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Realistic concentrations of Bisphenol-A trigger a neurotoxic response in the brain of *zebrafish*: Oxidative stress, behavioral impairment, acetylcholinesterase inhibition, and gene expression disruption

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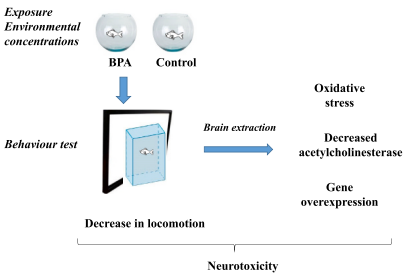
HIGHLIGHTS

- Bisphenol-A disrupted the swimming behavior of *Danio rerio*.
- Exposure to Bisphenol-A prompted oxidative stress in the *Danio rerio* brain.
- Acetylcholinesterase is inhibited in the *Danio rerio* brain after acute exposure.
- Upon Bisphenol-A exposure, mbp, α1-tubulin, and manf expression increased.
- Realistic concentrations of Bisphenol-A prompted neurotoxicity in fish.

GRAPHICAL ABSTRACT



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ABSTRACT

Bisphenol A (BPA) is a micro-pollutant found in various environmental matrices at concentrations as low as ng/L. Recent studies have shown that this compound can cause oxidative damage and neurotoxic effects in aquatic organisms. However, there is a lack of research investigating the effects of BPA at environmentally relevant concentrations. Therefore, this study aimed to assess the neurotoxic effects of acute BPA exposure (96 h) at environmentally relevant concentrations (220, 1180, and 1500 ng/L) in adult zebrafish (*Danio rerio)*. The Novel Tank trial was used to evaluate fish swimming behavior, and our results indicate that exposure to 1500 ng/L of BPA reduced the total distance traveled and increased freezing time. Furthermore, the evaluation of biomarkers in the zebrafish brain revealed that BPA exposure led to the production of reactive oxygen species and increased acetylcholinesterase activity. Gene expression analysis also indicated the overexpression of *mbp, α1-tubulin,* and *manf* in the zebrafish brain. Based on our findings, we concluded that environmentally relevant concentrations of BPA can cause anxiety-like behavior and neurotoxic effects in adult zebrafish.

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1. Introduction

Bisphenol A (BPA) is a compound widely used to manufacture plastics, especially polycarbonate. In addition, it is used to manufacture polyvinyl chloride (PVC), dental adhesives, and thermal printing paper (Chen et al., 2016). Due to the widespread use and disposal of products containing BPA, this compound is introduced into the environment through diverse mechanisms, including incomplete elimination during wastewater treatment, leaching from discarded BPA-containing materials, such as polycarbonate and recycled paper, and during its processing in manufacturing activities (Im and Loffler, 2016).

According to recent research, BPA has been detected in various environmental matrices at concentrations ranging in nanograms per liter (ng/L). For instance, Yamazaki et al. (2015) found 1–1950 ng/L of BPA in surface waters collected from select rivers in Japan, Korea, China, and India. Consequently, it poses a potential threat to hydrobionts. However, despite the extensive investigation into the toxicological impacts of BPA, additional information regarding the harmful effects of BPA at environmental concentrations of ng/L is still required. Our previous study pointed out that BPA at concentrations between 220 and 1500 ng/L generated zebrafish embryotoxicity, teratogenicity, and oxidative stress (Heredia-García et al., 2022). Thus, there is mounting evidence that this pollutant, at environmentally relevant concentrations, may compromise aquatic organisms' health and overall fitness.

BPA has been categorized as an endocrine disruptor (Talpade et al., 2018). Although the main affectation of these disruptors is at the reproductive system level, the brain also depends on estrogen for proper development (Seralini and Jungers, 2021); thus, BPA could induce damage in the CNS (Bless et al., 2016). The investigation conducted by Chen et al. (2017), which employed zebrafish as a bioindicator, revealed that BPA bioaccumulated in the brain at a concentration of 1 μg/L. Furthermore, the findings demonstrated that BPA induced overexpression of myelin (*MBP/gen*) in the central nervous system (CNS) and led to significant inhibition of acetylcholinesterase (AChE). Likewise, Sahoo et al. (2020) employed behavioral assays in zebrafish to assess the neurotoxic effects of BPA at a concentration of 17.52 μM. The findings revealed that quercetin exhibited a protective effect by reducing oxidative stress in the brains of exposed organisms. The Tang et al. (2020) study utilized the *Tegillarca granosa* bioindicator, which was subjected to environmental concentrations of BPA at 10 and 100 ng/L. The outcomes revealed toxic effects, as manifested by reduced expression of genes encoding modulatory enzymes and neurotransmitter receptors, such as TRAF6, TAB2, IKKα, NFkB, GABAT, and MAO, thus indicating the occurrence of neurotoxicity. The studies mentioned earlier propose that BPA triggers its effects via an oxidative stress response in fish, which may cause the hypothesized neurotoxic effects. There are numerous investigations on the neurotoxic effects of BPA in different aquatic species; however, these studies use concentrations exceeding those present in the environment and employ other biomarkers compared to those used in our research.

The present study assessed the neurotoxicity in adult zebrafish by evaluating their behavior, oxidative stress, and acetylcholinesterase (AChE) activity after exposure to environmentally relevant concentrations of BPA (220, 1180, and 1500 ng/L). The selection of these concentrations was based on a prior investigation carried out by our research group, where we observed that this compound could induce embryotoxic, teratogenic, and oxidative damage effects (Heredia-García et al., 2022). We hypothesize that BPA, at these concentrations, may impact the fish's behavior and trigger a significant elevation of oxidative damage biomarkers in the zebrafish brain.

2. Method

2.1. Test substance

Bisphenol-A, 99% of purity, (2,2-Bis(4-hydroxyphenyl)propane, 4,4′-Isopropylidenediphenol) used in the study was acquired from Merck (Darmstadt, Germany).

2.2. Test organisms

Adult zebrafish of the AB strain, aged twelve months and of both sexes, with an average length of 4.5 ± 0.5 cm and weight of 3.2 ± 0.3 g, were selected for this study. The individuals were sorted by sex, and an equal number of males and females were placed into each system. Before experimentation, the zebrafish were acclimatized for six weeks in individual glass aquaria at one organism per liter stocking density. The water parameters were maintained at a constant temperature of 28 ± 1 °C and free of chlorine, sterilized by UV light and carbon filtration. Additionally, the dissolved oxygen (9.8 ± 0.5 mg/L), pH (7.4 ± 0.17), conductivity (373 ± 30 S/cm), nitrate content (2.6 ± 0.2 mg/L), and nitrite content (0.028 ± 0.007 mg/L) were monitored and controlled throughout the study. To provide nutrition, Spirulina flakes (Ocean Nutrition) and *Artemia* sp. *Nauplii*, a type of crustacean in brine, were used.

