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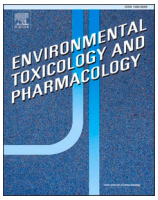

Multi-biomarker approach to assess the toxicity of carbamazepine, a neuropharmaceutical, in the female fish *Astyanax lacustris* (Teleostei: Characidae)

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ABSTRACT

Carbamazepine (CBZ) is a pharmaceutical commonly used in the treatment of epilepsy and bipolar disorder and has been detected in different aquatic ecosystems worldwide. Considering its possible role in altering nervous system and reproduction, this study aimed to evaluate the effects of CBZ on molecular and cellular biomarkers of the teleost *Astyanax lacustris*. Results demonstrated that CBZ, in environmentally relevant concentrations (500 ng L^{-1}) increases *fshβ* gene expression levels, decreases muscle protein content and hepatic LPO (500 ng L^{-1} and 1250 ng L^{-1} of CBZ). Nonetheless, no effects were observed towards enzymatic activities, steroid plasma levels and/or lipid content. Considering that *A. lacustris* inhabits clean and polluted environments, it is possible to suggest that animals possess a level of tolerance to stressors, allowing them to maintain reproductive functions regardless of environmental challenges.

1. Introduction

Contaminants of emerging concern (CECs), such as pesticides, polycyclic aromatic hydrocarbons, pharmaceuticals and personal care products (PPCPs), are threats to aquatic ecosystems. Due to their ubiquitous presence and persistence, concentrations ranging from low ng L^{-1} to high μg L^{-1} have been reported worldwide and in different biological matrices (Chaves et al., 2021; Edwards et al., 2017). According to Batucan et al. (2022), those compounds are termed micropollutants and their environmental and biological impacts are still largely unknown.

Amongst the most detected CECs in freshwater ecosystems, PPCPs can be highlighted. They are biologically active compounds whose properties and pharmacological activities are designed to target specific physiological pathways in humans and in animals (Boxall et al., 2012). Despite their importance for human and wildlife welfare, whenever those compounds reach ecosystems, they are able to interact and induce toxicity to several organisms (Boxall et al., 2012; Ebele et al., 2017). Many pharmaceuticals can also act as endocrine disruptive compounds (EDCs) and their effects might be to mimic, to antagonize or to alter endogenous steroid hormones, impacting directly in reproductive pathways (Carnevali et al., 2018). Exposure to diclofenac reduced the levels of 17β-estradiol and testosterone hormones in the male fish *Astyanax lacustris in vivo* (Godoi et al., 2020) and ibuprofen reduced the levels of those hormones in an *ex vivo* approach in this same species (Branco et al., 2021). However, no effects in plasma level of steroids were observed in female *A. lacustris* after exposure to both drugs (Godoi et al., 2024). Those compounds are analgesic and anti-inflammatory, and their main mechanisms of action are related to the inhibition of two cyclooxygenases enzymes (namely, COX1 and COX2) involved in the synthesis of prostaglandins (Batucan et al., 2022; Bushra and Aslam, 2010).

Even though diclofenac and ibuprofen are relevant, many other pharmaceuticals are present in aquatic ecosystems and can be even more toxic and dangerous for the aquatic biota (Freitas et al., 2015; Gutiérrez-Noya et al., 2020; Xia et al., 2017). For instance, carbamazepine (CBZ) which is an anticonvulsant drug widely used for the treatment of epilepsy and bipolar disorders (Chen and Lin, 2012), has exhibited toxic effects on bivalves (Aguirre-Martínez et al., 2018; Mezzelani et al.,

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2020) and various fish species (Calcagno et al., 2016; Galus et al., 2014; González-Mira et al., 2018). With its increasing consumption, CBZ has been detected in freshwater ecosystems at varying concentrations such as 21 μg L^{-1} in Japan (Sim et al., 2011), 0.652 μg L^{-1} in Brazil (Thomas et al., 2014), and 1.07 μg L^{-1} in Germany (Brezina et al., 2017). Although environmental levels are apparently low in Brazilian regions, the persistent release of CBZ and its potential biotransformation within aquatic ecosystems raise significant concern.

While CBZ effects on bivalves and other invertebrate species have been reported, there is still a lack of studies regarding CBZ's toxicity to teleost species, mainly tropical fish, and even less information on females. Exposure of zebrafish (*Danio rerio)* embryos, *in vivo*, to environmentally relevant CBZ concentrations (1, 2 and 5 μg L^{-1}) has been shown to induce alteration in behavior and growth (Qiang et al., 2016). The *in ovo* nanoinjection of CBZ (12 ng per egg) in *Oryzias latipes* can impair embryonic development, notably affecting heart beat rate, shrinkage of the yolk sack, eye development and induction of hemorrhage (Nassef et al., 2010a). In adult fish, differently, authors demonstrated that CBZ might induce liver histopathological changes (Yan et al., 2018), alteration of the fatty acid metabolism (González-Mira et al., 2018) and impairment of the reproduction process, observable through hormonal alteration and offspring production (Fraz et al., 2018; Galus et al., 2014). However, research on reproduction has been predominantly focused on male teleost species, revealing oxidative stress in spermatozoa of *Cyprinus carpio* exposed to CBZ (Li et al., 2010a), as well as reduction of body and plasma 11-ketotestosterone (11-KT) levels in *D. rerio* following CBZ exposure (Fraz et al., 2018).

To investigate the toxic effects of CBZ on a female tropical fish, a biomarker evaluation approach can be employed. Biomarkers are considered potential tools in ecotoxicology and they allow the understanding about the adverse biological responses that might be elicited when the organisms are exposed to certain chemicals (Depledge and Fossi, 1994; van der Oost et al., 2003). Chen et al. (2014), for example, demonstrated that some biochemical biomarkers, such as the enzymes superoxide dismutase (SOD), glutathione *S*-transferases (GST) and glutathione reductase (GR), as well as heat-shock proteins (HSPs) were altered in clams *Corbicula fluminea* due to CBZ exposure, confirming that biomarkers are valuable tools in ecotoxicological studies. Similarly, Li et al. (2009) demonstrated that, in rainbow trout *Oncorhynchus mykiss*, CBZ exposure for 42 days impacted directly the activities of SOD, catalase (CAT) and GST which are considered important biomarkers related to pollution, and disrupted the Na^+K^+ATPase pump in the white muscle of the fish.

