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Long-term effects of embryonic exposure to benzophenone-3 on neurotoxicity and behavior of adult zebrafish

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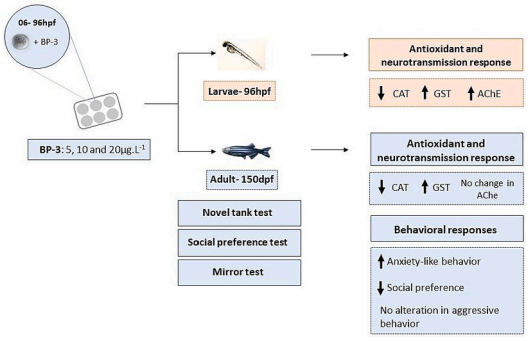
HIGHLIGHTS

- BP-3 exposure at the early life stages of zebrafish led to late behavioral alterations.
- Zebrafish exposed to BP-3 during early life stages presented increased anxious-like behavior in the adult phase.
- Zebrafish exposed to BP-3 presented less social behavior in adulthood.
- Antioxidant enzymatic activities were altered in fish exposed to BP-3 and the alterations remained in the adult phase.

GRAPHICAL ABSTRACT



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ABSTRACT

Benzophenone-3 (BP-3) is the most widely used ultraviolet filter (UV filter) in industries to avoid UV radiation damage. BP-3 is added to most sunscreens to protect the skin, hair, and lips from sun rays. It results in continuous discharge into aquatic environments, leading to aquatic biota and human's continuous exposure. Consequences of BP-3 exposure on the physiology and behavior of aquatic animals, mainly zebrafish, have been investigated, including their neurotoxic effects. However, little is known about its consequences in long-term developmental endpoints. This study aimed to investigate the long-term effects of embryonic BP-3 exposure on biomarkers of neurotoxicity in zebrafish. For this, we exposed embryos to 5, 10, and 20 $\mu g \bullet L^{-1}$ BP-3 concentration and let fish grow to adulthood (5mpf). We evaluated anxiety-like behavior, social preference, aggressiveness, and enzymatic activity of the antioxidant defenses system and neurotoxic biomarkers (Glutathione S-transferase -GST, catalase -CAT, and acetylcholinesterase -AChE) in adult zebrafish. Enzymatic activities were also investigated in larvae immediately after BP-3 exposure. Animals early exposed to BP-3 presented anxiety-like behaviors and decreased social preference, but aggressiveness was not altered. In general, exposure to BP-3 leads to altered enzymatic activity, which persists into adulthood. GST activity increased in embryos and adults, while CAT activity decreased in both life stages. AChE activity enhanced only at the larval stage (96 hpf). The long-term behavioral

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and biochemical effects of BP-3 highlight the need for abolishing or restricting the compound from personal care products, which are continually disposed into the environment and threaten the biota and human health.

1. Introduction

For over 40 years, benzophenone-3 (BP-3, oxybenzone, or 2-hydroxy-4-methoxybenzophenone) has been the most widely used ultraviolet filter (UV filter) in sunscreens due to its capacity to absorb UVA, UVB, and the less frequent, UVC rays (Wnuk et al., 2022). In addition to sunscreens, BP-3 is commonly found in other personal care products (PCPs), such as shampoos, moisturizers, perfumes, and makeup. Its wide use by industries has led to the continuous discharge into aquatic environments, including wastewater effluents, rivers, lakes, and seas worldwide. Therefore, it is considered a pseudo-persistent pollutant (Downs et al., 2016) to which the aquatic biota and humans are continuously exposed (Lee et al., 2021; Yang and Ying, 2013).

BP-3 has a lipophilic characteristic, therefore, it may accumulate in tissue (Wnuk and Kajta, 2021). In humans, the main ways of absorption are via dermal contact, the gastrointestinal tract (ingestion), and inhalation, leading to the systemic circulation of BP-3. The compound metabolites have already been found in human urine, breast milk, and placental tissues (Velanganni and Miltonprabu, 2020; Wang et al., 2021; Zhang et al., 2013). However, the other animals of the global biota are also subjected to BP-3 contamination. Aquatic organisms such as corals, algae, microcrustacean, insects, and fish were already shown to suffer the effects of BP-3 disposed into the environment (Esperanza et al., 2019; He et al., 2019; Lee et al., 2018, 2020; Molins-Delgado et al., 2016; Muñiz-González and Martínez-Guitarte, 2020).

Several studies investigating BP-3's effects on organisms pointed out its endocrine disruption potential, showing estrogenic and anti-androgenic activity. In zebrafish, Kinnberg et al. (2015) showed that BP-3 leads to an altered sex ratio, increasing the number of females in the population, affects gonadal maturation in females and increases vitellogenin induction in males. Additionally, BP-3 exposure resulted in prolonged hatching time and reduced hatching rate in zebrafish embryos, while it affected estradiol biosynthesis and gonadotropin release by altering the expression of genes related to hormonal syntheses and receptor levels (Meng et al., 2020).

Besides its effects as an endocrine disruptor, BP-3 may have other mechanisms of action and toxicity. For example, zebrafish embryos exposed to BP-3 presented reduced number of enteric neurons (Wang et al., 2021), lower levels of thyroid hormones (Lee et al., 2018), decreased axonal growth and cell proliferation, and increased brain cell apoptosis (Tao et al., 2020). BP-3 also increased ROS production and upregulated the expression of genes such as catalase (*cat*) and superoxide dismutase 2 (*sod 2*) (Moreira et al., 2023).

The neurotoxicity induced by chemical pollutants is currently being investigated and zebrafish has become a popular model organism for neurodevelopmental and neurobehavioral toxicology studies. Embryo and adult behavioral changes are well established and easy to identify, while the embryonic development is highly sensitivity to pollutants exposure. Therefore, zebrafish behavioral assays are commonly used to identify the neurotoxic effects of environmental pollutants (Kalueff et al., 2016; Pilehvar et al., 2020). For instance, endocrine disruptors are known to reduce neuronal membrane permeability, and effect the functioning of ion channels and the mitochondria, causing oxidative stress, cell death, and neurotoxicity (Tao et al., 2022). However, the consequence of BP-3 exposure to the brain function and its mechanisms of neurotoxicity are still not fully comprehended, and the long-term effects of BP-3 exposure are not known.