2.3. Ethical approval

The Ethics and Research Committee of the Autonomous University of the State of Mexico (UAEM) reviewed and approved this research protocol to ensure that experiments follow institutional animal care standards (approval ID: RP.UAEM.ERC.148.2022). In addition, the provisions of the official Mexican standard on the breeding, care, and use of laboratory animals (NOM-062-ZOO- 1999) were also considered.

2.4. BPA exposure and sample collection

The systems for exposure to BPA were chosen considering the Elizalde-Velázquez et al. (2022a),b method. In brief, 120 fish were distributed among four glass tanks, where each tank had a volume of 30 L and housed 30 testing organisms, yielding a ratio of 1:1. Each system was assigned one of three concentrations tested by the BPA ($C_1 = 220$, $C_2 = 1180$, and $C_3 = 1500$ ng/L), while another system was designated as the control treatment. The identical and unaltered protocol was executed thrice across three autonomous trials, using 360 organisms for the complete research. The renewal of each system's medium was carried out daily throughout the entire duration of the test. Following the exposure of the organisms, water samples were collected for analysis. After 96 h, the organisms were transferred to individual fish tanks for behavioral tests. Subsequently, they were euthanized and dissected following the AVMA Guidelines on Euthanasia 2020 edition to obtain their brains. To euthanize the fish, the hypothermic shock was induced by placing them in icy water (2–4 °C). A total of thirty brains were gathered for each concentration, in addition to a control group, and these were pooled in Eppendorf tubes containing phosphate buffer (pH = 7.4). The Eppendorf tubes were then frozen at −80 °C for subsequent analysis to determine levels of oxidative stress and acetylcholinesterase activity.

2.4. Analysis of the test substance in water and brains of zebrafish

The technique depicted by Wu et al. (2017) was followed for sample treatment. 10 mL of water samples were collected from each test system and stored at −20 °C until quantification. The water samples were taken at 96 h. Before quantification, water samples, including exposure solutions, were sieved to a 0.45 μm membrane. Brain samples were homogenized with 200 μL of phosphate buffer (0.1 M pH 7.4), 100 μL of internal standard C-BPA (200 μg/L), and 1 μL of dichloromethane. The homogenates were centrifuged at 16 000×*g* for 10 min, and 100 μL of the supernatant was assigned to vials for LC-MS/MS analysis. BPA and C-BPA contents were quantified in aqueous mediums and brain samples using an HP Series 1260 high-performance liquid chromatograph

(Agilent Technologies) and a G6460 triple quadrupole electrospray ionization mass spectrometer (Agilent Technologies). A C18 column was used and kept at 40 °C with a 0.4 mL/min flow rate. The mobile phase started with 40% methanol and 60% water; then, at 7 min, the gradient increased to 100% methanol and was maintained for 3 min. The gas was dried at 350 °C, and the capillary voltage was 4.5 kV. Using the BPA concentration in both the brain tissue and the surrounding environment, the bioconcentration factor (BCF) was calculated with the following formula: BCF = [concentration in brain tissue]/[concentration in surrounding environment] (García-Medina et al., 2022).

2.6. Novel Tank test

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, as stated below. Briefly, fish were individually selected at random 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 at a temperature of 28 ± 1 °C. Movements were documented for 360 s with a GigE camera (HT-GE134GC-T-CL, 139 FPS maximum frame rate, United States) placed in front of the experimental tank. The videos were analyzed by the ToxTrac program (Organism Video Tracking Application, 2.91 version), which divided the screen into two zones (top and bottom) and determined the entire distance transited (cm), the time of permanence at the top and bottom (s), latency (s), frequency of transitions between zones and freezing time (s) (Cachat et al., 2010). Novel Tank test was performed three times in three independent experiments, and for each experiment, we used a total of 30 organisms per concentration, including the control group (n = 90 per concentration).

2.7. Oxidative stress test

The previously extracted brains were homogenized using a rotor-stator homogenizer (Ultra-Turrax, IKA, Germany). The resulting homogenate was distributed into two vials. Vial 1 contained 300 μL of homogenate and 300 μL of 20% trichloroacetic acid and was centrifuged at 11 495 rpm/15min to evaluate the levels of lipoperoxidation (LPX), hydroperoxides content (HPC), and protein carbonyl content (PCC). Meanwhile, vial 2 contained 700 μL of homogenate and was centrifugated at 12 500 rpm/15 min to assess the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), as well as the total protein content. The procedure for both vials is presented in Table 1. All results were normalized against total protein levels. The brain samples from three independent experiments were analyzed to assess oxidative stress biomarkers. Each biomarker was evaluated three times in all samples, yielding nine results per biomarker and concentration (n = 9 per concentration).

Table 1
Oxidative stress techniques.

Tube	Biomarker	Tube content	Quantification	Method
1	LPX	50 μL supernatant, 450 μL Tris-HCl 150 mM, 1 mL TCA-TCB	nM of reactive carbonyls 535 nm	Buege and Aust, 1978
	HPC	50 μL supernatant, 900 μL reaction mix	nM of malondialdehyde 560 nm	Jiang et al. (1992)
	PCC	150 μL precipitate, 150 μL DNPH/HCl 10 Mm, 500 μL ATC 20%, ethanol:acetate de etilo 1:1	nM of cumene 366 nm	Levine et al., 1994
2	SOD	1 mL guanidine 6 M, 50 μL supernatant, 260 μL carbonate buffer, 200 μL adrenaline 30 Mm	U SOD 480 nm	Misra and Fridovich (1972)
	CAT	30 μL supernatant, 420 μL isolation buffer, 300 μL H2O2 20 Mm	mM of H2O2 240 nm	Radi et al. (1991)
	GPX	100 μL supernatant, 290 μL reaction buffer, 100 μL H_2O_2 20 Mm	U/L of GPx 340 nm	Gunzler and Flohe-Clairborne (1985)
	Total proteins	12 μL reductase glutation, 13 μL supernatant, 75 μL distillated water, 1.25 mL Bradford reactive	mg protein 595 nm	Bradford (1976)

TCA-HCl = Tris hydrochloride. TCA-TBA = thiobarbituric-trichloroacetic acid. DNPH = 2,4-Dinitrophenylhydrazine. TCA stands for trichloroacetic acid. EDTA = Ethylenediaminetetraacetic acid. NADPH = Nicotinamide adenine dinucleotide phosphate.

