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Fluoxetine-induced neurotoxicity at environmentally relevant concentrations in adult zebrafish *Danio rerio*

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ABSTRACT

Fluoxetine (FLX) exerts its therapeutic effect by inhibiting the presynaptic reuptake of the neurotransmitter serotonin. Nonetheless, at high concentrations of this drug, adverse effects occur in the brain of exposed organisms. Bearing this into account, the objective of this study was to evaluate the neurotoxic effects of the fluoxetine through the evaluation of behavior (Novel tank test), determination of oxidative stress, and determination of acetylcholinesterase (AChE) activity in adult zebrafish *Danio rerio*. For this purpose, *Danio rerio* adults were exposed to three environmentally relevant concentrations (5, 10, 16 ng L^{-1}) of FLX for 96 h. Our results demonstrate fish presented a significant disruption in their behavior, as they remained long-lasting time frozen at the top of the tank. Since we observed a significant reduction of AChE activity in the brain of fish, we believe the above described anxiety-like state is the result of this enzyme impairment. Moreover, as FLX-exposed fish showed a significant increase in the levels of oxidative damage biomarkers, we suggest this AChE disruption is associated with the oxidative stress response fish exhibited. Based on our findings, we believe the environmentally relevant concentration of FLX alters the redox status of the brain, impairing this way the behavior of fish and making them more vulnerable to predation.

1. Introduction

Pharmaceuticals are a very diverse group of chemical compounds that are widely used for the treatment of diseases. However, current pollutant removal methods are not effective in removing them completely from the environment (Aus der Beek et al., 2016). Consequently, many of them are often detected in surface water and hospital, municipal and industrial effluents. Psychiatric pharmaceuticals are among the most widely used and detected pollutants in aquatic ecosystems.

Fluoxetine is among the most common antidepressant used for the treatment of depression, anorexia, bulimia, and obsessive-compulsive disorder (Weinberger and Klaper, 2014; Castillo-Zacarías et al., 2021). Due to confinement by COVID-19, its consumption steadily increased, leading to a significant increase in its entrance into the aquatic environment. Consequently, FLX has been reported to be present in aquatic environments at different concentrations that are usually in the ng L^{-1} range. The highest FLX concentration in fresh surface water was 300 ng L^{-1}, detected in samples from Crabtree Creek, (North Carolina, United States) (Mceachran et al., 2018), while the lowest concentration was 0.03 ng L^{-1}, reported in the Guadalquivir river basin (southern Spain) (López-Serna et al., 2013).

Previous studies have reported some alterations due to the presence of antidepressants in aquatic species, such as algae (Brooks et al., 2003), annelids (Hird et al., 2016), insects (Sánchez-Argüello et al., 2009), crustaceans (De Castro-Català et al., 2017), amphibians (Conners et al., 2009), mollusks (Lazzara et al., 2012) and fish (Abreu et al., 2015; Nielsen et al., 2019; Zindler et al., 2019, 2020a, 2020b; Martin et al., 2020). Concerning FLX, (Duarte et al., 2020) reported that FLX (0.3 and 3 μg L^{-1}) induced growth retardation, inhibition of detoxification

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mechanisms (antioxidant enzymes), increased lipoperoxidation, DNA, and liver damage in *Argyrosomus regius* fish. Moreover, Dorelle et al., (2020) reported that exposure of *Cichlasoma dimerus* fish to 2–20 μg L^{-1} of FLX for 5 days induced an anorectic effect with a direct impact on fish growth and reproduction. In addition, Hossain et al., (2020) reported that FLX (0.05–100 μg L^{-1}) caused alterations in the escape mechanism in *Faxonius virilis*.

Despite the growing number of studies suggesting the risk posed by FLX to water bodies, the assessment of its neurotoxic effects at environmentally relevant concentrations has been poorly studied. Behavioral bioassays measure the behavior of an organism, qualitatively or quantitatively, to detect and analyze some external stimulus or as an indicator of an internal physiological or psychological state. Most assays are associated with mental disorders: anxiety, aggressive, compulsive (Maximino and van der Staay, 2019). There are several advantages of incorporating behavior in ecotoxicology studies: a) behavior is a multi-level indicator of biological effects (Peterson et al., 2017), b) it is one of the most sensitive indicators of exposure impact, with some estimates placing it at 10 and 1000 times more sensitive than measures of lethality (Gerhardt, 2007; Peterson et al., 2017), and c) behavior is considered an early warning tool (Hellou, 2011). The Novel Tank test is a test that allows modeling anxiety, oxidative stress, and the effect of anxiolytics. The evaluated parameters such as total distance (cm), distance traveled at the top and bottom (cm), latency (s), time at the top and bottom (s), frequency of bottom-to-bottom transitions (dive test), top-to-bottom, and duration of freezing episodes (s) are indicative of anxiety and stress effects due to exposure to this type of drugs (Haghani et al., 2019).

Zebrafish is a model organism for the study of neuroactive compounds using cellular, molecular and genetic approaches (Stewart et al., 2015; Cunha et al., 2016). Effects of FLX in other biomarker models exposed to high concentrations and at early stages during chronic exposure have been reported in the literature. Therefore, the present study aimed to evaluate the adverse effects induced by FLX on adult zebrafish exposed to environmentally relevant concentrations (5, 16, and 40 ng L^{-1}) by using behavioral biomarkers Novel Tank test at 96 h of exposure, biomarkers of oxidative stress: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), lipoperoxidation (LPX), protein carbonylation (POX) and hydroperoxide content (HPX) at 96 h of exposure) in adult zebrafish brain and finally brain acetylcholinesterase activity (AChE).