Pre-natal BP-3 exposure were observed in mice, and it increased apoptosis and altered estrogen receptor expression levels in neocortical cells, leading to neurotoxicity in embryos and causing dysregulation of the epigenetic status (Wnuk et al., 2018). In zebrafish, a recent study evaluated the effects of early BP-3 exposure on neurodevelopment and neurobehavior in early life stages (Tao et al., 2020). However, there are no cues of BP-3 long-term effects. Thus, we carried out a primary investigation on the long-term effects of embryonic BP-3 exposure at environmental relevant concentrations, focusing on neurotoxicity biomarkers: oxidative stress, AChE and behaviors in zebrafish. We hypothesized that BP-3 elicits consequences that persist into adulthood, such as impaired anxiety-like and social behaviors, and permanently disturbs the activity of the antioxidant system and AChE activity on the encephalon of adult zebrafish.

2. Materials and methods

2.1. Animals housing and embryo collection

To obtain embryos used in BP-3 exposure and later in behavior assays, adult zebrafish (*Danio rerio*), wild-type (WT), were obtained from a local farm and held at the fish vivarium at Physiology and Behavior Department, Federal University of Rio Grande do Norte (UFRN), Brazil, in a recirculation water system with controlled conditions. The water was filtered by mechanical, biological, and chemical processes, and pH (~6.7), temperature (28 °C), oxygen (~6.7 mg/L), and conductivity (160μS) were constantly checked. The photoperiod was kept at 14 h light/10 h dark, lights on at 06:00 am. All fish care and experimental protocols were approved by the Animal Use Ethics Committee of the UFRN (CEUA, certificate number 211.075/2019).

Adult zebrafish were placed in breeding tanks (2 male, 1 female) containing a removable partition, with holes separating males and females but allowing chemical contact between individuals. The partition was removed in the first hours of light, and spawning occurred naturally. One hour after spawning, embryos were collected from different breeding groups, counted, mixed, and randomly placed in Petri dishes with water from the fish vivarium.

2.2. Embryo exposure to BP-3

Fertilized embryos were observed in a stereo microscope with a camera at 40× magnification (Laborana^{R}), and only embryos with no abnormal morphology (Kimmel et al., 1995) were used for experiments.

For chemical exposure, a stock solution (25 $\mu g.mL^{-1}$) was prepared by dissolving BP-3 (98%purity; Sigma-Aldrich, Brazil, CAS number: 131–57-7, Code: PHR1074) in DMSO (99,9 %, Sigma-Aldrich, Brazil, CAS number, 67–68-5, Code, 472301) and stored in an opaque bottle at −20 °C. Zebrafish embryos were distributed in 12 well plates (2 embryo/well) and exposed to BP-3 solution from 6 to 96 hpf, following Tao et al. (2020). An average of 40 embryos were exposed to each BP-3 concentration, resulting in 5 experimental groups: Water, DMSO (0.08 %), BP-3 5, BP-3 10, and BP-3 20, containing a BP-3 concentration of 5, 10 and 20 $\mu g \bullet L^{-1}$). Tao et al. (2020) found that this concentration range is environmentally relevant and results in mortality rates are similar to control groups. Moreover, the tested concentrations were also based on scientific studies showing UV filters' presence in several different water sources worldwide, in which oxybenzone was the most common filter found and the one present in the highest concentration (Tsui et al., 2014; Gago-Ferrero et al., 2012; Ramos et al., 2016; Balmer et al., 2005; Cuderman and Heath, 2007; Loraine and Pettigrove, 2006; Stackelberg et al., 2004; da Silva et al., 2015; Brausch and Rand, 2011; Langford et al., 2015; Ekpeghere et al., 2016).

The media was renewed every 48 h to maintain BP-3 concentrations in each group during the exposure period, including the control water and solvent groups, so animals were handled equally. After the exposure

period, larvae were used for two goals: 1) 96hpf larvae were immediately stored at −4 °C for further biochemical analysis ($n = 100$, details in Section 2.4); 2) 96 hpf larvae were transferred to 2 L tanks without the pollutant and let grow to 15dpf ($n = 100$) and after that, were transferred to 10 L tanks also without the pollutant (distributed in two replicas of each group), where they remained until 150 dpf to further behavioral and biochemical analysis.

2.3. Behavioral assays

To analyze the long-lasting effects of early BP-3 exposure on 150 dpf zebrafish (adults), anxious-like behavior, sociability, and aggressiveness were evaluated using the following protocols: Novel tank, social preference, and mirror test, respectively (Access supplementary material to the schematic drawings of the experimental tanks). Experimental fish were submitted to all protocols (Control and DMSO groups $n = 11$; BP-3 groups $n = 13$). Fish was first tested to the novel tank and social preference on the same day, and 24 h later, they were submitted to the mirror test. Behaviors were recorded using a Logitech HD Webcam positioned 1 m away and in front of the tanks, and the videos were analyzed using ANY-maze software (Stoelting Co, USA, version 6.33). Room and tank water conditions were kept similar throughout the tests: room temperature: 23 °C, luminosity: 240 lx, sound: 65.4 dBA, vibration: 0.2 m/s^{-2}, tank water temperature: 28 °C, tank water conductivity: 160μS and tank water pH: 6.7.

2.3.1. Novel tank

Zebrafish anxiety responses to novelty exposure are robust and widely explored in pharmacological and toxicological studies. The novel tank protocol is one of the most used for evaluating locomotor activity and anxiety behavior in zebrafish (Aparna and Patri, 2021; Egan et al., 2009). In this experiment, animals were individually transferred to an unknown rectangular tank, filled to 2.8 L water, and immediately recorded for 6 min, following Moreira and Luchiari (2022). All the tank sides were covered with white paper to minimize external effects, excepted for the front wall, allowing animal records. The tank was virtually divided into three equal horizontal areas (bottom, middle and top). A virtual line was established on the bottom of the tank to analyze locomotor activity and anxious-like behavior, accessed by the following parameters: mean speed while moving, total distance traveled, distance from the bottom, time in the bottom area and latency to the first entry in the top area.

2.3.2. Social preference

In natural conditions, zebrafish are social animals, that is, they prefer to live near conspecifics (Fu et al., 2021; Ogi et al., 2021). To test the effects of BP-3 on social preference in adult zebrafish, we used three identical tanks positioned side by side, and filled up to 2.8 L water level. Focal fish were individually placed in the middle tank, while one of the side tanks was empty (only water) and the other contained a group of six conspecifics (1male:1female) of similar sizes, used as social stimulus. The conspecific and the empty tank were randomly positioned on the right and left of the central tank to avoid bias. Whiteboards were placed between the middle and side tanks' walls to prevent visual contact between focal fish and the shoal during acclimation, which lasted 6 min. After that, whiteboards were removed and fish behavior was recorded for 6 min (Moreira and Luchiari, 2022). The middle tank was virtually divided into three equal vertical areas, where the social area was defined as the nearest to the social stimulus. A virtual line was also established on the social area and the following parameters were registered: mean speed while moving, total distance traveled, distance from the social area, and time in the social area.

