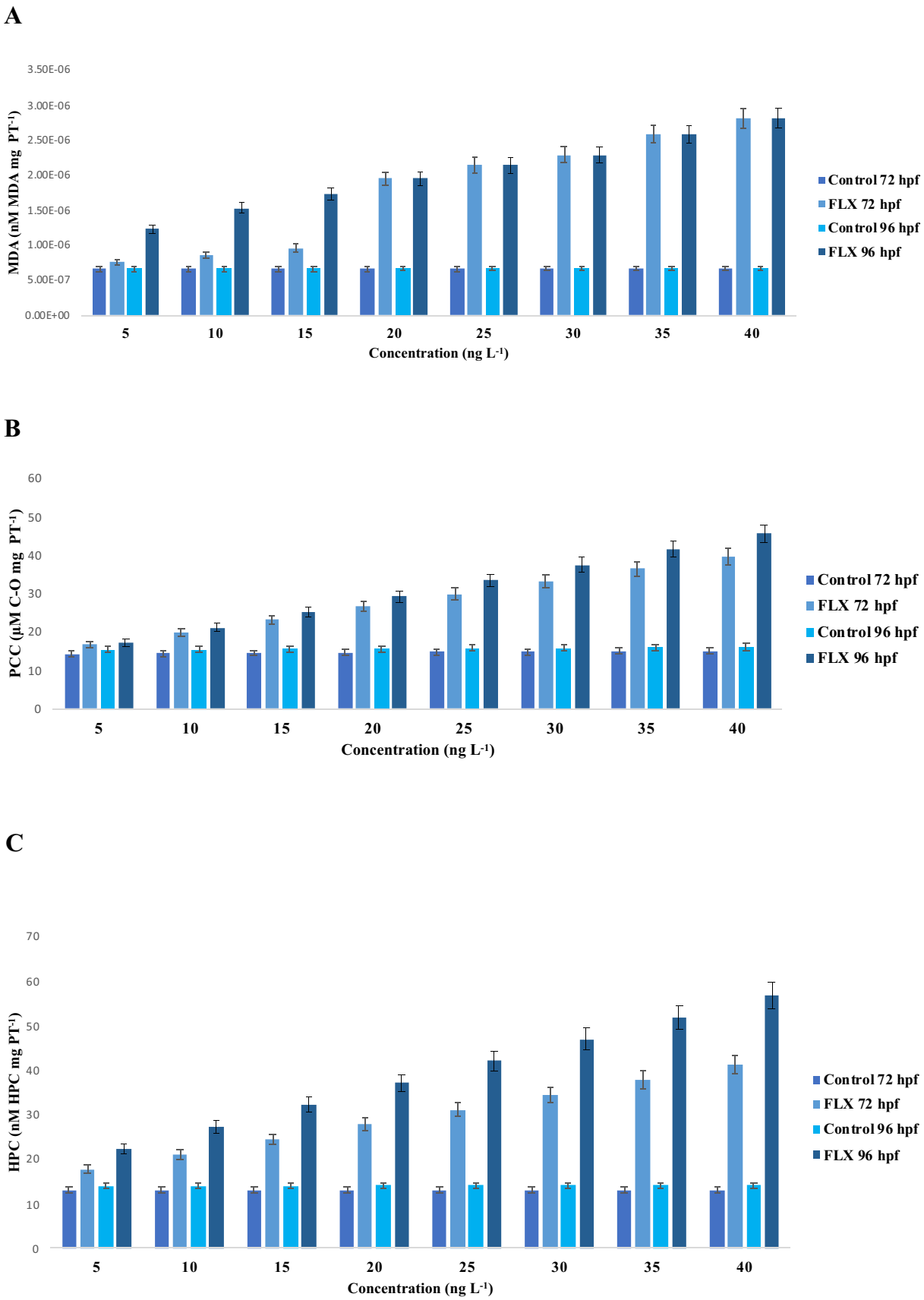

Fig. 5. A) Lipid peroxidation (LPX) ($F(8.244) = 134.325$; $n = 9$; $p < 0.001$), B) Protein carbonyl content (POX) ($F(8.244) = 23.278$; $n = 9$; $p < 0.001$) and C) Hydroperoxide content (HPX) ($F(8.244) = 73.132$; $n = 9$; $p < 0.001$) in embryos of zebrafish *D. rerio* exposed to eight different concentrations of FLX at 72 and 96 hpf. Values are the mean of three replicates ± SE.