2. Method

2.1. Ethical statement

The Ethics and Research Committee of the Autonomous University of the State of Mexico (UAEMex) has reviewed and approved this protocol (approval ID: RP.UAEM.ERC.132.2020). For this purpose, the provisions of the Mexican official standard on the breeding, care, and use of laboratory animals (NOM-062-ZOO- 1999) were taken into account.

2.2. Test substances

The analytical standard of fluoxetine, N-methyl-3-phenyl-3-[4(trifluoromethyl)phenoxy]propan-1 amine, was purchased from Sigma-Aldrich (St. Louis, MO). The purity of the FLX standard was ≥ 98% (C17H18F3NO, molecular weight 309.33 g/mol (CAS number 54910–89–3).

The remaining reagents used to determine oxidative stress biomarkers and acetylcholinesterase activity were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Zebrafish housing systems

Wild-type zebrafish adults (AB strain; 5 fish/l) were acclimated for two months in aquaria supplied with aerated, dechlorinated, charcoal-filtered, and UV-sterilized tap water (27 ± 1 °C 14 h light: 10 h dark). Dissolved oxygen (9.8 ± 0.5 mg L^{-1}), pH (7.4 ± 0.17), conductivity (373 ± 30 μS cm^{-1}), nitrate content (2.9 ± 0.3 mg L^{-1}) and nitrite content (0.030 ± 0.009 mg L^{-1}) were monitored and controlled during zebrafish maintenance, as well as during subsequent experiments.

2.4. Exposure systems

For exposure, we allocated a total of 40 adult zebrafish (5 months old) (*D. rerio*) in 4 experimental tanks (10 individuals per tank). From these ten organisms per system, we make sure to place five male and five female fish, whose size and weight were 3.5 ± 0.2 cm and 475 ± 25 mg. Three concentrations of the antidepressant fluoxetine and a control fluoxetine-free (0, 5, 16 and 40 ng L^{-1}) were applied to each of the 4 experimental tanks. Water renewals were performed every third day, and FLX concentrations were adequately restored. Feeding was suspended 24 h before the Novel Tank Test to avoid the presence of organic particles in the medium. After 96 h of exposure, fish were transferred to individual behavioral tanks and acclimatized for 10 min in the new environment before each trial to avoid interference from handling stress. All tanks were covered during experimental trials, and observations were made in high definition to minimize any stress or bias caused by eye contact/human presence.

2.5. Novel Tank test and method of euthanasia

To ensure fish did not suffer from any disturbance during the behavioral test, we performed this in an isolated room, at the same time of day, under the same controlled temperature and light conditions and as it is stated below. Briefly, fish were individually selected at random, netted and placed in a rectangular tank (approximately 21.2 (cm) x 21.2 (cm) x 25.2 (cm) x 25.2 (cm)) with a capacity of 15 L, for 6 min. The behavior of the fish was recorded and subsequently analyzed with Tox Track Ink motion tracking software. The following anxiety behavior parameters were analyzed: total distance (cm) traveled, distance traveled at the top and bottom (cm), latency to enter the top (s), time spent at the top and bottom (s), frequency of top>bottom, bottom>top transitions and duration of freezing. A fish with an anxious behavior will be less likely to explore the top of the tank and will show increased freezing behavior. Fifteen minutes after the novel tank test, we individually placed each fish from the behavioral test in a 50 mL beaker to induce hypothermic shock (2–4 oC) according to the AVMA Guidelines on Euthanasia 2020 Edition. Death was determined by visually examining complete cessation of opercular (gill) movement and lack of response to tactile stimulation. The fish were then dissected, and the brains extracted (pull of 10 brains for each concentration of FLX) were placed in an Eppendorf tube with 1.5 mL of phosphate buffer solution (PBS, pH 7.4) and stored at − 80 °C until analysis of acetylcholinesterase activity and determination of oxidative stress biomarkers. The behavioral test above described was performed three times in different and independent experiments to calculate the mean of three autonomous results and depict it in boxes and whiskers charts. The brains of fish from three independent experiments were extracted, collected, and used as we describe below for each subsequent test. Thus, we used a total of 120 organisms for the whole study.

2.6. Oxidative stress biomarker determination

Samples were separated into two Eppendorf tubes and treated according to the methods established by Elizalde-Velázquez et al., 2021ab. Briefly, Tube 1 contained 300 μL from the homogenate and 300 μL of a solution of trichloroacetic acid (TCA, 20%). Tube 2 contained 700 μL from the homogenate. All tubes were maintained at − 20°C until these were used. Tube 1 was centrifugated at 11 495 rpm for 15 min at 4 °C and the precipitate was used to determine the protein carbonyl content

(PCC), while the supernatant was used examined to establish the degree of LPX and the hydroperoxide content (HPC). Tube 2 was centrifugated at 12 500 rpm for 15 min at 4 °C and the supernatant was used to determine the activity of antioxidant enzymes: CAT, superoxide dismutase (SOD), and glutathione peroxidase (GPx).