2.3.3. Mirror test

Naturally, zebrafish tend to respond aggressively when encountering a conspecific and congener individual, showing marked displays like chasing and biting, to establish hierarchical relationship (Audira et al., 2018; Tao et al., 2022). We conducted a mirror test to investigate the effects of early exposure to BP-3 on the aggressive behavior of adult zebrafish. For this, we used a rectangular tank containing 2.8 L water and a mirror positioned by the glass on one side of the tank. The side occupied by the mirror was randomized for each experimental fish. Animals were individually transferred to the tank to acclimate for 3 min. At this moment, a whiteboard was positioned between the tank wall and mirror, to prevent fish from accessing its image. After acclimation, the whiteboard was removed and behavior was recorded for 6 min (Moreira and Luchiari, 2022).

The tank was virtually divided into three equal vertical areas to analyze fish behavior, and the aggressiveness area was considered the nearest to the mirror. Mean speed while moving, total distance traveled, distance from the aggressiveness area and time in aggressiveness area were registered. In addition, the number of aggressive displays, characterized by rapid frontal movements directed to the mirror (face to face) with fins erected, was accounted for by a blind trained observer.

2.4. Enzymatic activity assays

To evaluate the effects of embryonic BP-3 exposure in larva and adult zebrafish the activity of catalase (CAT), glutathione S-transferase (GST), and acetylcholinesterase (AChE) were measured. These enzymes are considered classic biomarkers related to antioxidant response (CAT, GST) and neurotransmission (AChE) (Muniz et al., 2021).

To analyze the toxicity induced by BP-3 on larvae, after the exposure period, 96hpf animals were transferred to microtubes (2 pools of 10 organisms/experimental group) containing a ratio of 100 μL of phosphate-buffered saline (pH 7.2) per larvae, and quickly frozen, then stored at −4 °C for up to 3 days, for further enzymatic activities.

For adults, after behavioral assays, fish were euthanized on ice, and encephalon were collected and transferred to microtubes containing 0.5 mL of phosphate-buffered saline (pH 7.2) and stored at −4 °C for up to 3 days. Adult encephalon (6 encephalon/experimental group) were individually macerated using a pistil and then centrifuged (10,000 ×g for 20 min, 4 °C). The same procedure was performed for larvae pools to obtain the post-mitochondrial supernatant, which was used to determine the content of soluble proteins and the activity of selected enzymes. The protein content was determined according to the Bradford method (1976). The activity of the tested enzymes was calculated based on the total soluble protein content of the sample under analysis. Then, the activity of the antioxidant enzymes glutathione S-transferase (GST) and catalase (CAT) were determined according to Domingues and Gravato (2018), while the activity of acetylcholinesterase (AChE) was measured according to the procedure described by Ellman et al. (1961). Procedures were adapted for 96-well microplates, using 50 μL of homogenate incubated with 250 μL of reaction solution (0.075 M acetylcholine and 0.01 M 5.5′-acid dithiobis- [2-nitrobenzoic] [DTNB]) for 5 min, at 25 °C. The formation of thiocholine, a degradation product of acetylcholine, was determined with a spectrophotometer (Multiskan G0, Thermo Fischer, USA) at 414 nm. All samples processing occurred in a room protected from light, and all analyses were performed in quadruplicates.

2.5. Statistical analysis

Data were analyzed for normality and homoscedasticity using Shapiro-Wilk and Levene tests, respectively. One-way ANOVA and Kruskal-Wallis were performed to compare behavioral parameters and enzymatic activities between groups, followed by their respective post hoc tests (Dunnett and Dunn's test) when necessary. For biochemical analysis each technical quadruplicate was considered a statistical sample. Pearson or Spearman (when nonparametric data) correlation tests were performed to show correlations between enzymes activity and BP-3 concentration.

All statistical analyses were performed with a significance level of $p < 0.05$ and Graphpad-Prism software version 7.0 was used.

3. Results

3.1. Behavioral tests

3.1.1. Novel tank

Regarding locomotor parameters, One-way ANOVA showed no statistical difference in total distance traveled ($F_{(4,55)} = 1.48$; $p = 0.21$) (Fig. 1a) and mean speed while moving ($F_{(4,56)} = 1.71$; $p = 0.15$) between experimental groups and control (Fig. 1b).

Regarding the parameters that indicate anxiety-like behaviors, One-way ANOVA showed statistical significance for distance from the bottom ($F_{(4,53)} = 8.37$; $p < 0.0001$), and the Dunnett test indicated that fish exposed to BP-3 10 showed lower distance from the bottom during the test compared to the control group ($p < 0.0001$) (Fig. 1c). One-way ANOVA also indicated statistical significance in time at bottom of the tank ($F_{(4,53)} = 7.70$; $p < 0.0001$). Dunnet test showed that fish exposed to BP-3 10 spent more time at the bottom compared to the control group ($p < 0.0001$) (Fig. 1d).