2.8. Total protein determination

Total protein content was determined to standardize the evaluation of oxidative stress biomarkers in the brains of zebrafish exposed to BPA. To accomplish this, homogenized brain samples (50 μL) in phosphate buffer (pH 7.0) were mixed with deionized water (150 μL) in a test tube. Subsequently, 5 mL of Bradford reagent, which consists of Coomassie blue (0.1 g), 96% ethanol (50 mL), and H3PO4 (100 mL), dissolved in deionized water to make 1 L, was added to the test tube. The contents were mixed and left to rest for 5 min, after which the absorbance was read at 595 nm. The resulting data were extrapolated using a standard bovine serum albumin curve. The Bradford method, first described by Bradford in 1976, was employed for this determination.

2.9. Acetylcholinesterase activity test

The methodology proposed by Ellman et al. (1961) was applied to analyze acetylcholinesterase activity. Ten brains, which had previously been extracted and homogenized, were chosen for each concentration. These samples were centrifugated at 10 000 rpm for 15 min at 4 °C using a Centurion Scientific K241R instrument. Next, 400 μL of the supernatant was mixed with 2.6 mL of phosphate buffer (pH 8.0, 0.1 M), 0.1 mL of DTNB (5,5-dithiobis-2-nitrobenzoate, 0.1 M), and 25 μL of the substrate (acetylthiocholine iodide 0.075 M) using glass test tubes. Changes in absorbance were measured at 412 nm and recorded every minute for 5 min. The results were expressed in enzymatic activity units (U mol of substrate per minute). Acetylcholinesterase activity was assessed by analyzing brain samples from three distinct experiments. Furthermore, each sample was evaluated thrice, resulting in nine outcomes per concentration, including the control group (n = 9 per concentration).

2.10. RT-qPCR

After dissection of the fish, the brains were placed in an Eppendorf tube free of RNases that contained RNALater (QIAGEN) to prevent RNases from degrading the nucleotides. The ratio of RNALater added to Eppendorf was 10 μL:1 mg of tissue, considering data included in the manufacturer's manual. All Eppendorf were stored at −20 °C until RNA extraction. RNA extraction was carried out in the molecular biology room, which has a laminar flow hood that works with positive pressure

to prevent the infiltration of environmental contaminants. The samples were thawed in an ice bath, and each tissue was transferred to a new tube containing lysis solution (RNeasy QIAGEN). Subsequently, a volume of ethanol (70%) equal to that of the sample was added, and the sample was transferred to an RNeasy Mini Kit (QIAGEN) column, which was then centrifuged at 10 000 rpm for 15 s. Hereafter, 700 μL of RWI buffer (RNeasy QIAGEN) and 500 μL of RPE were added to the samples, and all samples were centrifuged at 10 000 rpm for 15 s and 2 min, respectively. The continuous flow on each washing was disposed of following the instructions in the manufacturer's manual. The column was transferred to a new tube, and 50 μL of RNase-free water was added and centrifuged for 1 min at 10 000 RPM. RNA quality was assessed by agarose gel electrophoresis (1%), and purity was assessed by spectrophotometry (NanoDrop, 2000/2000c Thermo Scientific) through 260/280 and 260/230 ratios. The RNA obtained was stored at −20 °C until use. The RNA samples were thawed in an ice bath, and 2.0 μL of 7x gDNA Wipeout Buffer (QuantiTect Reverse Transcription Kit QIAGEN), 10.0 μL of the RNA template, and 2.0 μL of RNase-free water (QuantiTect Reverse Transcription Kit QIAGEN) were placed in an Eppendorf tube free of RNases. These tubes were incubated for 2 min at 42 °C and immediately placed on ice to subsequently add 1.0 μL Quantiscript reverse transcriptase (QuantiTect Reverse Transcription Kit QIAGEN), 4.0 μL Quantiscript RT Buffer 5x (QuantiTect Reverse Transcription Kit QIAGEN), 1.0 μL RT Primer Mix (QuantiTect Reverse Transcription Kit QIAGEN). All above was mixed and incubated at 42 °C for 15 s and 93 °C for 3 min for reverse transcription inactivation. Once the reverse transcription was completed, the cDNA quality was evaluated by agarose gel electrophoresis (1%) purity by spectrophotometry (NanoDrop, 2000/2000c Thermo Scientific) through 260/280 and 260/230 ratios. The qPCR reaction was performed by adding 25 μL of the 2x QuantiTect SYBR Green PCR (QIAGEN). The kit contains a HotStartTaq DNA polymerase, a modified form of a 94 kDa recombinant DNA polymerase isolated initially from Thermus aquaticus and cloned in *Escherichia coli*. In addition, 1.0 μL of each primer, 4.0 μL of cDNA, and 19 μL of RNase-free water were added to vials. All genes evaluated are shown in Table 2. All reagents were mixed, and the qPCR was run using the following conditions: 94 °C for 15s, followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The qPCR equipment used was a Gene Q Rotor (QIAGEN). All samples were normalized against the *β-actin* gene (housekeeping). mRNA expression changes were calculated using the $2^{-\Delta\ \Delta Cq}$ method (Pfaffl, 2001; Pfaffl et al., 2002; Schmittgen and Livak, 2008). Once the qPCR cycles were completed, a dissociation curve was performed to distinguish between specific and non-specific products, using the following conditions: 10s at 95 °C, 10s at 65 °C and 10s at 95 °C with 0.2 °C increments. Data were collected at each increment of the melting curve. qPCR reactions were performed in triplicate for all samples. In addition, samples from all three experiments were evaluated (n = 9). As a negative control, samples to which no temper was added were used, and RNase-free water was placed instead.

2.10. Statistical analysis

The behavioral and gene expression data were subjected to statistical analysis using a one-way ANOVA followed by the Student-Newman-Keuls post hoc test, while normality was evaluated through the Shapiro-Wilk test. The data were processed using the SigmaPlot 12.3 program. On the other hand, the biomarkers for oxidative stress and acetylcholinesterase were analyzed through a Kruskal-Wallis nonparametric one-way ANOVA, followed by a Student-Newman-Keuls post hoc test. Furthermore, a Pearson correlation analysis was conducted using R software between the behavioral endpoints and the other biomarkers.

3. Results

3.1. Bioconcentration factor

Table 3 shows that although the bioconcentration values for the three concentrations tested in the study were low (between 0.068 and 0.141), BPA could be taken up in the zebrafish brain at 96 h.