Based on the above, the main objective of this study was to evaluate the effects of the neuropharmaceutical CBZ, a Contaminant of Emerging Concern (CEC), on biomarkers of toxicity in *A. lacustris* females. *A. lacustris*, previously classified as *Astyanax altiparanae* is an omnivorous teleost species that shows a great phenotypic plasticity (Siqueira-Silva et al., 2015), being a potential candidate as a bioindicator in ecotoxicological studies in the tropics (Abdalla et al., 2019; Assis et al., 2021; Branco et al., 2021; Godoi et al., 2024, 2020; Muñoz-Peñuela et al., 2024, 2021; Pinheiro et al., 2021, 2020).

2. Material and methods

2.1. Animals

Female fish *A. lacustris* were purchased from an aquaculture farm located in Paraibuna, SP, Brazil. Animals were transported to the animal facility of the Institute of Biosciences at the University of São Paulo, USP. Once in the laboratory, animals (N = 175) were maintained for 4 days in black boxes containing 300 L of aerated freshwater at the temperature of 25 ± 2 ºC. Photoperiod (14 L: 10 D) and room temperature (25 ± 2 ºC ºC) were constantly maintained and feeding was also provided with commercial extruded fish feed (32 % crude protein), *ad libitum*, every day. Water was renewed (70 %) every 48 h.

2.2. Chemical

Carbamazepine was purchased from Sigma-Aldrich (Saint Louis, MO, USA) with a purity of ≥ 98 % and was dissolved prior to use in 0.001 % m/v of DMSO (dimethyl sulfoxide, Synth, São Paulo, SP, BR) due to its low solubility in water (18 mg L^{-1}). The internal standard carbamazepine-d_{10} was obtained from Cerilliant (Texas, USA). Formic acid and ammonium formate (purity N98 %) were obtained from Merck KGaA (Darmstadt, Germany). Methanol and acetonitrile (both HPLC grade) were purchased from Tedia (Fairfiled, OH, USA).

2.3. Experimental design

After 4 days, female fish were separated into five groups (6 animals per aquarium), as follows: 1. Control; 2. Dimethyl sulfoxide (DMSO); 3. 250 ng L^{-1} of CBZ, 4. 500 ng L^{-1} of CBZ and 5. 1250 ng L^{-1} of CBZ. The complete experiment was conducted in triplicates (three aquaria per group, totalizing 15 aquaria). Each aquarium, which was covered with brown ethylene-vinyl acetate (E.V.A.), contained 80 L of constantly aerated freshwater at the temperature of 25 ± 2 ºC. Room temperature and photoperiod were maintained as previously described. Water quality parameters were monitored throughout the experimental period and did not differ among aquaria and/or experimental groups (7.38 mean pH and 4.64 mg L^{-1} mean dissolved oxygen). Water (70 %) was renewed in every 48 h until the end of the exposure period to maintain the water quality and the CBZ concentration. Different concentrations of CBZ were chosen based on environmental levels reported for this pharmaceutical worldwide, including the levels observed for Guarapiranga reservoir (250 ng L^{-1}), located in São Paulo, Brazil (Shihomatsu et al., 2017) and the concentration observed for other Brazilian regions (Thomas et al., 2014) (500 ng L^{-1}) and one higher concentration (1250 ng L^{-1}) simulating future scenarios. Thus, nominal concentrations were 250 ng L^{-1}, 500 ng L^{-1} and 1250 ng L^{-1} of CBZ. Animal experimentation was previously approved by the Ethics Committee on Animal Use (CEUA) of the Institute of Biosciences of the University of São Paulo - USP, process nº 392/2021.

After 7 days of exposure, animals were anesthetized using eugenol (100 mg L^{-1}) and morphometrical measurements (total length and body mass) were annotated. About 1 mL of blood was collected from the caudal vein of each animal and the samples were centrifuged (655.2 g, 5 min at 4 ºC) to separate the plasma. The plasma samples were immediately preserved and stored at −70 ºC until further analysis. Subsequently, the anesthetized females were euthanized following the CONCEA (National Council for the Control of Animal Experimentation) guidelines and the tissues (brain, pituitary gland, gills, liver, ovaries and epaxial muscle) were dissected. The liver and ovary samples were weighed to calculate the gonadosomatic (GSI) and hepatosomatic (HSI) indexes, and subsequently all tissues were stored at −70 ºC.

2.4. Water analysis

Throughout the 7-day exposure period, approximately 5 mL of water was collected from each aquarium at different time points to measure the concentration of CBZ. Sampling occurred at specific time points: 0 h (immediately after the addition of CBZ), 48 h (before and after the water renewal), 96 h (before and after the water renewal), and 144 h of CBZ exposure. Afterwards, the water samples were filtered (0.22 μm) and stored at 4 ºC. Quantification of CBZ was performed using a validated on-line solid-phase extraction-ultra high-performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS) method as described previously (Pivetta et al., 2020). Carbamazepine-d_{10} at a concentration of 1000 ng L^{-1} was used as internal standard. The linear range was from 100 to 1500 ng L^{-1} and the precision less than 10 %. The limit quantification (LOQ) of the method was 100 ng L^{-1} of CBZ in the water. The results of the water analysis are presented on Table 1.

Table 1
Quantification of CBZ in water sampled of the aquaria at different time points: 0 h, 48 h (before and after the water renewal), 96 h (before and after the water renewal) and 144 h of exposure.