The degree of LPX was assessed with the method reported by Buege and Aust, (1978). Results are expressed as nM of malondialdehyde, obtained by the measured environmental concentration of 1.56×10^5/M/cm per mg of protein per g of tissue. The PCC was ascertained by the method described by Levine et al., 1994, with some modifications. Results are expressed as nM of reactive carbonyls, obtained by the measured environmental concentrations of 21, 000/M/cm per mg of protein per g of tissue. The HPC was quantified by the method of Jian et al., 1992 and results are expressed as nM of cumene per mg of protein per g of tissue. CAT activity was estimated by the method of Radi et al., 1991 and results are expressed as mM of H_2O_2 per mf of protein per g of tissue. SOD activity was established by the method of Misra and Fridovich, 1972 and results are expressed as U SOD per mg of protein per g of tissue. GPx activity was measured by the method of Gunzler and Flohe-Clairborne, 1985 and results are expressed as U/L of GPx per mg of protein per g of tissue. All biomarkers results were normalized against total proteins, which were measured by the method of Bradford, 1976. Furthermore, the experiments were replicated three times.

2.7. Determination of acetylcholinesterase activity

For the determination of AChE activity, we used the method established by Ellman et al., 1961. Briefly, tubes were defrosted, homogenized, and centrifugated at 10,000 g for 15 min (4 °C). 400 µL of the supernatant were mixed with 2.6 mL of phosphate buffer (pH 8.0, 0.1 M) and then 0.1 mL of DTNB (5,5-dithiobis-2-nitrobenzoate, 0.1 M) were added. Changes in absorbance were measured at 412 nm and were recorded for 5 min. Results were expressed in enzymatic activity units (U, µmol of substrate per minute).

2.8. Determination of FLX in brain by LC-MS/M

Before the quantification, each brain sample (24 – 25 mg) was homogenized in the IS solutions, 50 mM acetate buffer (pH 4), methanol, and acetonitrile. Thereafter, samples were ultrasonically treated for 15 min and centrifuged at 4 °C, 12,000 g for 15 min. The supernatant was transferred into a glass tube and diluted with Milli-Q water. The water-diluted sample (methanol content not exceeding 5%) was loaded onto an Oasis HLB cartridge (30 mg, 1 mL) preconditioned with methanol and Milli-Q water. Analytes retained in the cartridge were eluted with methanol, and the solvent was evaporated to dryness under a flow of N2. The residue was reconstituted in methanol: Milli-Q water (4:6, v/v) and then filtered using a cellulose membrane syringe filter. Ten microliters of the totally 20–2000-fold diluted brain extracts were injected in an instrument described below. Instrumental analysis was performed on an ultra-high-performance liquid chromatography system(1290 Infinity I LC System, Agilent Technologies, Waldbronn, Germany) coupled to a quadrupole time-of-flight mass spectrometer (6550 iFunnel Q-TOF LC/MS, Agilent Technologies) operating in electrospray ionization (ESI) positive mode with multiple reaction monitoring (MRM). Data acquisition was performed with Analyst software(B.07.00, Agilent Technologies). Ten microliters of extract were injected into an Agilent Poroshell-120-EC-C18 analytical column (2.7 µm, 100 mm × 2.1 mm; Supelco, Bellefonte, USA) with 5 mM ammonium acetate in Milli-Q water/methanol (4:6, v/v) as the mobile phase set at a flow rate of 0.4 mL min-1. The main ion source parameters were as follows, curtain gas: 30 psi; collision gas: 8 psi; ion spray voltage: 5500; source gas temperature: 700 °C; ion source gas 1: 60 psi; ion source gas 2: 50 psi. Information on MRM transitions and compound-dependent parameters including declustering potential (91), collision energy (49), cell exit potential (20), retention time (3.95), Monitor ion (m/z) (310−44), detection limits (0.020) and quantification limits (0.063). The method was validated with samples spiked with the native compound. Since we extracted the brains of fish from three independent experiments, we performed the analysis for each one of the pools of the brain we got from each exposure. Moreover, we analyzed each pool of brain per triplicate. Thus, we got three different measures for this experiment which were presented as mean values ± S.E.M of 9 subsamples (n = 9). Each subsample represents each of the measures we got for each treatment group. The limits of quantification for each FLX concentration are shown in Table 2.

2.9. Statistical analysis

All data was evaluated using a non-parametric ANOVA followed by a post hoc test (Student Newman Keuls) and expressed as the mean ± standard deviation (SD). Sigma Plot 12.1 software was used for statistical analysis.

3. Results

3.1. Results of the Novel Tank test

The parameters evaluated in the Novel Tank test are shown in (Fig. 1). According to our results, a decrease in the total distance traveled is observed. This indicates a decrease in exploratory behavior. Moreover, it can be observed that as the FLX concentration increases, the prevalence at the top, expressed as latency time (s) at the top, becomes more noticeable. Consequently, there is a decrease in the number of superior-inferior, inferior-superior transitions, representing a longer duration of freezing episodes or freezing which is defined as the cessation of movements. These findings allow us to report that the fish present episodes of anxiety and stress due to acute exposure to environmentally relevant concentrations of FLX.

3.2. Biomarkers of oxidative stress

The antioxidant activity of SOD, CAT and GPX in the brain of adult zebrafish (*D. rerio*) was reported in Fig. 2A. Here, it can be observed that the activity of the enzymes is affected by exposure to FLX. For SOD, CAT, and GPX, a significant increase in activity is observed as the FLX concentration increases, showing a greater response at a concentration of 40 ng L^{-1}.

The levels of LPX, POX, HPX in adult *D. rerio* exposed to FLX are represented in Fig. 2B. In these biomarkers of cellular oxidation, an increase is observed compared to the control group for the three concentrations of FLX. For LPX and HPX, a significant increase is observed as the concentration increases, showing a greater damage response at 40 ng L^{-1}. For POX, a significant increase is observed for the concentration of 16 ng L^{-1} and a decrease for 40 ng L^{-1} compared to the control group.