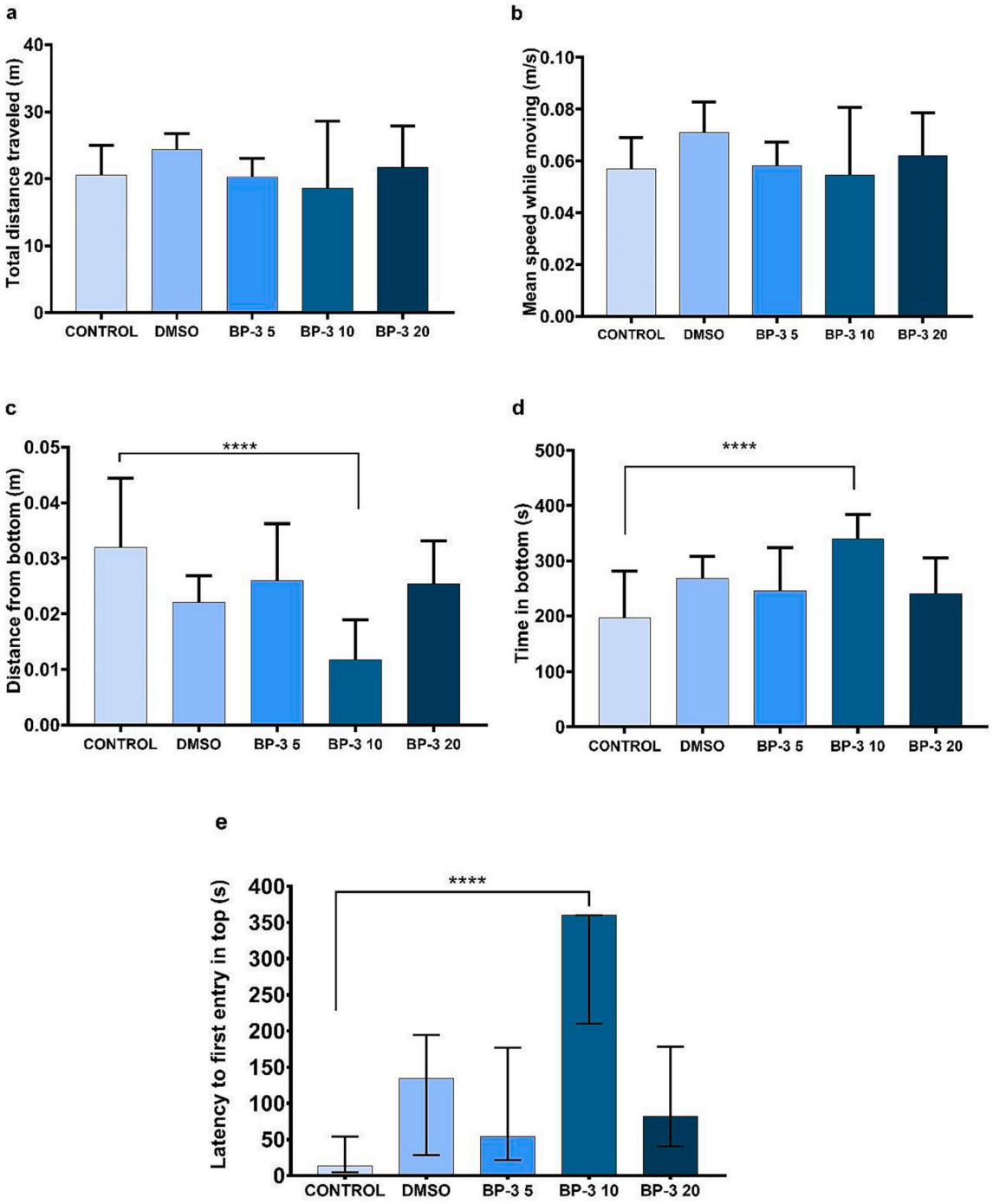

Fig. 1. Zebrafish locomotor activity and anxiety-like behavior in Novel Tank test, during 6 min. Five months age fish from Control (n = 11), DMSO ($n = 11$), BP-3 5 (n = 13), 10 ($n = 13$) and 20 μgL^{-1} (n = 13) were tested for behavioral response after exposure to BP-3 on the embryonic phase (from 6 to 96hpf). a) Total distance traveled (m), b) mean speed while moving (m/s), c) distance from the bottom (m), d) time in the bottom (s), e) latency to the first entry in the top area (s). Data are expressed as mean ± S.D. (graphs a-d), and as median and I.Q.R. (graph e). **** indicate statistical significance at $p \leq 0.0001$(ANOVA for c and d, Kruskal-Wallis for e).

Kruskal-Wallis evaluated latency to the first entry at the top area and showed statistical significance between group (H = 22.52; df = 4; p = 0.0002). Dunn's test indicated that fish exposed to BP-3 10 treatment showed higher latency to entry the top area compared to the control group ($p < 0.0001$) (Fig. 1e).

3.1.2. Social preference

As observed in the novel tank test, One-Way ANOVA also showed no statistical significance for total distance traveled ($F_{(4,54)} = 0.91$; p = 0.46; Fig. 2a) and mean speed while moving ($F_{(4,55)} = 1.10$; $p = 0.36$; Fig. 2b) between groups in the social preference test.

One-Way ANOVA showed statistical significance for distance from the social area ($F_{(4,50)} = 3.29$; $p = 0.01$), and Dunnett test indicated that BP-3 10 group showed a lower distance from the social area than the control group (p = 0.01) (Fig. 2c). Kruskal-Wallis showed statistical significance for time in social area (H = 9.89, df = 4; $p = 0.0422$) and Dunn's showed that animals from BP-3 10 group spent less time in social area than the control (Fig. 2d).

3.1.3. Mirror test

A mirror test was used to assess aggressive behavior in exposed fish. Regarding locomotor parameters, One-way ANOVA showed statistical significance in total distance traveled ($F_{(4,56)} = 5.12$; $p = 0.0014$) (Fig. 3a) and mean speed while moving ($F_{(4,56)} = 4.94$; $p = 0.0017$) between experimental groups and control (Fig. 3b). Dunnett test indicated that fish exposed to BP-3 10 presented lower total distance traveled ($p = 0.0033$) (Fig. 3a) and mean speed while moving ($p = 0.004$) (Fig. 3b) compared to control.

Regarding distance from the aggressive area, time in the aggressive area and number of attacks against the mirror, One-way ANOVA showed no statistical significance between groups ($p = 0.1146$, $p = 0.1959$ and p = 0.8471, respectively) (Fig. 3c, 3d and 3e).

3.2. Enzymes activity

The effects of BP-3 on enzymatic activity in larvae and adults are presented in fig. 4. In larvae collected immediately after BP-3 exposure, One-way ANOVA showed statistical significance for GST ($F = _{(4,35)} = 3.19$; $p = 0.0245$), CAT ($F_{(4,34)} = 7.99$; $p = 0.0001$) and AChE ($F_{(4,35)} = 12.43$; $p \leq 0.0001$) activity. The post hoc Dunnet indicated that GST activity increased in larvae exposed to BP-3 10 ($p = 0.0094$) and BP-3 20 ($p = 0.0252$) compared to control (Fig. 4a). The test also indicated that larvae exposed to BP-3 20 showed decreased CAT activity compared to the control ($p = 0.0001$) (Fig. 4b). In contrast, AChE activity increased in larvae exposed to BP-3 10 ($p < 0.0001$) and BP-20 ($p = 0.0064$) (Fig. 4c).