Exposure Period	Samples	Mean concentration of CBZ ± SD, (ng L^{-1})
0 h	Control (water)	< LOQ
	Control (DMSO)	< LOQ
	CBZ 250 ng L^{-1}	251 ± 7.0
	CBZ 500 ng L^{-1}	509.3 ± 11.3
	CBZ 1250 ng L^{-1}	1151.7 ± 77.5
48 h (before the water renewal)	Control (water)	<LOQ
	Control (DMSO)	<LOQ
	CBZ 250 ng L^{-1}	243.7 ± 5.5
	CBZ 500 ng L^{-1}	504.3 ± 7.8
	CBZ 1250 ng L^{-1}	1219.7 ± 34.8
48 h (after the water renewal)	Control (water)	<LOQ
	Control (DMSO)	<LOQ
	CBZ 250 ng L^{-1}	166.3 ± 48.4
	CBZ 500 ng L^{-1}	564.7 ± 67.6
	CBZ 1250 ng L^{-1}	1233.7 ± 14.7
96 h (before the water renewal)	Control (water)	<LOQ
	Control (DMSO)	<LOQ
	CBZ 250 ng L^{-1}	165.3 ± 53.2
	CBZ 500 ng L^{-1}	504.3 ± 7.8
	CBZ 1250 ng L^{-1}	1219.7 ± 34.8
96 h (after the water renewal)	Control (water)	<LOQ
	Control (DMSO)	<LOQ
	CBZ 250 ng L^{-1}	196.7 ± 19.4
	CBZ 500 ng L^{-1}	468.7 ± 19.8
	CBZ 1250 ng L^{-1}	1150.0 ± 53.5
144 h	Control (water)	<LOQ
	Control (DMSO)	<LOQ
	CBZ 250 ng L^{-1}	214.7 ± 23.8
	CBZ 500 ng L^{-1}	477.0 ± 18.0
	CBZ 1250 ng L^{-1}	1235.7 ± 34.8.

aLOQ: limit of quantification, 100 ng L^{-1}. SD: standard deviation.

2.5. Morphometrical analysis

At the end of the exposure period, each fish was weighed and measured. Therefore, total body mass and length were recorded. After anesthesia and euthanasia, animals were dissected, and the liver and the ovaries were quickly weighed to obtain the tissue mass. For the gonadosomatic (GSI) and hepatosomatic (HSI) indexes, the following equations were used: GSI = [ovaries mass/total body mass] x 100; and HSI = [liver mass/total body mass] x 100. The indexes were evaluated according to Vazzoler (1996).

2.6. Acetylcholinesterase activity (AchE)

Acetylcholinesterase activity was measured in both the brain and muscle tissues of fish to evaluate the potential neurotoxic effects of CBZ. Tissues samples were homogenized in 0.1 M potassium phosphate buffer (pH 7.5, 1:10 w/v) and, subsequently, centrifuged at 17.949 g, during 20 min at 4 ºC. Following centrifugation, the pellets were discarded, and the resulting supernatant was used for the evaluation of acetylcholinesterase activity, using the protocol stablished by Ellman et al. (1961) and modified by Alves-Costa et al. (2007). The assay was conducted in 96-well microplates and absorbance measurements were performed using a spectrophotometer at 415 nm (Spectramax 250 spectrophotometer, Molecular Devices). Results were expressed as nM·min^{-1}·mg of $protein^{-1}$.

2.7. Real-Time: q-PCR

Total RNA of the pituitary samples was isolated using the PureLink™ RNA Mini kit (Invitrogen, Thermo Fisher Scientific), following manufacturer's instructions. From there on, the samples were treated with Turbo™ DNAse (Invitrogen, Thermo Fisher Scientific) to clear RNA samples from contamination with DNA. RNA concentration (A260 nm) and purity (A260/280 nm and A230/260 nm) was assessed using a spectrophotometer (NanoDrop™ One Microvolume UV-Vis Spectrophotometer, Thermo Fisher Scientific). Only samples meeting quality criteria (1.6 ≤ 260 nm/280 nm ≥ 2.0) were included in subsequent analysis. cDNA synthesis was performed using SuperScript™ II Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific) and with 1 µg of RNA per sample as template. Pituitary cDNA samples were diluted (1:10 v/v) and were used to evaluate the expression of the housekeeping and target genes by RT-PCR which was performed using 12.5 µL of Power SyBR Green PCR Master Mix (Applied Biosystems), 0.5 µM primer and 2 µL of cDNA. The cycle conditions were as follows: 2 min at 50 °C, 10 min at 95 °C followed by forty cycles of 15 sec at 95 °C and 40 sec at 60 °C and were performed in a Step One Real Time-PCR System (Applied Biosystems). Primer for ribosomal protein L7 (*rpl7)* were designed based on the transcriptome of *A. lacustris* (Lima et al., 2024), and elongation factor 1 (*ef1-α),* luteinizing hormone *(lhβ)* and follicle stimulating hormone (*fshβ)* were previously standardized by de Jesus et al. (2017). Primer sequences are listed in Table 2. PCR efficiency was determined for each primer by performing a standard curve from serial dilutions of pooled cDNA. Gene expression was calculated according to Livak and Schmittgen (2001), through the analysis of the $2^{-\Delta\Delta Ct}$.

2.8. Energetic substrates

Total lipid content was evaluated in the liver, muscle and ovaries of the female fish. Briefly, lipids were extracted with chloroform, methanol and water (2:1:0.5 v/v/v), according to Folch et al. (1957) and modified by Parrish (1999) for aquatic organisms. Quantification was performed by an enzyme-colorimetric method proposed by Frings et al. (1972). Cod liver oil (Sigma-Aldrich, Saint Louis, MO, USA) was used for the calibration curve and samples were measured using a spectrophotometer (Spectramax 250 spectrophotometer, Molecular Devices) at 540 nm. Data were expressed as mg·g^{-1}.

Total protein of samples (brain, gills, liver, muscle and ovaries) was extracted with perchloric acid (6 % for precipitation) and solubilized with potassium hydroxide (2.5 %), following the protocol described by Milligan and Girard (1993). Protein concentration was evaluated through a colorimetric assay, using a spectrophotometer (Spectramax 250 spectrophotometer, Molecular Devices) at 660 nm, as described by Lowry et al. (1951). A calibration curve was obtained using bovine serum albumin (Sigma-Aldrich, Saint Louis, MO, USA) and results were expressed in mg·g^{-1}.

Table 2
List of the primers used for qPCR.