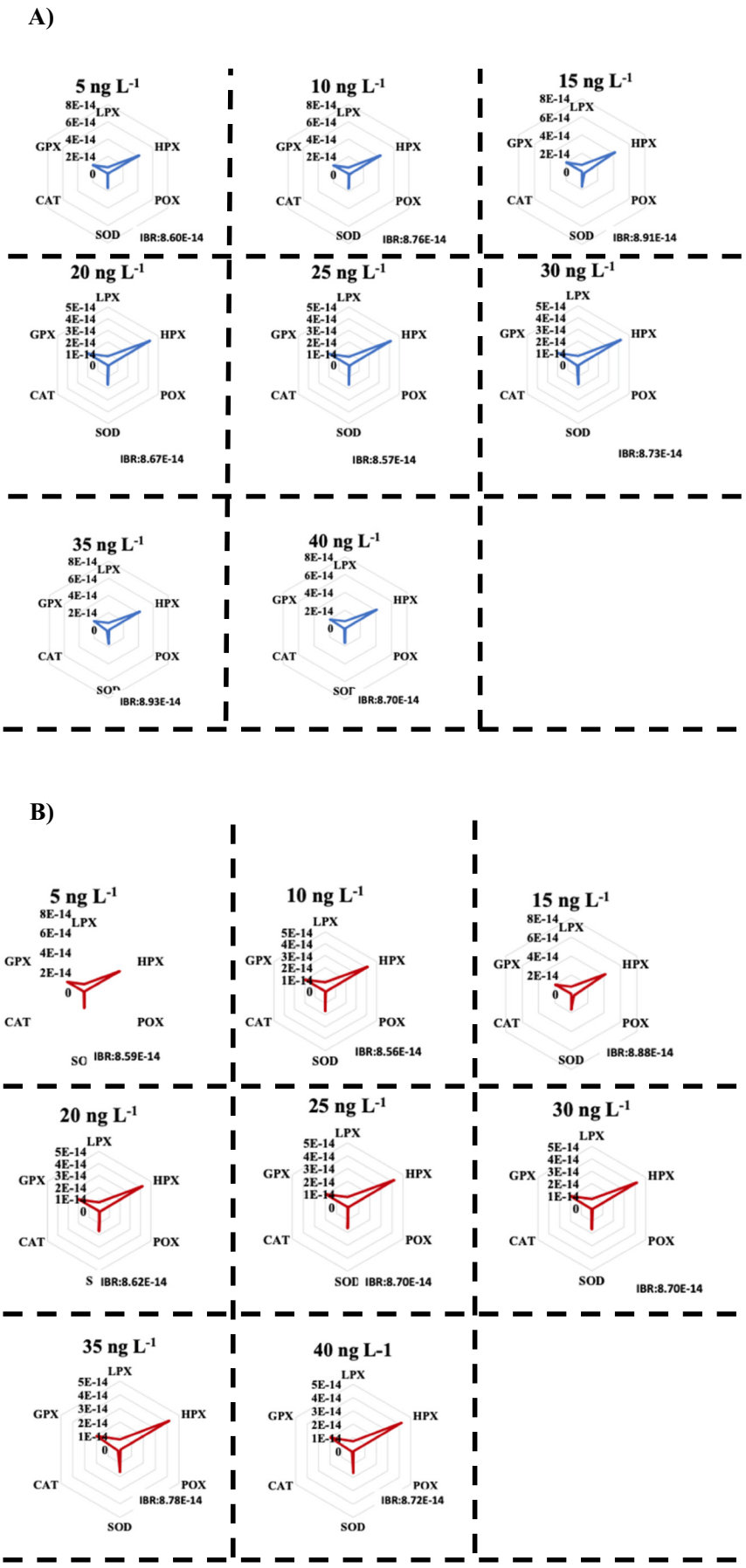

Fig. 6. The star plot for the calculated IBRv2 index of oxidative stress biomarkers in *D. rerio* embryos exposed to FLX at A) 72 hpf and B) 96 hpf.