For GST and CAT activity in adult zebrafish encephalon, Kruskal-Wallis and One-way ANOVA pointed out that GST (H = 27.45; df = 4; $p < 0.0001$) and CAT ($F_{(4,103)} = 25.30$; $p < 0.0001$) activity were statistically significant, respectively. Dunn's test indicated that GST activity increased in animals early exposed to BP-3 10 ($p = 0.0028$) and BP-3 20 ($p = 0.0100$) (Fig. 4d). The Dunnet test showed that CAT also increased activity in BP-3 10 group ($p = 0.0097$), while it was reduced in BP-3 20 compared to the control group ($p < 0.0001$) (Fig. 4e).

Regarding AChE activity, One-way ANOVA showed no statistical significance between exposed groups and control animals ($F_{(4,112)} = 0.6771$; $p = 0.6093$) (Fig. 4f).

Regarding the analysis of the correlation between BP-3 concentrations and enzyme activity carried out on zebrafish larvae, the Pearson test indicated a significant positive correlation between BP-3 concentration and GST activity ($r = 0.427$, $p = 0.015$) and a negative correlation between BP-3 concentration and CAT activity ($r = -0.671$, $p < 0.001$). The Spearman test indicated a significant positive correlation between the BP-3 concentration and ACHE activity (rho = 0.781, p = 0.001). In adults, the Spearman Correlation test indicated a significant positive correlation between the variables BP-3 concentration and GST

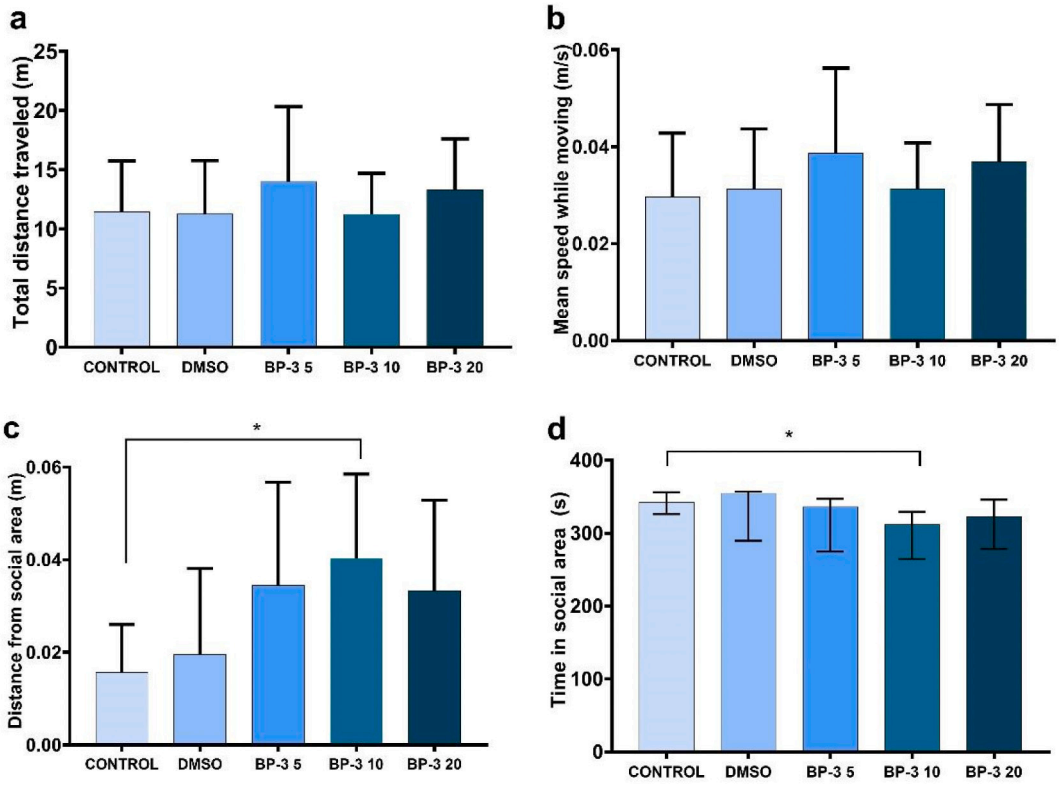

Fig. 2. Locomotor activity and affiliative behavior assessed in the social preference test registered during 6 min. Five months age fish from Control (n = 11), DMSO ($n = 11$), BP-3 5 (n = 13), 10 ($n = 13$) and 20 μgL^{-1} (n = 13) were tested for behavioral response after exposure to BP-3 in the embryonic phase (from 6 to 96hpf). a) Total distance traveled (m), b) Mean speed while moving (m/s), c) Distance from the social area (m) and d) time in the social area (s). Data are expressed as mean ± S.D. (graphs a-c) and as median and I.Q.R. (graph d) *indicates statistical significance at $p \leq 0.05$ (ANOVA for c and Kruskal-Wallis for d)