PRIMER	EFFICIENCY (%)	SEQUENCE
rpl7	93.1	F: 5' – GGCCCAGCTGGTTGTGATCGCA – 3' R: 5' – GCCTCCACCAGTTTGGCAAGAGC – 3'
ef1-α	98.2	F: 5' CACTGGTACCTCACAGGCTGACT – 3' R: 5' – CCAGCCTCAAACTCACCAACA – 3'
lhβ	98.7	F: 5' – TGCCCAAAATGCCTAGTGTTTC – 3' R: 5' – TCTTGTACACCGGATCCTTGGT – 3'
fshβ	99.3	F: 5' – GTCCTGATGATTCTGCTGCT – 3' R: 5' – GCATTCCTCGCTCTCCAC – 3'

2.9. Steroid analysis

The steroids 17β-estradiol (E_2) and testosterone (T) were evaluated in the fish plasma samples using ELISA (enzyme-linked immunosorbent assay) commercial kits (Cayman Chemical®). Previous to the analysis, samples were extracted with ethyl ether, as described by Assis et al. (2017) and diluted (4x for the testosterone assay and 3x or 8x for the 17β-estradiol assay). Following, samples were placed, in duplicates, in 96-well microplates proper for ELISA analysis and absorbance was recorded at 405 nm (for T) and 414 nm (for E_2) in a Spectramax 250 spectrophotometer (Molecular Devices). Concentration of steroid in plasma samples were calculated according to a calibration curve and are represented as pg mL^{-1}. The kits and protocol procedure were previously validated for *A. lacustris* by Kida et al. (2016) and Brambila-Souza et al. (2019). Intra- and inter- assay coefficient (CV) were below 20 % as recommended by Sink et al. (2008).

2.10. Antioxidant defense system

To evaluate the antioxidant defense system of *A. lacustris*, the activity of the enzymes SOD, CAT and GST were performed. Samples of gills, liver, muscle, and ovary were homogenized (1:10 for liver, muscle and ovary and 1:5 w/v for gills) in cold phosphate buffer (0.1 M, pH 7) and centrifuged (15.000 *g*, 20 min, 4 ºC). The resulting supernatant was collected and used for the enzymatic assay. For SOD activity, the protocol of McCord and Fridovich (1969) was used. In this assay, the reduction of cytochrome C through the inhibition of the xanthine/xanthine oxidase system was accessed using a spectrophotometer (Spectramax 250 spectrophotometer, Molecular Devices) with a wavelength of 550 nm. CAT activity was evaluated through the decomposition of hydrogen peroxide (H_2O_2) per minute at a wavelength of 240 nm (Spectramax 250 spectrophotometer, Molecular Devices), according to Beutler's method (Beutler, 1975). GST activity was measured by using the substrate 1-chloro-2,4-dinitro- benzene (CDNB, Sigma-Aldrich, Saint Louis, MO, USA) and reduced glutathione (5 mM), according to the protocol of Habig et al. (1974) and using a spectrophotometer (Spectramax 250 spectrophotometer, Molecular Devices) at 340 nm. Results were relativized by mg of protein.

2.11. Lipid peroxidation (LPO)

To observe whether CBZ induces lipid peroxidation, fish tissues (gills, liver and muscle) were analyzed using the TBARS Assay Kit (Thiobarbituric Acid Reactive Substances, TBARS, Cayman Chemical®). Briefly, this approach evaluates the formation of malondialdehyde (MDA) and thiobarbituric acid (TBA) adducts under high temperature (100 ºC), acid conditions and spectrophotometrically at 530–540 nm. Results were relativized by mg of protein.

2.12. Statistical analysis

Data are represented as mean ± standard error. All statistical analyses were performed with RStudio (R4.2.1) software for windows. Prior to analysis, assumptions regarding normality and homogeneity were assessed. Subsequently, a one-way analysis of variance (ANOVA) was employed. Following, a Tukey HSD post-hoc was conducted. In cases where the data did not meet parametric assumptions, a Kruskal-Wallis test followed by Dunn's test was applied. Statistical difference was used considering a significance level of 5 % ($p \leq 0.05$). For controls comparison, a *t*-test was employed with the same statistical difference being considered ($p \leq 0.05$).

3. Results

To assess the effects of CBZ, a serial biomarker approach was conducted. Initially, comparisons were made between the Control (water) and DMSO (vehicle) groups to ascertain if DMSO exerted any toxic effects. The data comparing the Control (water) and DMSO (vehicle) are presented in Supplementary Material 1. Overall, no statistical differences were observed between both groups (water *vs* DMSO). Consequently, data from the Control (water) group were excluded from the graphical representation. Instead, the DMSO (vehicle) was considered more appropriate for statistical comparison, as DMSO was the vehicle present in all CBZ treatments.

3.1. Morphometrical analysis

Regarding the morphometric parameters measured, it was observed that neither gonadosomatic (Fig. 1A) nor hepatosomatic (Fig. 1B) indexes were altered after 7 days of exposure of fish to CBZ ($p > 0.05$).

3.2. Acetylcholinesterase activity

AChE was measured in the brain (Fig. 2A) and in the muscle (Fig. 2B) of the female fish and no statistical differences were observed in any of the treatments or tissues evaluated ($p > 0.05$) after 7 days of exposure to CBZ.

3.3. Gene expression

The expression of the gonadotropin genes (*fshβ* and *lhβ*) evaluated in the pituitary was partially altered due to CBZ exposure (Fig. 3A and B). Females exposed to a concentration of 500 ng L^{-1} of CBZ increased the expression of *fshβ* after 7 days of exposure in comparison to the DMSO group (Fig. 3A, $p = 0.0204$). However, none of the other concentrations tested were significantly different ($p > 0.05$). Similarly, the mRNA levels of *lhβ* were not altered after CBZ exposure in any of the concentrations tested of CBZ (Fig. 3B).

3.4. Steroid analysis

The plasma concentration of testosterone and estradiol were also measured in females and after 7 days of exposure to CBZ, neither testosterone (Fig. 4A) or estradiol levels (Fig. 4B) were altered in none of the concentrations tested ($p > 0.05$).

3.5. Metabolic content

Total lipid content, evaluated in the liver (Fig. 5A) and in the muscle (Fig. 5B) were not altered by CBZ in any of the treatments tested. Similarly, protein content which was analyzed in the liver (Fig. 5C) was not changed in animals exposed to CBZ. Nonetheless, in the muscle of fish exposed to 500 ng L^{-1} of CBZ (Fig. 5D), a decrease of the total protein content was observed in comparison to animals from the DMSO group ($p = 0.033068$).

3.6. Antioxidant defenses

The activities of SOD, CAT and GST, evaluated in the gills (Fig. 6A, D and G), liver (Fig. 6B, E and H) and muscle (Fig. 6C, F and I) of female *A. lacustris* remained unchanged following exposure to CBZ. Neither 250 ng L^{-1}, 500 ng L^{-1} or 1250 ng L^{-1} of CBZ induced any significant alterations in the activity of these enzymes after 7 days of exposure.