The detection limit (LOD) was 0.4 ng/L.

3.2. Novel Tank test

Fig. 1 shows the behavioral parameters of zebrafish after exposure to environmental concentrations of BPA. Significant decreases in total distance traveled (TD) ($F(3) = 99.926$; $n = 90$; $p < 0.001$), frequency of top to-bottom transition (Ttb) ($F(3) = 95.410$; $n = 90$; $p < 0.001$) and bottom to top transition (Tbt) ($F(3) = 92.801$; $n = 90$; $p < 0.001$) were observed compared to the control group. On the other hand, significant increases were observed in the distance traveled in the top (Dtop) ($F(3) = 102.788$; $n = 90$; $p < 0.001$) bottom (Dbott) ($F(3) = 66.950$; $n = 90$; $p < 0.001$), in time duration in the top (Ttop) ($F(3) = 100.719$; $n = 90$; $p < 0.001$) bottom (Tbott) ($F(3) = 100.719$; $n = 90$; $p < 0.001$), latency (Lty) ($F(3) = 46.496$; $n = 90$; $p < 0.001$) and freezing (Fz) ($F(3) = 73.305$; $n = 90$; $p < 0.001$) compared to the control group. Latency refers to the time it takes for the fish to reach the top for the first time after introduction to the tank. As shown in supplementary material, no significant differences were observed in the behavior of male and female adult fish exposed to BPA (Fig. 1S).

3.3. Oxidative stress test

According to our data, the enzymatic activity of SOD ($H(3) = 112.737$; $n = 9$; $p < 0.001$), CAT ($H(3) = 112.343$; $n = 9$; $p < 0.001$), and GPX ($H(3) = 112.737$; $n = 9$; $p < 0.001$) (Fig. 2) in all BPA treatment groups showed a significant increase in comparison to the control group. Moreover, similar to the activity of antioxidant enzymes, levels of all oxidative damage biomarkers in all treatment groups significantly increased in relation to the control group LPX ($H(3) =$

Table 3
LC-MS/MS Analysis of BPA concentrations in water and brain samples, and BCF in zebrafish brains at 96 Hours.

Nominal exposure concentrations (ng/L)	Exposure concentration (ng/L)	Concentrations in brains of zebrafish (ng/Kg)	BCF
Control	ND	ND	–
220	190.03 ± 1.24	18.79 ± 6.43	0.098
1180	1049.32 ± 4.643	71.72 ± 3.21	0.068
1500	1298.53 ± 14.61	183.64 ± 23.544	0.141

ND not detected.
"-" represents no data.

Table 2
Sequence of genes tested.

Gen	Access number	Forward primer	Reverse primer	Reference
mbp	AW076969	AATCAGCAGGTTCTTCGGAGGAGA	AAGAAATGCACGACAGGGTTGACG	Chen et al. (2017)
α1-tubulin	NM_194 388	AATCACCAATGCTTGCTTCGAGCC	TTCACGTCTTTGGGTACCACGTCA	Gu et al. (2022)
manf	BC124316	AGATGGAGAGTGTGAAGTCTGTGTG	CAATTGAGTCGCTGTCAAAACTTG	Chen et al. (2017)
β-actin	NM_131 031	GTTTAGGTTGGTCGTTCGTTTGA	AAGTGCGACGTGGACA	Gonzalez et al. (2005)

mbp = myelin basic protein gene; **α1-tubulin** = alpha-1- tubulin gene; **manf** = mesencephalic astrocyte derived neurotrophic factor gene; **β-actin** = Beta-actin gene.