3.3. Acetylcholinesterase activity (AChE)

AChE activity in the brain of fish exposed to FLX is shown in Fig. 3. From this figure, it can be observed that AChE activity in the brain of fish decreased in a concentration-dependent manner. Thus, significant differences were found between the treatment groups and the control group. Moreover, differences in AChE activity between treatment groups were also significant.

3.4. Correlation of Pearson between biomarkers of oxidative stress and neurotoxicity

Fig. 4 shows a graph with values ranging from − 1 (red) to 1 (blue). The colors indicate the type of correlation between the variables, thus a red coloration indicates a negative correlation while a blue coloration indicates a positive correlation. On the other hand, the intensity of the

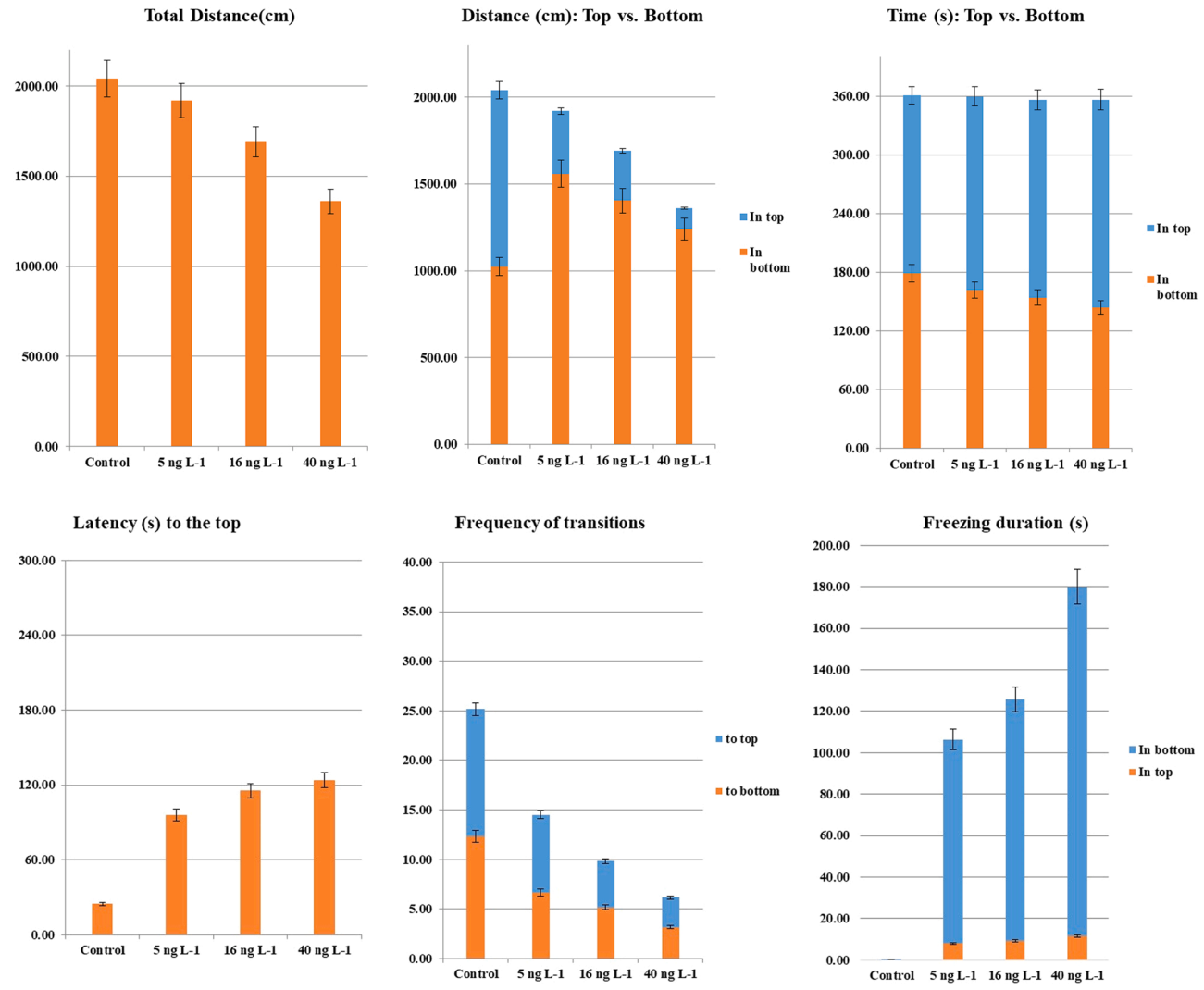

Fig. 1. Behavioral changes observed in fish exposed to environmentally relevant concentrations of FLX. Values are the mean each experiment was performed in triplicate ± SEM. Significant differences relative to: * control group; one-way ANOVA($p < 0.05$).

color denotes the strength of correlation between the variables. According to the above mentioned, we can express that at the concentration of 5 ng L^{-1} of FLX a positive and strong correlation is observed between the biomarkers of oxidative damage, behavior, and acetylcholinesterase activity. Furthermore, for the concentration of 16 ng L^{-1} and 40 ng L^{-1}, a positive and strong correlation is observed between the biomarkers evaluated.