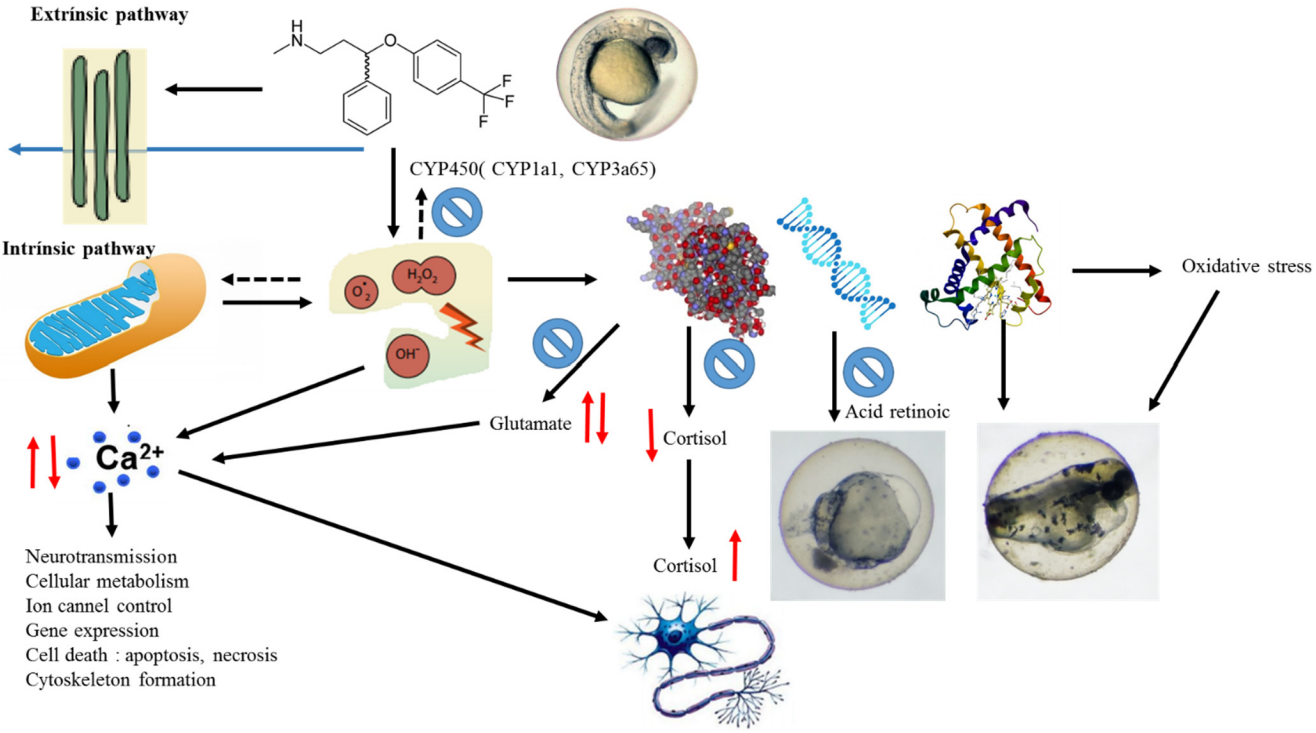

Fig. 7. Proposed mechanism by which FLX induces oxidative stress on *D. rerio* embryos.

FLX induced an increase in LPX levels. Finally, Aliko et al. (2021) indicated that exposure of common toad tadpoles *Bufo bufo* to 5 μg L^{-1} FLX, 10 μg L^{-1} IBU and their mixture were able to induce ROS species in embryos, and consequently, affect the development of tadpoles.

In summary and based on previous studies, we proposed a possible mechanism through which FLX can induce oxidative stress in zebrafish embryos (Fig. 7). FLX can induce oxidative stress through its demethylation, which is carried out by enzymes of the CYP450 family (CYP1a1, CYP3a65) present in zebrafish embryos. Once FLX was demethylated, ROS are generated, which leads to inhibition of bio-transforming CYP450 enzymes. Moreover, ROS generated can also produce oxidation of biomolecules such as nucleic acids, lipids, and proteins, leading to changes in the synthesis of retinoic acid which is indispensable for embryonic development, as in low quantities generates malformations in embryos. ROS generated by FLX biotransformation may also affect cortisol synthesis, altering neuronal functions that are expressed in behavior. Finally, ROS can also decrease the amount of calcium in the cell by the presence of H_2O_2, generating damage to the mitochondria and affecting the functions of neurotransmission, cell metabolism, control of ion channels (Na^+, K^+), gene expression, cell death, the functionality of the cytoskeleton, and the stimulation of glutamate receptors. Therefore, environmentally relevant concentrations of FLX (5 to 40 ng L^{-1}) present in water bodies can generate oxidative stress either by intrinsic or extrinsic pathway, which represents a risk for species that are in contact with this type of drug.

5. Conclusions

The lethal concentration 50 of FLX for embryos was 30 ng L^{-1}, while the effective concentration of malformation was 16 ng L^{-1}. The main teratogenic effects found were: pericardial edema, delayed hatching, spinal alterations such as scoliosis, and craniofacial malformations. These malformations described are the result of oxidation of biomolecules and alteration of the signaling pathways in which ROS participate. Thus, we can conclude that environmentally relevant concentrations of fluoxetine (5–40 ng L^{-1}) can induce oxidative stress in *D. rerio* embryos, which leads to abnormal development in organisms, and then to their death. Future studies should evaluate the toxic effects that environmentally relevant concentration of FLX can generate in the larval stage and in adulthood of zebrafish.

CRediT authorship contribution statement

JMOH and GAEV performed all the exposure experiments.
LMGO, JMOH and GAEV were involved in the conception.
LMGO, GHG, JDCV and HIF were involved in the design and interpretation of the data and the writing of the manuscript with input from ODG, NSR and MGM.

Declaration of competing interest

The authors report no declarations of interest.

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