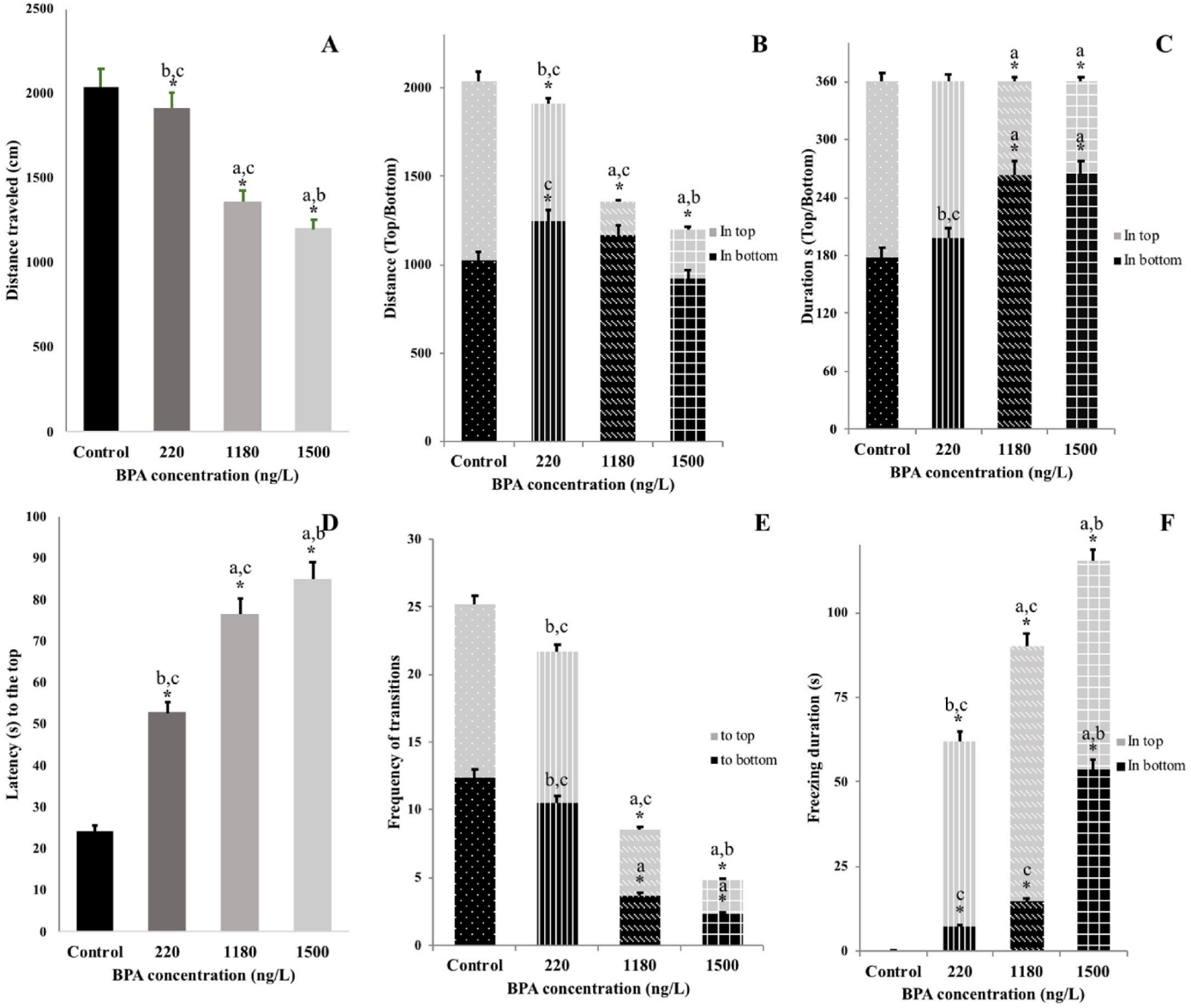

Fig. 1. Behavioral endpoints (A = Distance traveled, B = Distance at top/bottom, C=Time at top/bottom, D = Latency, E = Frequency of transitions, F=Freezing) evaluated in zebrafish adults exposed at 96 h to BPA (C_1 = 220, C_2 = 1180, C_3 = 1500 ng/L). Values represented as mean ± SEM, n = 90 per concentration. Asterisks denote meaningful dissimilarities compared to the control group ($p < 0.001$). Letters indicate significant differences between treatment groups (a: 220 ng/L BPA, b: 1180 ng/L, c: 1500 ng/L).Student-Newman-Keuls post hoc test.

112.343; n = 9; $p < 0.001$), PCC (H (3) = 102.357; n = 9; $p < 0.001$), and HPC (H (3) = 87.684; n = 9; $p < 0.001$). The comparison of oxidative stress results between males and females is shown in Fig. 2S. Again, no significant differences were observed between the sexes.

3.4. Acetylcholinesterase test

The alterations in AChE activity in the zebrafish brains are presented in Fig. 3. The figure indicates that BPA, at a concentration of 220 ng/L, did not produce significant changes in AChE activity compared to the control group. However, as the concentration of BPA increases, the activity of this enzyme is inhibited. Notably, at concentrations of 1180 ng/L and 1500 ng/L, there was a significant reduction in AChE activity compared to the control group (H (3) = 20.748; n = 9; $p < 0.001$). Our results indicated male and female fish had almost the same degree of AChE inhibition after BPA exposure (Fig. 3S).

3.5. qPCR

In the gene expression determination shown in Fig. 4, meaningful increases over the control group were observed for *mbp* (F (3) = 111.757; n = 9; $p < 0.001$), *α1-tubulin* (F (3) = 106.134; n = 9; $p < 0.001$) and *manf* (F (3) = 107.388; n = 9; $p < 0.001$). The gene expression of all genes remained invariable between the sexes (Fig. 4S).

3.6. Pearson correlation

The Pearson's correlation plot presented in Fig. 5 displays the relationship between oxidative stress biomarkers, AChE levels, and BCF in zebrafish exposed to BPA for 96 h concerning their behavioral changes. The plot's colors reflect the correlation type between the variables; the color's intensity indicates the correlation's strength. Blue represents a positive correlation between the variables, while a red represents a negative correlation. Positive correlations were observed between bottom time, latency, and freezing behaviors with stress biomarkers,