3.5. Determination of FLX in brain by LC-MS/M

Table 1 reports the concentration of FLX in the zebrafish brain. It can be seen that as the concentration of FLX increases, the amount of FLX in the brain increases. At a concentration of 40 ng L^{-1}, a greater amount of FLX was quantified concerning the control group, which is below the LOQ.

4. Discussion

In the present study, zebrafish behavior was observed and quantified using the Novel Tank test. This test is considered highly sensitive for assessing the effect of emerging contaminants on zebrafish behavior and determining whether the organism exhibits episodes of anxiety or stress due to FLX exposure. FLX is a selective serotonin reuptake inhibitor that can increase serotonin concentrations in the brain by blocking the serotonin transporter (Corcoran et al., 2010). Previous studies have observed behavioral effects of fluoxetine (i.e., aggression and appetite) in fish.

Our results showed that fish suffered from episodes of anxiety which were evidenced by a decrease in the total distance traveled, a reduction of time in the upper part, a higher latency to do exploration in the upper part, a decrease in the number of transitions to the upper part and finally an increase in freezing episodes. Thus, 5, 16, and 40 ng L^{-1} of FLX may alter the swimming behavior of fish. In agreement with our results, (Egan et al., 2009) reported that exposing zebrafish adults to 50 μg L^{-1} FLX for 2 weeks induced a decrease in top time, a shorter top scan latency, and a higher number of top transitions employing the Novel tank test. Moreover, Ofoegbu et al. (2019) reported that FLX (0.1–1.0 μg L^{-1}) induced a concentration-dependent increase in locomotor activity and a decrease in feeding in the freshwater Planarian organism *Schmidtea mediterranea*. Similarly, Farias et al. (2018) reported that zebrafish larvae exposed to 0.88, 15.8, 281.2, 500 μg L^{-1} of FLX for 120, 144, and 168 h reduced total swim distance, total swim time, and an alteration in larval behavior quantified by a light/dark test using Zebrabox (ZEB 478 Viewpoint). Finally, Martin et al. (2020) reported that 18 and 215 ng L^{-1} of FLX induced a monotonic reduction of behavior in adult *Gambusia holbrooki* mosquitofish. This was done using the Dark and Light assay in which they noted a preference for a dark environment which is closely related to episodes of anxiety.