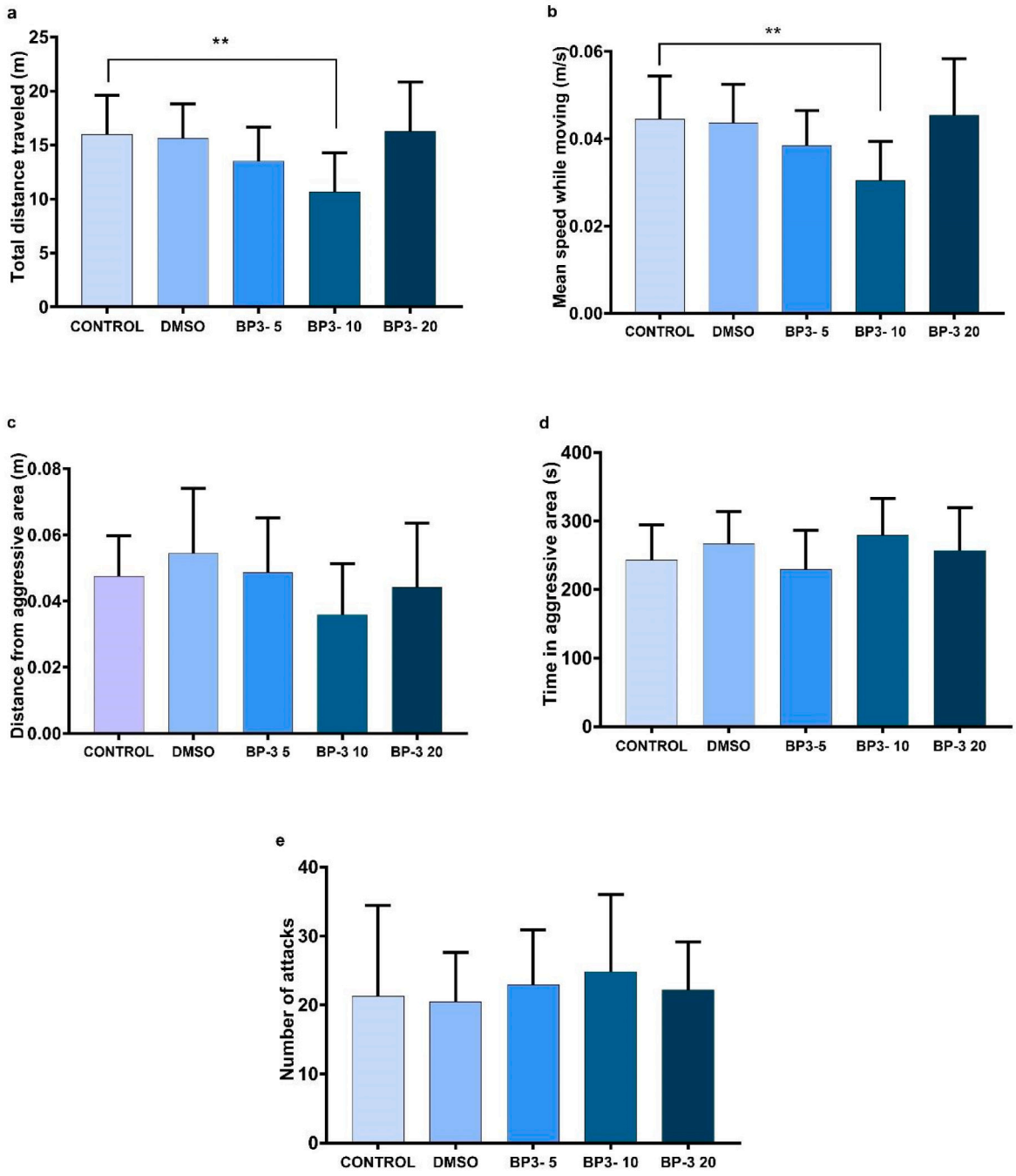

Fig. 3. Locomotor activity and aggressive behavior registered during the mirror test (6 min). Five months age fish from Control (n = 11), DMSO ($n = 11$), BP-3 5 (n = 13), 10 ($n = 13$) and 20 μgL^{-1} (n = 13) were tested for behavioral response after exposure to BP-3 in the embryonic phase (6 to 96hpf). a) Total distance traveled (m), b) mean speed while moving (m/s), c) distance from the aggressive area (m) d) time in the aggressive area (s) and e) number of attacks to the mirror. Data are expressed as the mean ± S.D. and asterisk indicates statistical significance compared to the control (**$p \leq 0.01$).

activity (rho = −0.462, $p < 0.001$) and a significant negative correlation with CAT activity (rho = −0.394, p < 0.001). The Pearson correlation test did not indicate a significant correlation between BP-3 concentration and AChE activity ($r = -0.070$ $p = 0.507$) (figures as supplementary material).

4. Discussion

The present study demonstrated that embryonic exposure to BP-3 induced behavioral impairment and altered enzymatic activity in adult zebrafish. Embryos were exposed to different concentrations of BP-3 from 6 to 96 h post-fertilization, which comprises the critical phase of neurodevelopment (Linney et al., 2004). After 5 months, we evaluated fish anxiety-like behavior, sociability, and aggressiveness. We also evaluated the effects of BP-3 on encephalon enzymatic activity in larvae immediately after BP-3 exposure and in adult fish after 5 months of the exposure. CAT and GST, which participate in the oxidative stress response, and AChE, which controls acetylcholine levels and is a neurotoxic indicator, were analyzed. Overall, acute exposure to BP-3 brought increased anxiety-like and reduced social behavior, elevated GST and reduced CAT activity in adult individuals tested 5 months after exposure.

Studies have explored the ecotoxicological effects of BP-3 on fish, yielding fundamental insights. However, more information still needs to