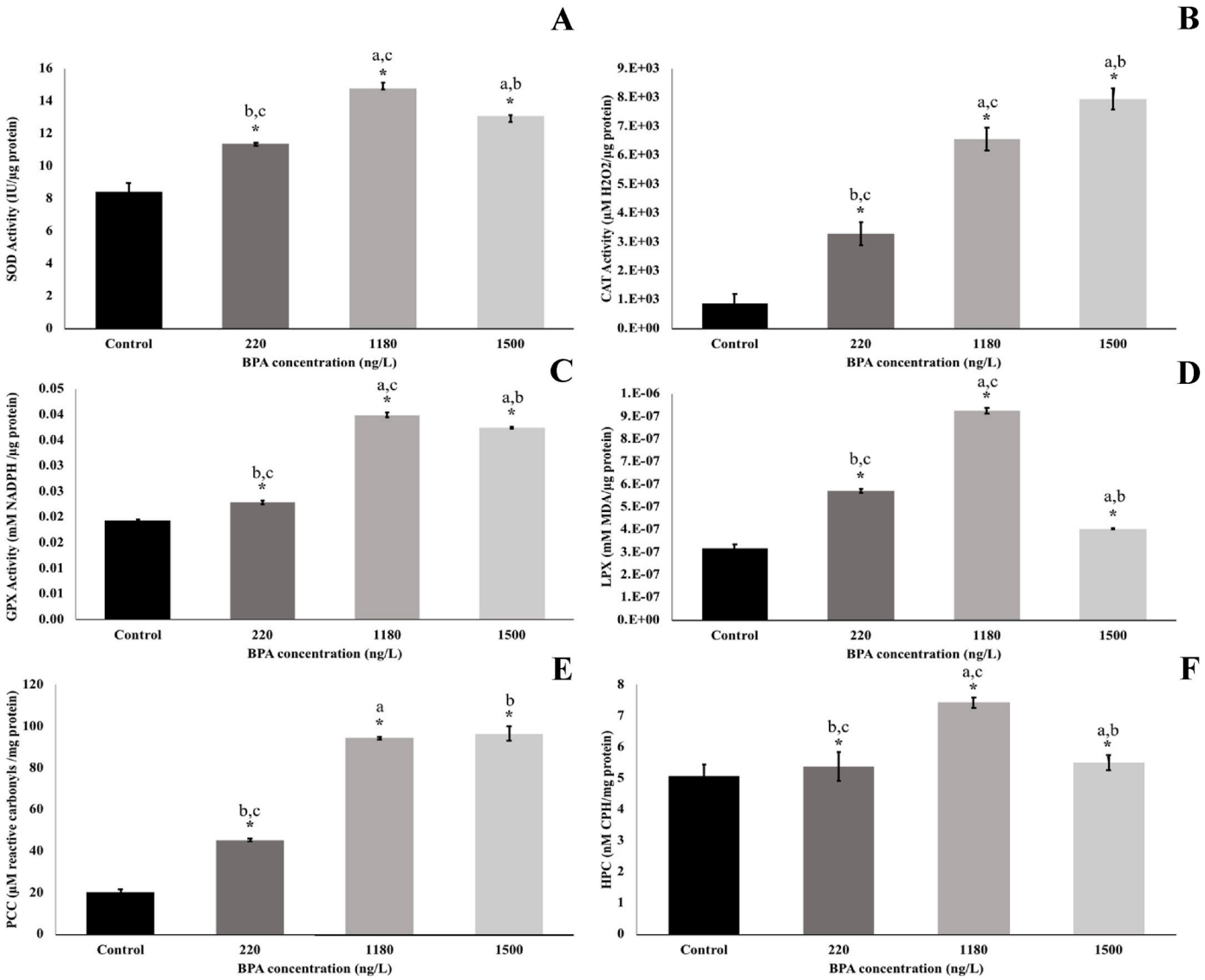

Fig. 2. Oxidative stress (A = SOD, B= CAT, C = GPX, D = LPX, E = PCC, F= HPC) prompted in zebrafish brains exposed to environmental concentrations of BPA (C_1 = 220, C_2 = 1180, C_3 = 1500 ng/L) at 96 h exposure time. Values represented as medians ± interquartile range, n = 9 per concentration. Asterisks denote meaningful dissimilarities compared to the control group ($p < 0.001$). Letters indicate significant differences between treatment groups (a: 220 ng/L BPA, b: 1180 ng/L, c: 1500 ng/L). Student-Newman-Keuls post hoc test.

whereas negative correlations were observed with bioconcentration factor and acetylcholinesterase. Specifically, acetylcholinesterase was positively associated with the bioconcentration factor and specific behavioral parameters, such as total swimming distance and the number of transitions between top and bottom, while negatively associated mainly with the biomarkers of oxidative stress.

4. Discussion

BPA is extensively used to produce plastics, dental adhesives, and thermal printing paper (Chen et al., 2017). However, the widespread use and disposal of BPA-containing products have resulted in the input of this compound into the aquatic environment (Im and Loffler, 2016). The above is noteworthy as the ubiquitous presence of BPA in water bodies may threaten water species integrity (Tang et al., 2020; Sahoo et al., 2020; Gyimah et al., 2021; Heredia-García et al., 2022). Even though there is plenty of information regarding BPA's harmful effects on fish, few studies have used environmentally relevant concentrations. Therefore, the primary objective of this study was to investigate the potential neurotoxicity induced by acute exposure (96 h) to BPA at environmentally relevant concentrations (220, 1180, and 1500 ng/L) in adult *D. rerio* (zebrafish).

From 2019 to date, different authors have reported exposure to BPA alters fish behavior, cognitive function, and equilibrium (Crowley-Perry et al., 2021; Gonzalez et al., 2021; Gu, 2022; Kim, 2020; Li et al., 2022; Naderi et al., 2022; Sahoo et al., 2021). For instance, Sahoo et al. (2020) documented that chronic exposure to a concentration of 17.52 µM of BPA significantly modifies the exploratory behavior of zebrafish by a boost in top-zone dwell and frequency of transitions, accompanied by a decrease in latency. Similarly, Gonzalez et al. (2021) observed anemonefish that chronically ingested BPA through a diet (100 µg/kg) showed a significant reduction in their aggressive behavior compared to the control group. Moreover, Naderi and colleagues (2022) discovered that sub-exposure to low levels of BPA (0.001 and 0.01 µM) during early life increased anxiety-like behavior, whereas fish exposed to higher concentrations of this compound (0.1 µM) exhibited social deficits and impaired object recognition memory. Concordantly to previous data, our outcomes confirmed BPA at environmentally relevant concentrations (220, 1180, and 1500 ng/L) produces anxiety-like behavior in *D. rerio*, characterized by a significant increase in the time fish remained

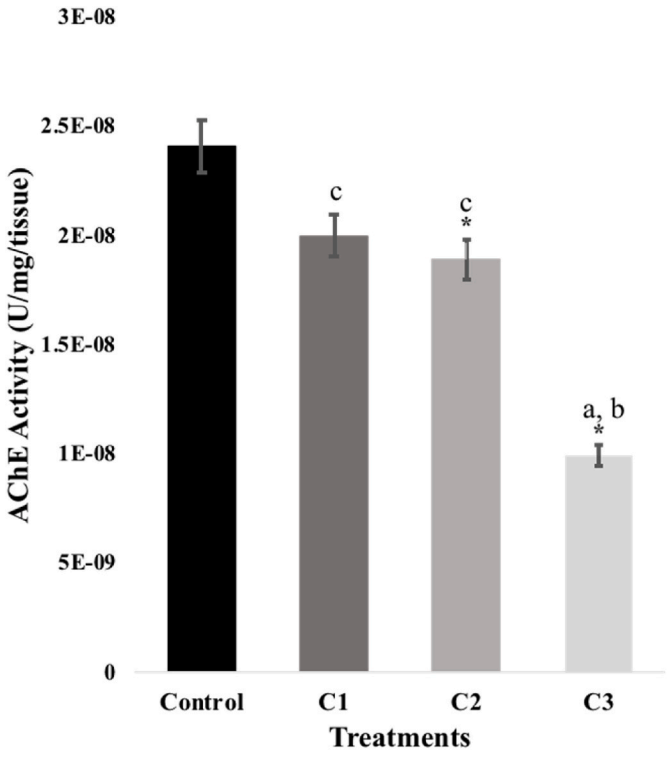

Fig. 3. Acetylcholinesterase activity (AChE) in adult zebrafish brains exposed to environmental concentrations of BPA ($C_1 = 220$, $C_2 = 1180$, $C_3 = 1500$ ng/L) at 96 h exposure time. Values represented as medians ± interquartile range, n = 9 per concentration. Asterisks denote meaningful dissimilarities compared to the control group ($p < 0.05$). Letters indicate significant differences between treatment groups (a: 220 ng/L BPA, b: 1180 ng/L, c: 1500 ng/L). Student-Newman-Keuls post hoc test.

freezing. Moreover, the results presented herein also reveal that acute exposure to BPA realistic concentrations does affect not only the most vulnerable stages of fish, such as embryonic or larvae but also the adult phase.