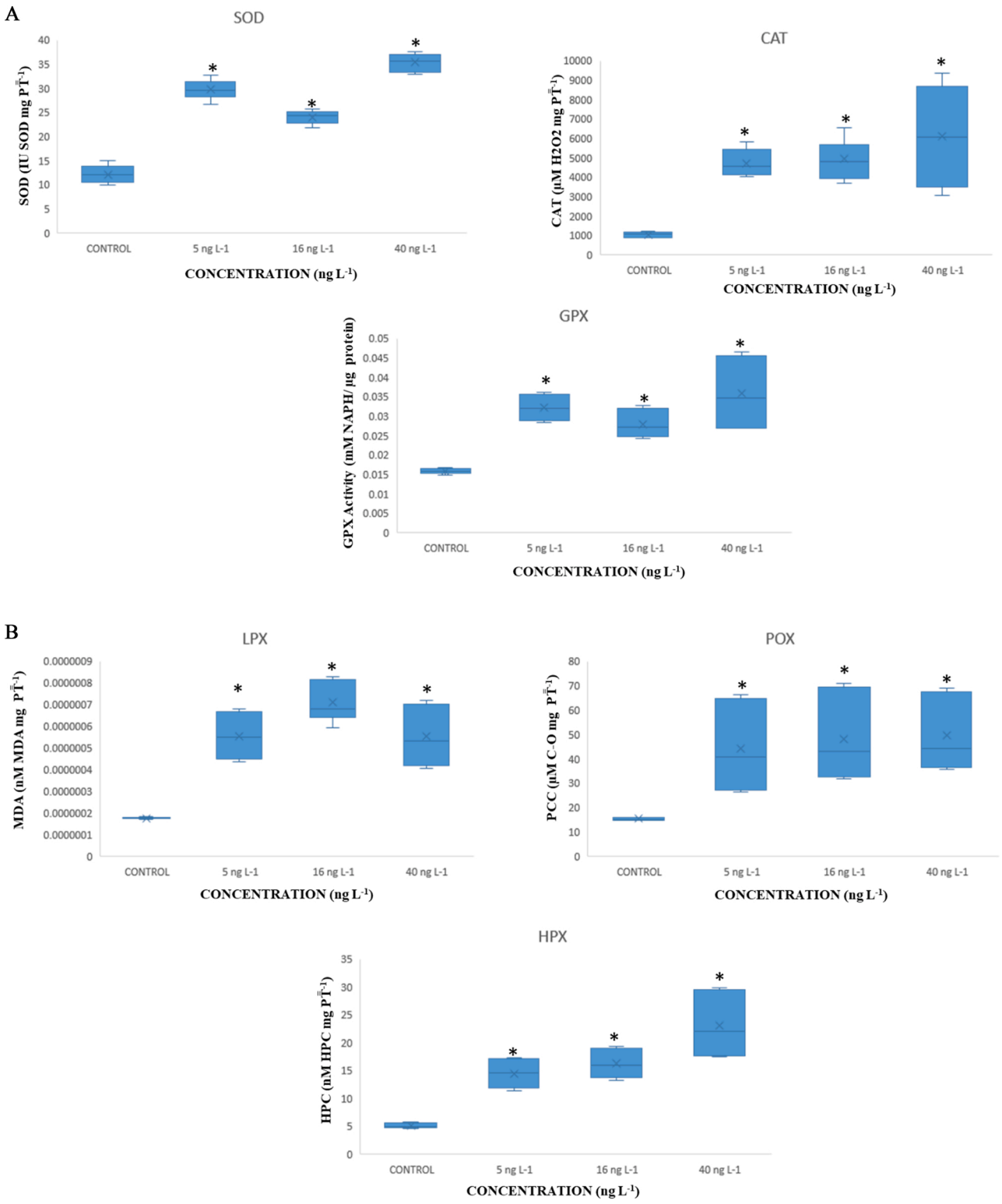

Fig. 2. Antioxidant activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) A, and levels of lipoperoxidation (LPX), carbonyl proteins (POX), hydroperoxides (HPX) B in the brains of *D. rerio* adults exposed to three different concentrations of FLX at 96 h. Values are the mean of three replicates ± SEM. * denote a significant difference compared to the control group; two-way ANOVA($p < 0.05$).

Serotonin has been established to play an important role in modulating locomotor behavior and mood in a wide range of vertebrates. Previous studies in adult teleosts, including zebrafish, have demonstrated an inverse relationship between serotonin levels and spontaneous swimming activity. Airhart et al. (2007) reported that the locomotor activity observed in zebrafish larvae correlated with a decrease in two serotonin receptor transcripts (SERT - serotonin and 5-HT1A - serotonin receptor transcript 1A) in the spinal cord after FLX exposure. All the above described is implicated with the generation of oxidative stress due to the exposure of this type of drug and the effects it has at the level of the Central Nervous System (CNS).

The central nervous system (CNS) has a high percentage of

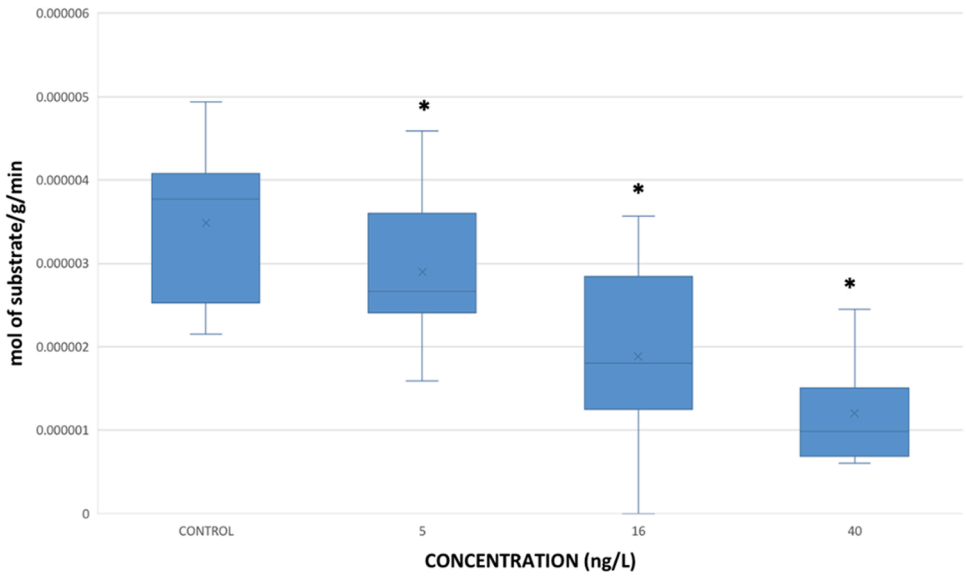

Fig. 3. Determination of acetylcholinesterase activity in *D. rerio* brains exposed to three environmentally relevant concentrations of FLX. * denote a significant difference compared to the control group; two-way ANOVA($p < 0.05$).

phospholipids (arachidonic acid, docosahexaenoic acid, inositol phosphate, and diacylglycerol). Phospholipids are molecules that are easily peroxidized forming free radicals. The main free radicals generated during the peroxidation of phospholipids are the superoxide radical (O2*), the peroxyl radical (ROO^-), the hydroxyl radical (OH), and (NO^-) (Caiaffo et al., 2016). These compounds and their by-products such as H_2O_2, generate oxidative effects on cellular molecules and components by breaking the balance between prooxidant and antioxidant processes. As a consequence, the condition known as oxidative stress is produced, which damages lipids, proteins, nucleic acids, and other cellular structures (Caiaffo et al., 2016).

Many organic pollutants can cause oxidative stress in fish. Lipoperoxidation is considered a good biomarker of oxidative stress damage in aquatic invertebrate organisms (Demidchik, 2015; Regoli and Giuliani, 2014). FLX induces oxidative stress through the generation of malondialdehyde (MDA). For example, (Huihui Chen et al., 2015) reported an increase in MDA levels in gills and digestive glands of adult Asian clam (*Corbicula fluminea*) exposed to 5 and 50 μg L^{-1} fluoxetine for 30 days. In another study done by Di Poi et al. (2016), MDA levels in a mixed sample of gills and digestive glands of juvenile oysters (*Crassostrea gigas*) increased approximately 4-fold after 14 days of exposure to 5.4 μg L^{-1} of fluoxetine. Their results are consistent with those obtained in the present study, where MDA levels were increasing as FLX concentration increased from 5 ng L^{-1} to 40 ng L^{-1}.