3.7. Lipid peroxidation (LPO)

Exposure to CBZ did not cause significant effects in the gills (Fig. 7A) or muscle (Fig. 7C) of fish in relation to LPO. However, in the liver (Fig. 7B), a reduction in the levels of LPO, evaluated by the TBARS method, was observed in fish exposed to 500 ng L^{-1} ($p = 0.0157$) and 1250 ng L^{-1} ($p = 0.0442$) of CBZ in comparison to the DMSO group.

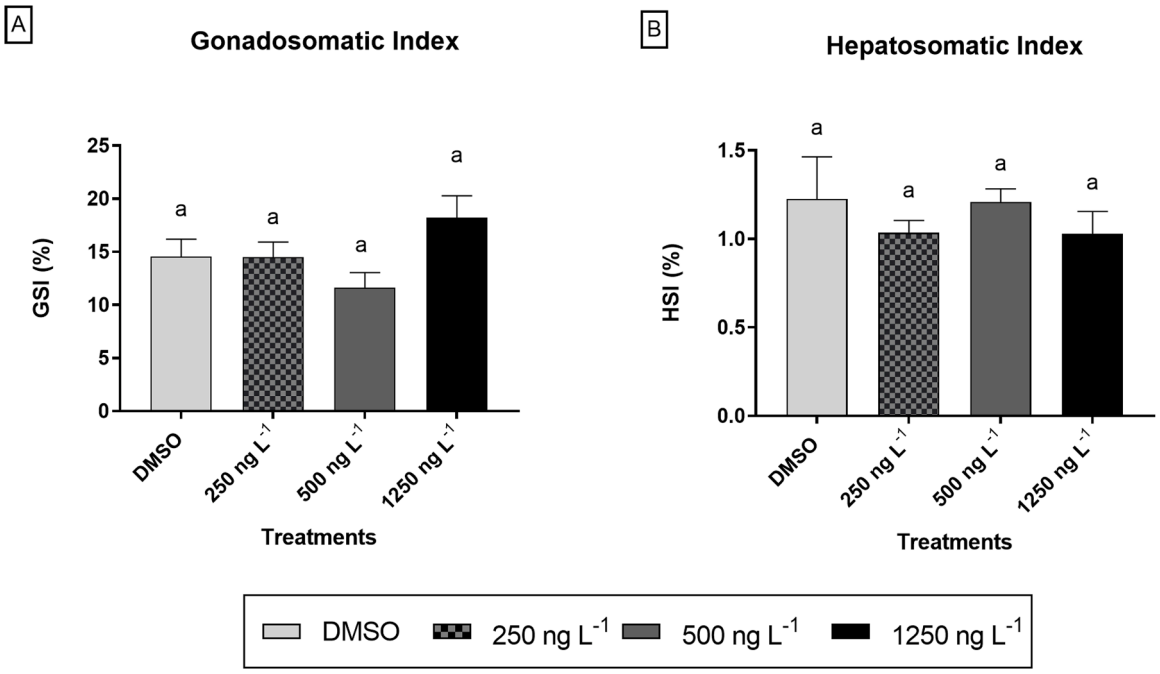

Fig. 1. Gonadosomatic (A) and Hepatosomatic (B) indexes of *Astyanax lacustris* females exposed for 7 days to CBZ. Similar letters represent no statistical differences ($p > 0.05$).

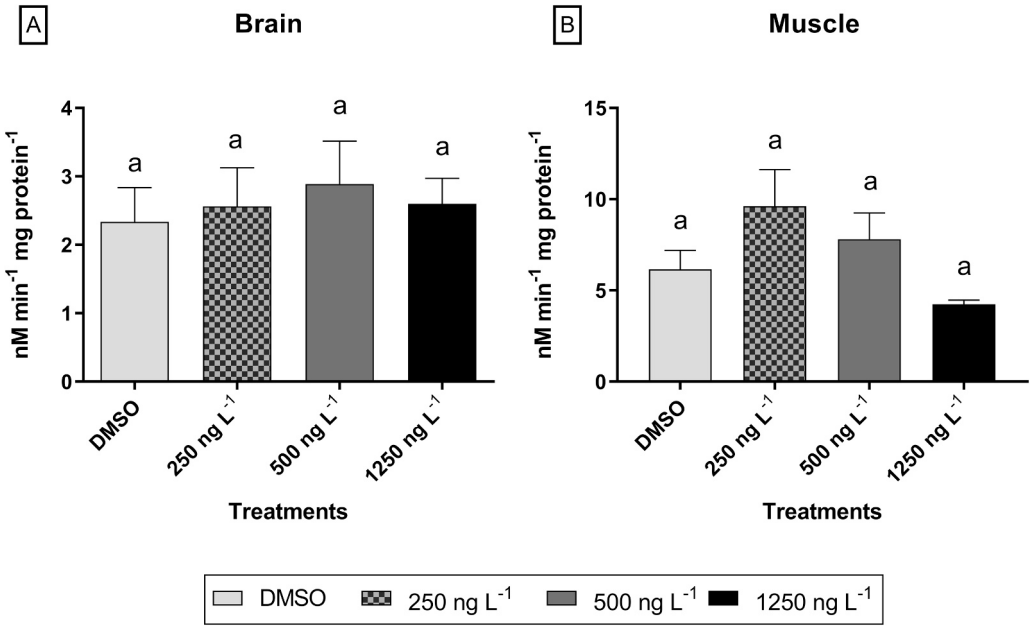

Fig. 2. AChE activity in the brain (A) and in the muscle (B) of the female teleost *Astyanax lacustris* exposed for 7 days to CBZ. Similar letters represent no statistical differences ($p > 0.05$).

4. Discussion

Pharmaceuticals, as a class of micropollutants, present different mechanisms of action and impact organism's molecular and cellular pathways, as well as organs in a distinct manner. Knowledge regarding the anti-inflammatories ibuprofen, diclofenac and also psychotropic drugs is growing and available to a limited number of species (Aguirre-Martínez et al., 2018; Branco et al., 2021; Godoi et al., 2024; Xia et al., 2017). However, toxicity of CBZ to organisms, mainly teleost species, is still poorly investigated. Considering that *A. lacustris* is native species from South America and shows an important role in ecosystems and in aquaculture, the impacts of this neuropharmaceutical on distinct biomarkers of the fish, were analyzed.