One likely mechanism through which BPA may impact fish behavior is oxidative stress, given its association with cognitive impairment (Elizalde-Velázquez et al., 2022a, 2022b). Moreover, it is known the brain is highly susceptible to damage caused by reactive oxygen species (ROS) due to elevated levels of polyunsaturated fatty acids, decreased antioxidant activity, and diminished capacity for cellular regeneration (Chandravanshi et al., 2018). In agreement with those above, our findings indicate that realistic concentrations of BPA (220, 1180, and 1500 ng/L) hinder the REDOX status of fish by elevating the antioxidant activity of SOD, CAT, and GPX and raising the levels of LPX, PCC, and HPC. Additionally, previous research also substantiates the notion that BPA incites oxidative stress in the cerebral cortex of fish (Pradhan et al., 2021; Sahoo et al., 2021; Akram et al., 2021; Afzal et al., 2022). For example, Akram et al. (2021) indicate low concentrations of BPA, specifically at levels of 1000 μg/L and 1500 μg/L, yielded a substantial increase in various oxidative stress biomarkers such as thiobarbituric acid reactive substance (TBARS) and a reduction in antioxidant enzyme activity within the brain. In the same way, Sahoo et al. (2021) pointed out that chronic waterborne exposure of zebrafish to BPA significantly decreased the levels of GSH reduced and augmented the ranks of LPX and PCC. Additionally, the findings of Afzal et al. (2022) indicated that exposure to BPA led to a substantial elevation in the levels of reactive oxygen species (ROS) and TBARS, as well as a reduction in the values of antioxidant enzymes SOD, CAT, and peroxidase (POD) in the treated groups (4.5 mg/L and 6.0 mg/L) in the brain of common carp. In contrast to prior investigations, our study employed significantly lower concentrations of BPA, yet none of the levels of antioxidant enzymes fell below those of the control group. However, it is paramount to note that the defense mechanism against oxidation (SOD and GPX) decreased at the highest concentration.

In 2017, Chen and colleagues established a correlation between gene expression levels of *gapdh, mbp, α1-tubulin,* and *manf* and the occurrence of locomotion impairments and neurotoxicity in zebrafish. The *mbp* gene is a nervous system gene used as a biomarker of myelination and expressed in oligodendrocytes (Lee and Fields, 2009). An *mbp*-increased expression indicates a protective effect of microglial cells (Ajmone-Cat et al., 2016) in response to an alteration at nervous system levels. The above suggests that the presence of free radicals will interact with the system's metabolic components, so regulation of *mbp* levels will be necessary. Fig. 4 shows that *mbp* was overexpressed in brain fish after acute exposure to all BPA environmentally relevant concentrations (220, 1180, and 1500 ng/L). Aside from the increase in *mbp* gene expression, the *α1-tubulin* gene, which is exclusively expressed in the nervous system and plays a crucial role in the formation of the microtubule cytoskeleton

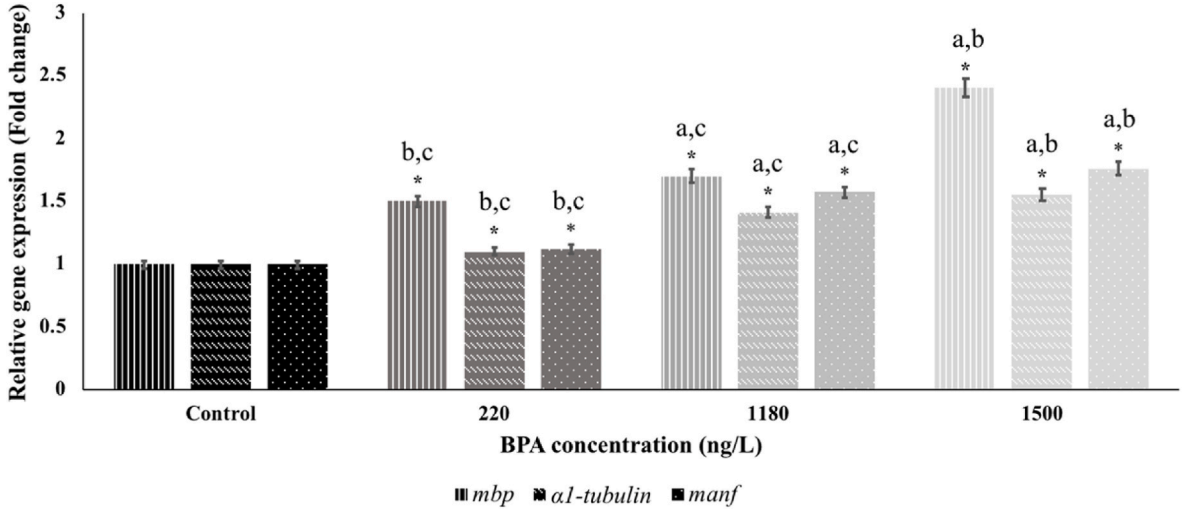

Fig. 4. Acute exposure to BPA ($C_1 = 220$, $C_2 = 1180$, $C_3 = 1500$ ng/L) at 96 h alters different genes related to neurotoxicity (*mbp, α1-tubulin*, and *manf)* in the zebrafish brain. Values were normalized compared to β-actin (used as a housekeeping gene) and represent the mean mRNA expression value ± SEM (n = 9 per concentration) relative to those of controls. Asterisks denote meaningful dissimilarities compared to the control group ($p < 0.001$). Letters indicate significant differences between treatment groups (a: 220 ng/L BPA, b: 1180 ng/L, c: 1500 ng/L). Student-Newman-Keuls post hoc test.