The best-known product of lipid peroxidation is malondialdehyde (MDA), a very toxic molecule that affects proteins and DNA (components of cellular structures) (Caiaffo et al., 2016). The findings found in this study reported that FLX induced an increase in the content of carbonylated proteins and hydroperoxides at the concentration of 40 ng L^{-1} FLX. FLX, through oxidative stress mechanism, can oxidize proteins and alter protein functionality by forming new low molecular weight aggregates. In DNA, the oxidation of puric and pyrimidic bases results in mutagenic events leading to apoptosis. Duarte et al. (2019) reported DNA damage and lipoperoxidation in *Pomatoschistus microps* after exposure to 0.1, 0.5, 10, and 100 μg L^{-1} of FLX. This supports that FLX has the ability to generate genotoxic effects even at low concentrations.

Oxidative stress tress damage also involves the alteration of antioxidant enzymes levels that are the first line of defense of organisms to protect themselves. The antioxidant defense system includes the coordinated effects of some enzymes such as SOD, CAT, and GPX. SOD is the enzyme responsible for catalyzing the dismutation of free radicals such as O_2 to H_2O_2, which is less reactive and can become degraded by other enzymes such as catalase or glutathione peroxidase. Herein, we demonstrated antioxidant activity of SOD, CAT, and GPX in fish exposed to FLX showed a significant increase compared to the control group. However, this response did not show a concentration-dependent manner tendency. In fact, we observed that at the middle concentration tested, all biomarkers showed a decrease compared to the lowest and highest concentration. The above may be explained by the augmented production of ROS in fish. For example, recent findings have shown that the reduction of CAT activity is associated with an increase in hydroperoxides content. Moreover, Caiaffa et al., 2016 reported that LPX production in the hippocampus and cerebral cortex increase during the depression as a result of a reduction in CAT and GPX levels. Similarly, Erkan Ozcan et al. (2004) reported that reduced levels of SOD, GPx, and CAT are the result of increased MDA production in organisms with anxiety and stress disorders. Thus, high levels of H_2O_2 and LPX in the brain of fish during anxiety-like states could reduce the levels of antioxidant enzymes.

A study by Gonzalez-Rey and Bebianno (2013) reported that exposure to 75 ng L^{-1} FLX induced decreased SOD activity in gills and intestinal glands of the mussel *Mytilus galloprovincialis*. Our findings report a decrease in SOD activity at the 16 ng L^{-1} concentration and a slight increase at the 40 ng L^{-1} FLX concentration after 96 h of exposure. Likewise, a study by Hongxing Chen et al. (2018) demonstrated that Chinese gudgeon fish *Pseudorasbora parva* exposed to 0, 50, and 200 μg L^{-1} of fluoxetine increased in CAT activity respectively in the gills. Additionally, Gonzalez-Rey and Bebianno (2013) reported that exposure of the mussel *Mytilus galloprovincialis* to 75 ng L^{-1} FLX induced CAT activity. Similarly, our results showed an increase in CAT and GPX activity at 40 ng L^{-1} in zebrafish brains after 96 h of FLX exposure. Franzellitti et al. (2014) observed an increase of GST activity in *M. galloprovincialis* gills after 7 days of exposure to 0.3–300 ng L^{-1} fluoxetine. Finally, Hongxing Chen et al. (2018) reported an increase of GST activity in gills of the Chinese gudgeon fish *Pseudorasbora parva* exposed to 0, 50, and 200 μg L^{-1} fluoxetine for 4 h.

The increase in ROS, mainly due to FLX exposure and the increase in MDA generated by lipid oxidation, reduced the activity of AChE. AChE is responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) to choline plus acetic acid and plays an important role in cholinergic neuronal function. Serotonin is known to increase