In humans, it is known since the 1990s that CBZ has its primary effects on the nervous system (Okada et al., 2002, 1997; Yan et al., 1992), affecting the Na^{+} and Ca^{+2} voltage-gated channels (Benes et al., 1999; Elliott, 1990) and the dopaminergic (Okada et al., 2002) and serotoninergic neurotransmitters (Yan et al., 1992). At this time it was also described that in rodents, CBZ has effects on y-aminobutyric acid (GABA) and it alters the levels of the luteinizing hormone (Wolf et al., 1993). Taking into account the conserved structure of the molecules involved in neurotransmission across the animal kingdom, effects of CBZ

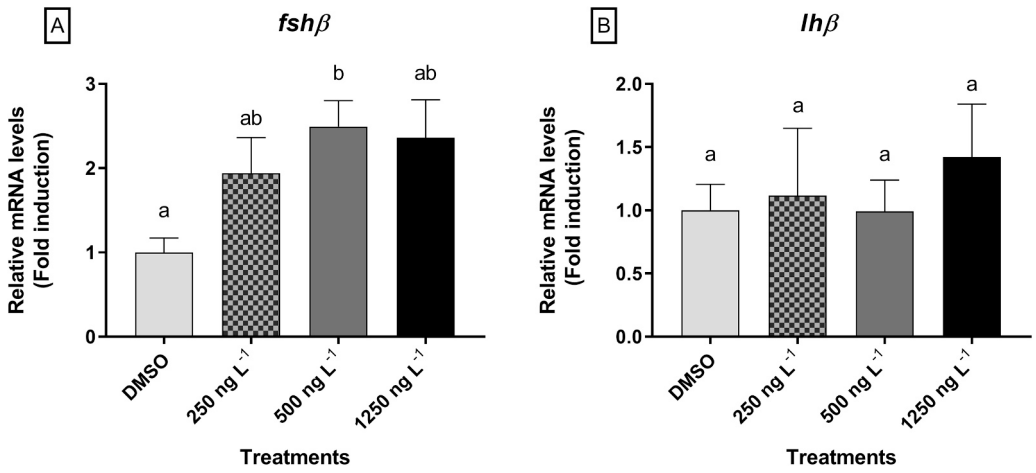

Fig. 3. Relative mRNA levels (fold induction) of the reproductive related genes *fshβ* (A) and *lhβ* (B) in the pituitary of the female teleost *Astyanax lacustris* exposed for 7 days to CBZ. Different letters represent statistical differences ($p \leq 0.05$).

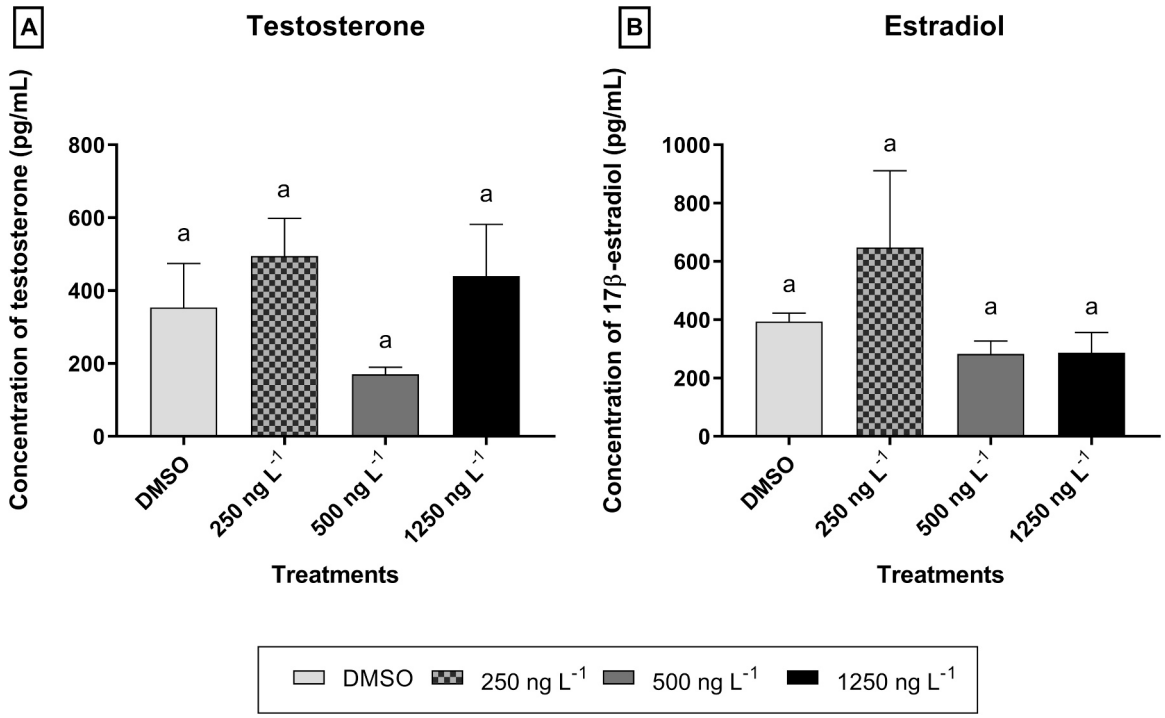

Fig. 4. Plasma concentration of testosterone (A) and estradiol (B) of the female teleost *Astyanax lacustris* exposed for 7 days to CBZ. Similar letters represent no statistical differences ($p > 0.05$).

in the brain of non-target aquatic organisms is expected. According to Garcia et al. (2012), CBZ can cross the blood-brain barrier and bioaccumulate in this tissue, where it negatively modulates behaviour related to swimming speed and food intake in *O. latipes* adults (Nassef et al., 2010b). Similarly, studies with *D. rerio* demonstrated that exposure to CBZ reduces aggressive behavior of males and their courtship (Fraz et al., 2019) and increases the anxiety response in females, concomitantly with the increase in the levels of serotonin (Xie et al., 2023). Even though the behavior analysis was not the goal of this study, we observed that animals exposed to CBZ at a concentration of 1250 ng L^{-1} were more lethargic than animals from the control groups (data not shown).