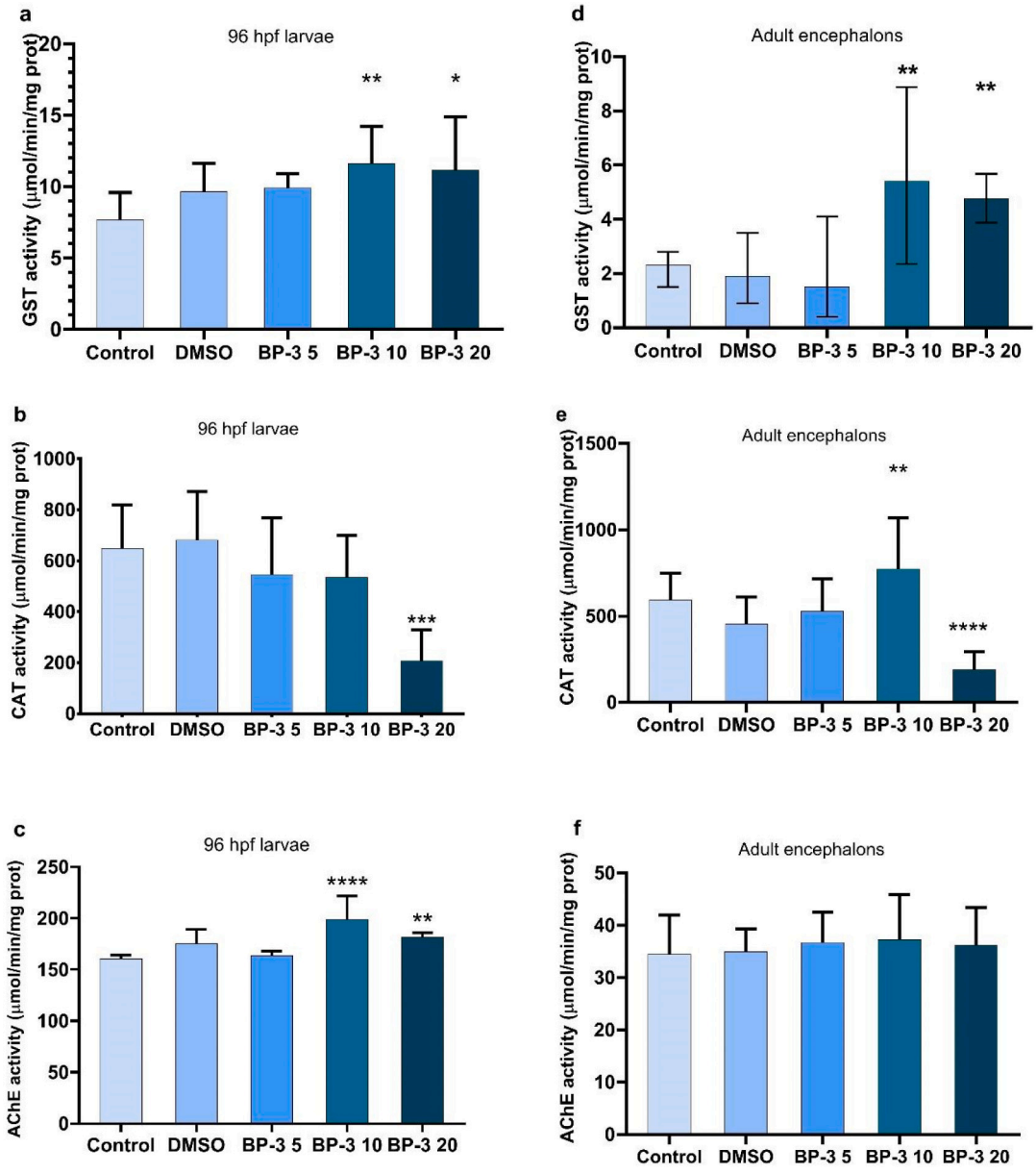

Fig. 4. Enzymatic activities of 96 hpf larvae (a-c) and adult zebrafish encephalon (5 mpf) (d-f) after embryonic exposure (6 to 96hpf) of BP-3. The antioxidant response was evaluated through the enzymatic activity of Glutathione S- transferase (GST) and Catalase (CAT). Neurotransmission was evaluated through Acetylcholinesterase (AChE) activity. Results are expressed as mean ± S.D. (a, b, c, e and f) and as median and I.Q.R (d). Asterisk indicates significance compared to the control (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

be available. In a revision, Ghazipura et al. (2017) analyzed the toxicity effects of BP-3 on fish, and the exposure resulted in reduced egg production, hatching rate, and testosterone levels, along with a down-regulation of steroidogenic genes. Zebrafish larvae in contact with sediment containing BP-3 (10 $\mu g.g^{-1}$) for 96 h presented reduced cardiac frequency and increased standard metabolic rate (Lucas et al., 2021). In another study, Almeida et al. (2021) investigated the effects of acute exposure (96 h) to BP-3 at environmentally relevant concentrations (10 to 1000 $ng \bullet L^{-1}$) on the gills and liver of *Poecilia reticulata*, finding substantial impacts, as circulatory disturbances, immunological depression, and genotoxicity. These studies confirm BP-3 properties, such as lipophilicity, photostability, and bioaccumulation potential, underscoring the adverse effects of BP-3 on fish and calling for further research to comprehend its ecotoxicity and impact on aquatic ecosystems.

Zebrafish behavior and biochemical markers, such as enzymes and neurotransmitters, have been used as endpoints to evaluate the sublethal effects of pollutants (de Farias et al., 2019). Depending on the developmental stage, exposure to pollutants and other chemicals can cause long-term changes in the physiology and behavior of organisms instead of animal death (Aluru, 2017). Tao et al. (2022) found that BP-3 exposure at 10 $\mu g \bullet L^{-1}$ during 6 to 24 hpf caused developmental neurotoxicity and impacted larval behavior, including increased spontaneous movement, decreased startle response, hyperactivity, decreased shoaling behavior, and reduced aggression. These authors showed decreased axonal growth, cell proliferation, and increased cell apoptosis in the larval brain. However, to date, no studies have been performed to assess the long-lasting effects of embryonic exposure to BP-3 in adult zebrafish. To our knowledge, this is the first investigation in this sense.

We observed that embryonic exposure to BP-3 10 $\mu g \bullet L^{-1}$ led to increased anxiety-like behavior in adults, evidenced by the reduced distance from the bottom of the tank, increased time at the bottom, and

high latency to enter the top area of the water column (Fig. 2). In the novel tank test, the novelty of the place imposes stress and anxiety, which are decreased when animals habituate to the tank (Pilehvar et al., 2020). Although animals showed anxious-like behaviors in this study, the locomotor parameters did not change, suggesting long-lasting anxiogenic action of BP-3 on 5mpf zebrafish, without sedation (Egan et al., 2009).

While the novel tank test evaluates the stress response, the social preference test approaches the affiliative response. Preference for social interactions and shoaling to conspecific is well-developed in zebrafish and essential to optimize foraging, avoid predation, and access mates (Landin et al., 2020; Norton et al., 2019). However, embryonic BP-3 exposure disrupted this natural behavior. Our results showed that adult fish presented decreased social preference (Fig. 3), which corroborates the acute effects of BP-3 observed in different life stages in zebrafish (Moreira and Luchiari, 2022; Tao et al., 2020). In a previous study, we observed impaired social behavior in adult zebrafish exposed to water containing BP-3 at 10 $\mu g \bullet L^{-1}$ concentration for 15 days (Moreira and Luchiari, 2022). Similar results were obtained by Tao et al. (2020) in larvae exposed to BP-3 at 11–12 dpf, which showed decreased social cohesion, and Bai et al. (2023) in chronically exposed zebrafish (from 6hpf to 5mpf) that presented a reduction in social preference. Therefore, results from the present study point to a long-lasting effect of BP-3 on the social preference in zebrafish, jeopardizing how individuals interact and benefit from grouping.

Regarding aggressive behavior, our results indicate that embryo exposure to BP-3 does not present aggressiveness loss in adulthood. In a previous study, zebrafish aggressive behavior was reduced by 15 days of exposure to BP-3 (Moreira and Luchiari, 2022), observed by fewer attacks and higher latency to approach the opponent.

The above-described behavioral alterations caused by BP-3 are not unique to this pollutant. Other pharmaceutical and personal care products (PPCPs) found as pollutants in the aquatic environment have been shown to impair behavioral responses in larvae and adult zebrafish. For instance, cetylpyridinium chlorid (CPC), an ingredient of oral products such as toothpaste and mouthwash (Dong et al., 2022), synthetic estrogen (Tamagno et al., 2022), and ammonia (Lin et al., 2022) disrupt social behavior in zebrafish. The disruption in behaviors reflects neurological changes that are not fully understood. In an ecological context, behavioral changes can decrease exploration, feeding, reproduction, and anti-predatory responses, jeopardizing individual or population fitness (Brodin et al., 2014; Goundadkar and Katti, 2017). A possible mechanism may involve the imbalance of the oxidative stress response in the embryo and adult brain.