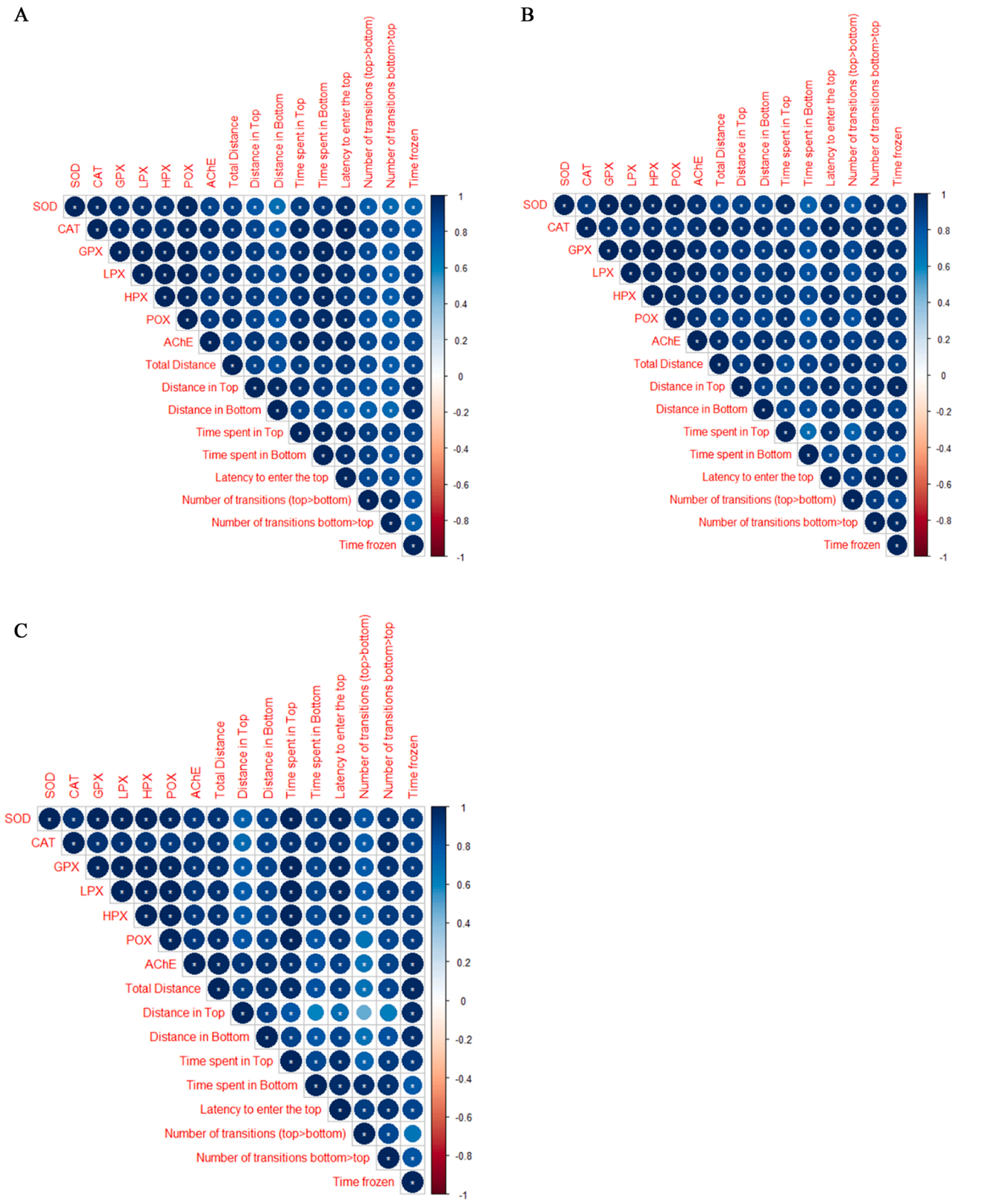

Fig. 4. Correlation of Pearson between behavioral changes, oxidative stress y acetylcholinesterase evaluated at FLX concentrations: A) 5 ng L^{-1}, B) 16 ng L^{-1}, C) 40 ng L^{-1}.

intracellular calcium (Ca^{2+}) levels and, in turn, stimulates acetylcholine release (Goldberg et al., 1992). Both serotonin and acetylcholine can intensify Ca^{2+} uptake, which can increase oxidative stress. Consequently, Ca^{2+} at high concentrations can induce mitochondrial damage (Görlach et al., 2015; Simmons and Koester, 1986). ROS production and induction of apoptosis can be triggered through net intracellular Ca^{2+} uptake or release from endoplasmic reticulum stores (Görlach et al., 2015). Our results demonstrated that FLX decreased the

Table 1
Concentration of FLX in the brain of zebrafish.

Nominal concentration of FLX	Measured concentration of FLX in the brain of zebrafish
Control	<LOQ
5 ng/L	0.466 ng/g
16 ng/L	1.492 ng/g
40 ng/L	3.732 ng/g

LOQ: limit of quantification (0.063 ng/g). LOD: limit of detection (0.020 ng/g)

acetylcholinesterase activity in the brain of zebrafish. Likewise, Munari et al., 2014 reported that FLX (1 – 5 μg L^{-1}) decreased the AChE in the gills of *Venerupis philippinarum* after 7 days of exposure. Moreover, de Farias et al., 2019 demonstrated that the AChE activity of *D. rerio* embryos was significantly inhibited at concentrations (6 μg L^{-1}) close to the maximum reported FLX concentration in surface waters. Tierney (2011) pointed out that a decrease in cholinesterase activity can cause progressive myopathy of skeletal muscles and, consequently, a loss of motility. Since our results showed that exposure of *D. rerio* adults to 5, 16, and 40 ng L^{-1} of FLX induced a slight decrease in the activity of AChE and a reduction of exploratory behavior in zebrafish, we suggest that FLX may cause a progressive myopathy of skeletal muscles. In summary, the presence of FLX can induce an increase of MDA in the brain, leading to the oxidation of lipids, proteins, and nucleic acids (DNA) present in the different organs and tissues of organisms and, consequently, cause the inhibition of AChE activity.

It has been shown that FLX has the ability to bioaccumulate in the fish brain (Brooks et al., 2009). For example, (Grabicova et al., 2014) and (Pan et al., 2018) reported that FLX bioconcentrates in the brain of *Oncorhynchus mykiss* (0.5–1.4 ng g^{-1}) and *Carassius auratus* (0.1 μg L^{-1}), respectively. These findings are consistent with our results, where FLX was quantified in the brain of fish at concentrations of up to 3.732 ng g^{-1}.

5. Conclusions

Fluoxetine at environmentally relevant concentrations (5, 16, and 40 ng L^{-1}) was able to alter behavior in adult zebrafish. These changes were related to the generation of anxiety episodes which were caused by an increase in serotonin levels, as well as by the formation of ROS. FLX also induced an alteration of acetylcholinesterase activity by an increase in reactive oxygen species. The findings of this investigation led us to the conclusion that FLX is a neurotoxic substance as it mainly alters neuroendocrine functions in the brain. Moreover, there is a strong correlation between the biomarkers evaluated as Novel Tank test (behavior), oxidative stress in the brain (SOD, CAT, GPX, LPX, POX, HPX), and AChE activity (neurotoxicity).

CRediT authorship contribution statement

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

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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