Considering that disrupted behavior was a response commonly observed after exposure to CBZ and that this neuropharmaceutical can induce neurotoxicity to aquatic organisms, effects on acetylcholinesterase activity might be expected as well. As observed by da Silva Santos et al. (2018), *D. rerio* exposed to CBZ decreased AchE activity both in brain and muscle. Similarly, in clams *Ruditapes philippinarum* and *C. fluminea*, the exposure to CBZ led to a reduction in the activity of AchE in the digestive gland (Aguirre-Martínez et al., 2018, 2016). In contrast, in the present study, no effects were observed in AchE activity, neither in the muscle nor in the brain. According to Mizuno et al. (2000), CBZ is able to induce a biphasic response in AchE, depending on its dosage. Since in this study we used environmentally relevant and low CBZ concentrations, the lack of responses may be related to the concentration itself.

Still, CBZ is able to affect the brain at transcriptomic levels. Some studies demonstrated that different genes might be modulated after exposure to this pharmaceutical. For example, genes encoding for somatolactin, prolactin and somatotropin were strongly altered in *Salmo*

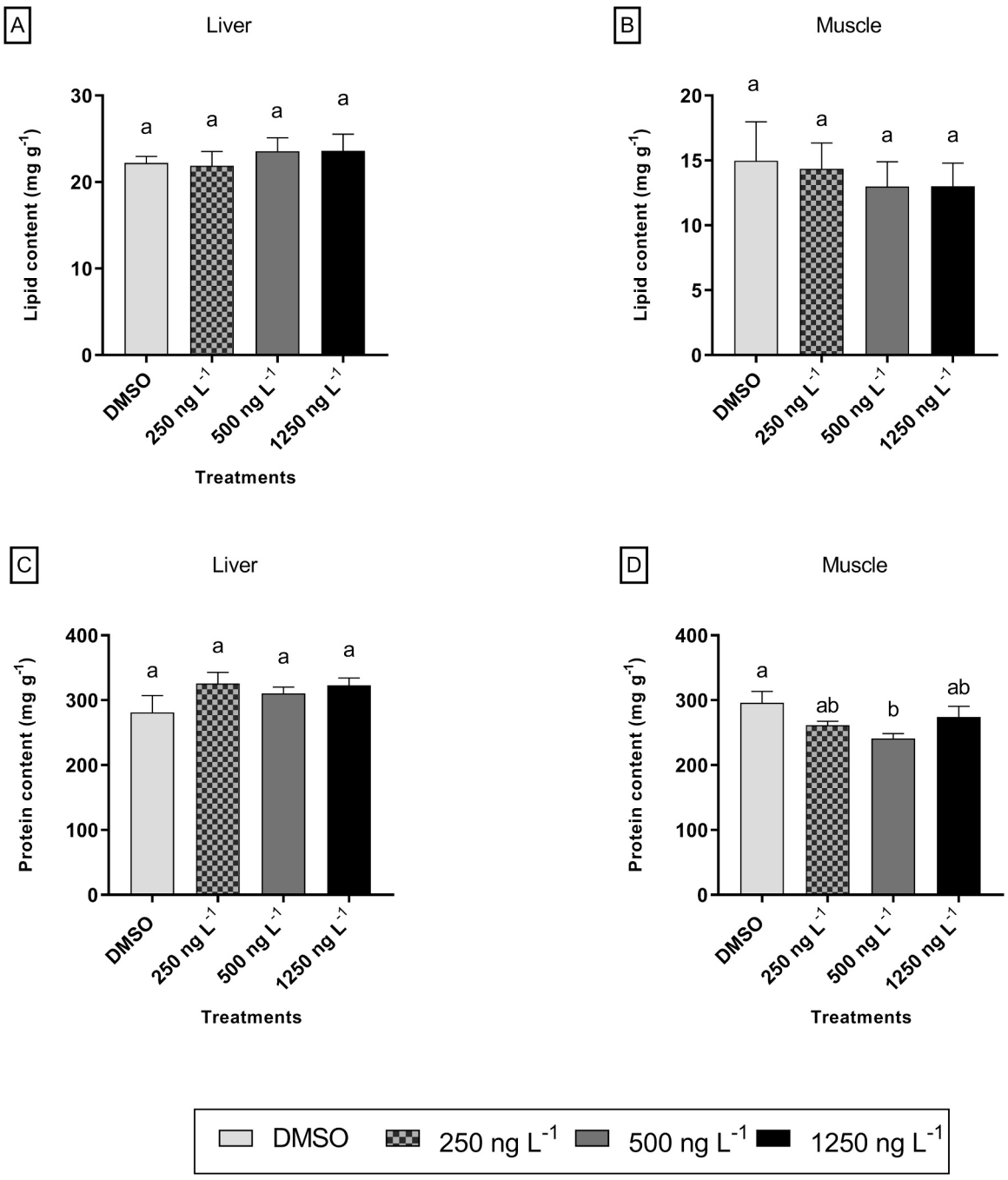

Fig. 5. Total lipid and protein concentration in the liver (A and C) and in the muscle (B and D) of *Astyanax lacustris* females exposed for 7 days to CBZ. Different letters represent statistical differences ($p \leq 0.05$).

salar exposed for 5 days to CBZ (Hampel et al., 2014). Genes related to the GABA-Glu pathway (*gad2, abat, gabrb2, gabrg2, gria1a* and *slc12a2*) and from the HPI (HPI, hypothalamic-pituitary-interrenal) axis (*crha, actha*) were also modulated in *D. rerio* (Xie et al., 2023). In fact, in the present study, alteration in the mRNA levels of females *A. lacustris* were also observed, as fish increased the expression of *fshβ* after 7 days of exposure to 500 ng L^{-1} of CBZ. Differently, the expression of *lhβ* remained unaltered in those organisms. Although *fshβ* and *lhβ* mRNA levels were not investigated in the study of Yan et al. (2018) authors showed that *gnrhr1α* and *hsd3β7,* two important genes from the steroidogenic pathway linked with the synthesis of gonadotropins, were upregulated in the Chinese rare minnow *Gobiocypris rarus* females exposed for 28 days to CBZ, indicating the potential of this neuropharmaceutical to impair reproduction. The lack of alteration in *lhβ* gene expression, and the increase in *fshβ* gene expression can be related to the different roles of both gonadotropins in fish reproduction. The luteinizing hormone (LH) is involved with the process of ovulation, so its production is normally limited to the pre-ovulatory period, while follicle-stimulating hormone (FSH) regulates vitellogenesis (via 17*β*-estradiol synthesis) (Alix et al., 2020), thus FSH synthesis is maintained in more regular and higher levels during the reproductive phase of the females that were used in the experiment (vitellogenic oocytes) (data not shown). In regard to the direct effect of the concentration, it can be suggested that CBZ interferes in the steroid hormone pathway, which later is compensated by maintaining the levels of the circulating hormone.