Zebrafish eleuthero-embryos exposed to concentrations from 1 to 10,000 $mg \bullet L^{-1}$ of BP-3 presented no differences in the transcription levels of CAT and SOD (Rodríguez-Fuentes et al., 2015). Sandoval-Gío et al. (2021) found increased AChE gene expression but decreased AChE activity in embryos exposed to 10 $\mu g \bullet L^{-1}$ BP-3, and no differences in SOD and CAT transcription levels. Thus, it seems that time of exposure and life phase are determining factors for different results. In our study, consistent results were obtained for the levels of antioxidant enzymes in larvae and adults: an increase in GST activity was observed both at 96hpf and 5mpf zebrafish (Fig. 4). CAT activity was decreased in larvae and adults exposed to 20 $\mu g \bullet L^{-1}$ BP-3 (Fig. 4). However, AChE activity increased in larvae exposed to 10 and 20 $\mu g \bullet L^{-1}$ BP-3, but the effects were not observed in adults.

Antioxidant enzyme activity variations are adaptive or defensive responses to ROS generation and the toxicity that results from it (Vieira et al., 2021). In the brain, increased ROS and antioxidant imbalance may cause oxidative damage to biomacromolecules, affecting neural functions and resulting in behavioral disorders (Lin et al., 2023). Moreira et al. (2023) found that zebrafish exposed to water containing BP-3 at 10 and 50 $\mu g \bullet L^{-1}$ of concentrations for 15 days presented increased ROS production and upregulation of catalase (*cat*) and superoxide dismutase 2 (*sod 2*) gene. Furthermore, learning and memory were reduced in animals exposed. These results suggested that BP-3 may lead to a redox status imbalance, causing impaired cognition.

GST is a detoxifying enzyme that activates cellular detoxification by eliminating exogenous compounds such as pharmaceutical products, heavy metals, pesticides, and pollutants (xenobiotics) (Glisic et al., 2015). Although we did not evaluate ROS production in this study, exposure to BP-3 at the embryonic phase caused an increase in GST, possibly as an adaptive response to oxidative stress. However, CAT activity was reduced, impairing antioxidative response, which may result in neurotoxicity and altered behavior. CAT reduction may occur when oxidative stress reaches high levels and substantially increases H_2O_2 production (Lin et al., 2023).

Another critical enzyme associated with the neurotoxic effects of pollutants is AChE (El-Nahhal, 2018; Olivares-Rubio and Espinosa-Aguirre, 2021). Some xenobiotics inhibit its activity, increasing acetylcholine (ACh) availability in the synaptic cleft (Aliakbarzadeh et al., 2022). Thus, the increased activity of AChE observed in larvae exposed to BP-3 suggests a mechanism of overcompensation to eliminate the neurotoxic effects of the excess AChE release after pollutant exposure. According to Yu et al. (2022), cell apoptosis also induces increased expression of AChE. BP-3 exposure and possibly increased brain cell apoptosis may enhance AChE activity. The neurotoxic effects of other pollutants and their effects on AChE activity have been studied. Zebrafish embryos exposed to pollutants such as Fenpropathrin, an insecticide commonly used in agriculture, and famoxadone-cymoxanil, a fungicide, also showed an increased AChE activity and altered behaviors (Cheng et al., 2020; Yu et al., 2022).

However, it is important to highlight that the entire encephalon of zebrafish was used for biochemical analyses in this study and, therefore, the responses of the different regions of the encephalon to the pollutant were not analyzed separately, and it should be considered in future studies. Additionally, studies addressing sex differences deserve attention. In this study, embryos were not separated by sex, and for the sake of comparison, adults were also not divided into males and females. Since sex can be a significant variable that influences the results, future experiments should consider it. The zebrafish provides a powerful model to test the neurotoxicity of environmental contaminants in different brain areas, resulting in detailed insights into the mechanisms of action of chemicals (Fitzgerald et al., 2021). Moreover, other mechanisms related to the effects of BP-3 on behavior must be investigated besides the oxidative stress caused by the pollutant. For example, an in vitro study using mouse neurons identified that BP-3 increased cell apoptosis and inhibited autophagy, two essential processes to guarantee the normal nervous system development (Wnuk et al., 2018). In zebrafish, increased apoptosis and decreased cell proliferation were observed in 24 h embryos exposed to BP-3 and correlated behavior changes (Tao et al., 2020). The endocrine-disrupting capability of BP-3 may be related to behavioral effects through development. Alteration in estrogen receptors and other steroid hormones associated with brain development and functioning should be considered in future studies due to the endocrine disruption attributed to high BP-3 concentrations (above those found in the environment) (Weiss, 2011; Wnuk and Kajta, 2021).

In this study, the altered antioxidant enzyme activities observed at the larval phase are assigned to BP-3 exposure, but epigenetics mechanisms may explain persistence in adulthood. It has been increasingly investigated, and there is evidence that exposure to environmental stressors during a sensitive stage of development may change the epigenetic status responsible for neurological disorders and physiological dysfunctions (Aluru, 2017). Epigenetics mechanisms associated with BP-3 early exposure must be investigated, including transgenerational studies.

The use of zebrafish as a model for neurobehavioral alterations caused by BP-3 exposure is of great importance for ecological evaluations, considering the effects on the survival and reproductive success of the species. However, the studies must be extrapolated to the translational context, considering the effects of embryological exposure to

BP-3. This compound is known to overcome blood-brain barriers and represents a risk to the pre-natal period (Kim and Choi, 2014; Tierney, 2011).

5. Conclusions

Embryonic exposure to BP-3 leads to abnormal behaviors in adulthood, including high anxiety and reduced sociality. Soon after exposure to BP-3, zebrafish larvae presented altered enzymes of the antioxidant defenses system and neurotoxic biomarkers (GST, CAT, and AChE), partially sustained after 5 months of the exposure, indicating persistent and long-lasting effects of the pollutant. The long-lasting behavioral and biochemical effects of BP-3 exposure highlight the urgent need to rethink its use in PCP, while finding ways to abolish its discharge into the environment.

CRediT authorship contribution statement

Ana Luisa Pires Moreira: Conceptualization, methodology, formal analysis, original draft, writing review and editing; Juliana Alves Costa Ribeiro Souza: formal analysis, data interpretation, and writing; Jéssica Ferreira de Souza: methodology and formal analysis; João Paulo Medeiros Mamede: methodology and formal analysis; Davi Farias: data interpretation and writing review; Ana Carolina Luchiari: Conceptualization, funding acquisition, supervision, data interpretation, writing review, and editing.

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.scitotenv.2023.168403.

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