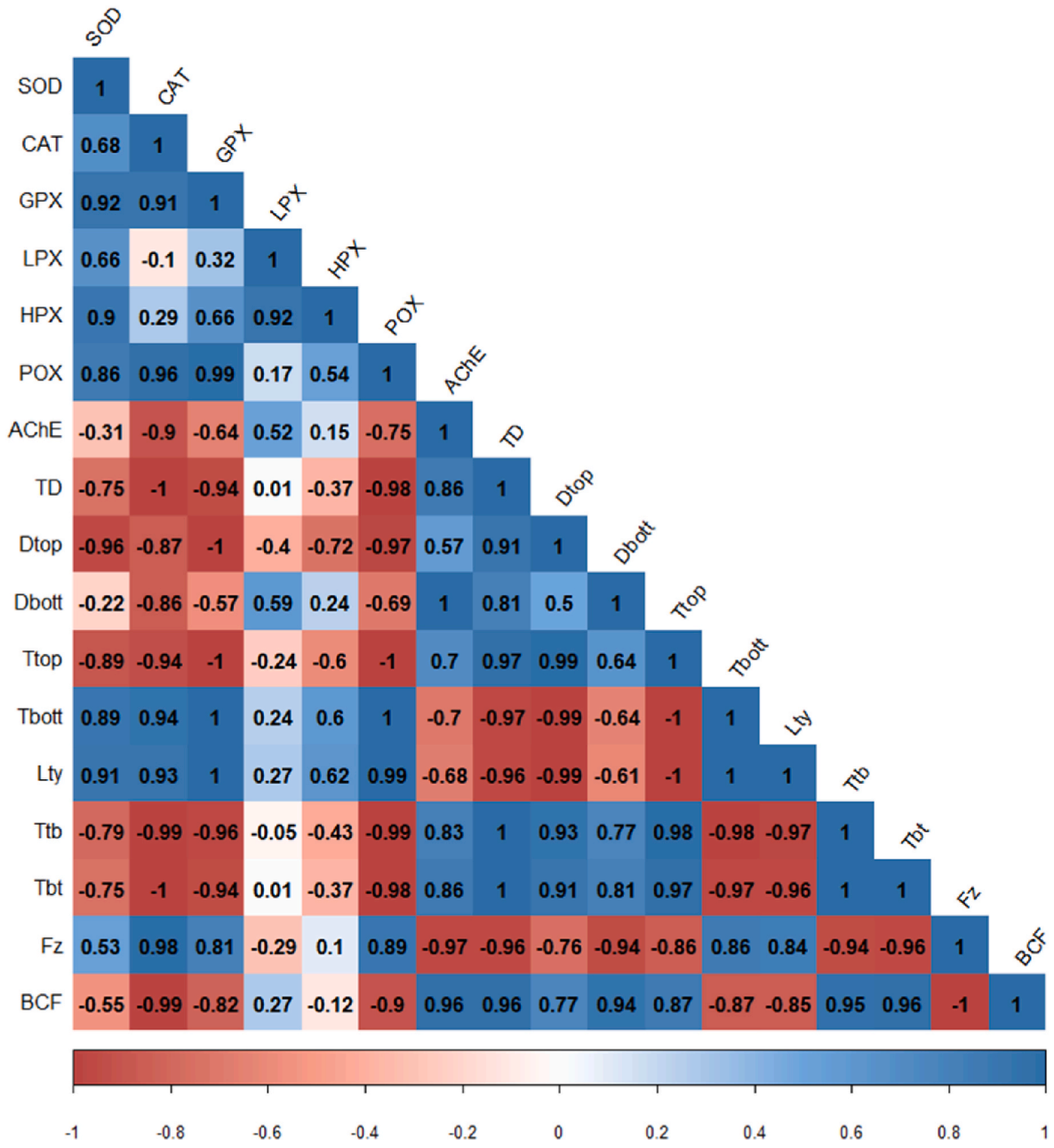

Fig. 5. Pearson correlation of the biomarkers evaluated in adult zebrafish exposed to environmental concentrations of BPA ($C_1 = 220$, $C_2 = 1180$, $C3 = 1500$ ng/L) at 96 h, showing the average value of the three concentrations. SOD (superoxide dismutase), CAT (catalase), GPX(glutathione peroxidase), LPX (lipoperoxidation level), HPX(hydroperoxides content), POX(carbonyl proteins), AChE (Acetylcholinesterase activity), TD (total distance), Dtop (distance in top), Dbott (distance in bottom), Ttop (time spent in top), Tbott (time spent in bottom), Lty (latency to enter the top), frequency of top to bottom transition (Ttb), frequency of bottom to top transition (Tbt), and Fz (freezing).

and regeneration of axons and dendrites (Wang et al., 2015), also exhibited overexpression. Based on the findings mentioned above, it is postulated that cellular regeneration would be imperative in the face of reactive oxygen species-induced damage. Herein, the expression of *manf* was observed to increase in a concentration-dependent manner, albeit its overexpression was comparatively lower than that of *mbp* and *α1-tubulin*. According to Chen et al. (2012), the *manf* gene plays a regulatory role in dopaminergic levels, which serve as biomarkers of neuronal damage due to their influence on cognition and motor control (Iversen and Iversen, 2007). The results indicate that an elevation in *manf* expression may mitigate the motor impairment observed in the behavioral graph (Fig. 1). These findings suggest a protective mechanism of *manf;* nonetheless, its efficacy appears inadequate in reducing immobility at higher concentrations of BPA.

Regarding cholinergic signaling, it can be said that the presence of AChE is fundamental to regulating normal brain function in zebrafish (Hanneman and Westerfield, 1989; Morley, 2005). In addition, AChE levels were also affected by BPA exposure and could be impacted since AChE actively participates in the modulation of motor and cognitive functions (Driscoll et al., 2009). AChE is responsible for hydrolyzing acetylcholine to generate choline and acetate (Behra et al., 2002). Therefore, when there is a decrease in AChE, we may expect a more significant presence of acetylcholine in the neuronal cleft (Tufi et al., 2016), harming the synapse between neurons. In Fig. 3, we observe a substantial decrease in AChE, suggesting an increased presence of acetylcholine. Luo et al. (2013) report that AChE activity is related to

behavioral parameters in mice upon BPA exposure. They observed that prolonged exposure decreased the presence of AChE in the hippocampus, and thus behavior was modified, generating anxiety. In the study by Chen et al. (2017), they also observed a decrease of AChE in zebrafish brains by exposure to BPA (0.78 μg/L), thus increasing acetylcholine levels generating an affectation in signaling, and with it, an abnormal muscle contraction and swimming behavior. They concluded that this compound affected the presence of this enzyme through indirect mechanisms, such as the inhibition of this enzyme. The present results may suggest that the decrease in AChE levels influenced the behavior of zebrafish. Also, our result indicates that BPA affected locomotor activity and altered neurochemical profiles in zebrafish adults.

5. Conclusions

Based on the findings of this study, it can be inferred that exposure of zebrafish to environmentally significant levels of BPA produces neurotoxic effects on the organism by interfering with locomotion, oxidative stress status, and acetylcholinesterase activity. The observed bioconcentration factor indicates the accumulation of BPA in the brain of zebrafish, leading to the production of reactive oxygen species that adversely impact various nervous system components. The identification of significant effects at ecologically relevant BPA concentrations serves as a cautionary note on the harmful impact of this compound on ecosystems. This research is expected to contribute to the public's understanding of the negative implications of plastic use for diverse species.

Credit author statement

Gerardo Heredia-García and Gustavo Axel Elizalde-Velázquez performed all the exposure experiments. Leobardo Manuel Gómez-Oliván and Gerardo Heredia-García were involved in the conception, Leobardo Manuel Gómez-Oliván, Gerardo Heredia-García and Gustavo Axel Elizalde-Velázquez were involved in the design and interpretation of the data and the writing of the manuscript with input from Hariz Islas-Flores, Sandra García-Medina, Marcela Galar-Martínez and Octavio Dublán-García.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.138729.

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