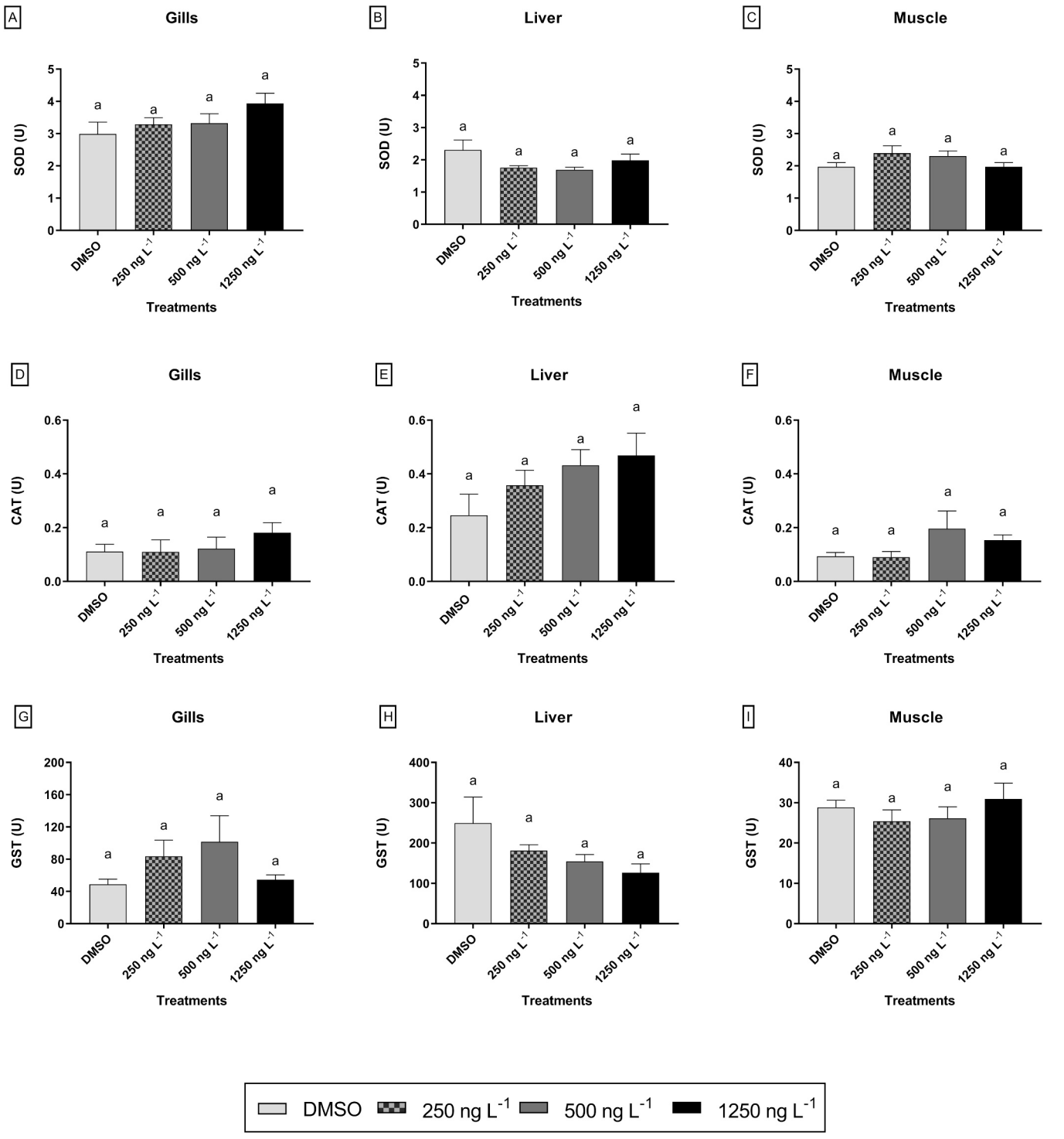

Fig. 6. Activity of the antioxidant enzymes SOD, CAT and GST in gills (A, D and G), liver (B, E and H) and muscle (C, F and I), respectively, of *Astyanax lacustris* females exposed for 7 days to CBZ. Similar letters represent no statistical differences ($p > 0.05$).

Indeed, many pharmaceuticals, such as diclofenac, ibuprofen and caffeine, have already been labeled as EDC, and can affect embryo development and reproduction in fish (Branco et al., 2021; Godoi et al., 2024, 2020). As for CBZ, its potential to be an EDC remains unclear. While in males, CBZ reduced the levels of 11-ketotestosterone (Fraz et al., 2019, 2018; Galus et al., 2014; Xie et al., 2023), increased vitellogenin content (VTG) (Xie et al., 2023), reduced the sperm velocity (Li et al., 2010a), altered the sperm morphology (Fraz et al., 2019) and quality (Li et al., 2010a), in females, the effects are still limited and controversial. In the study of Shi et al. (2019), *D. rerio* females exposed for 10 days to 0.1 and 10 μg L^{-1} of CBZ reduced both E_2 and VTG content. Differently, in the study of Yan et al. (2018), the exposure of *G. rarus* females for 28 days to 100 μg L^{-1} of CBZ increased the levels of E_2 and VTG, and decreased the levels of 11-ketotestosterone. Contrarily, in the present study, neither E_2 nor testosterone levels were altered after 7 days of exposure to 250 ng L^{-1}, 500 ng L^{-1} or 1250 ng L^{-1} of CBZ. Those differences observed among results might be mainly related to the concentrations of CBZ that fish were exposed to, being lower in the

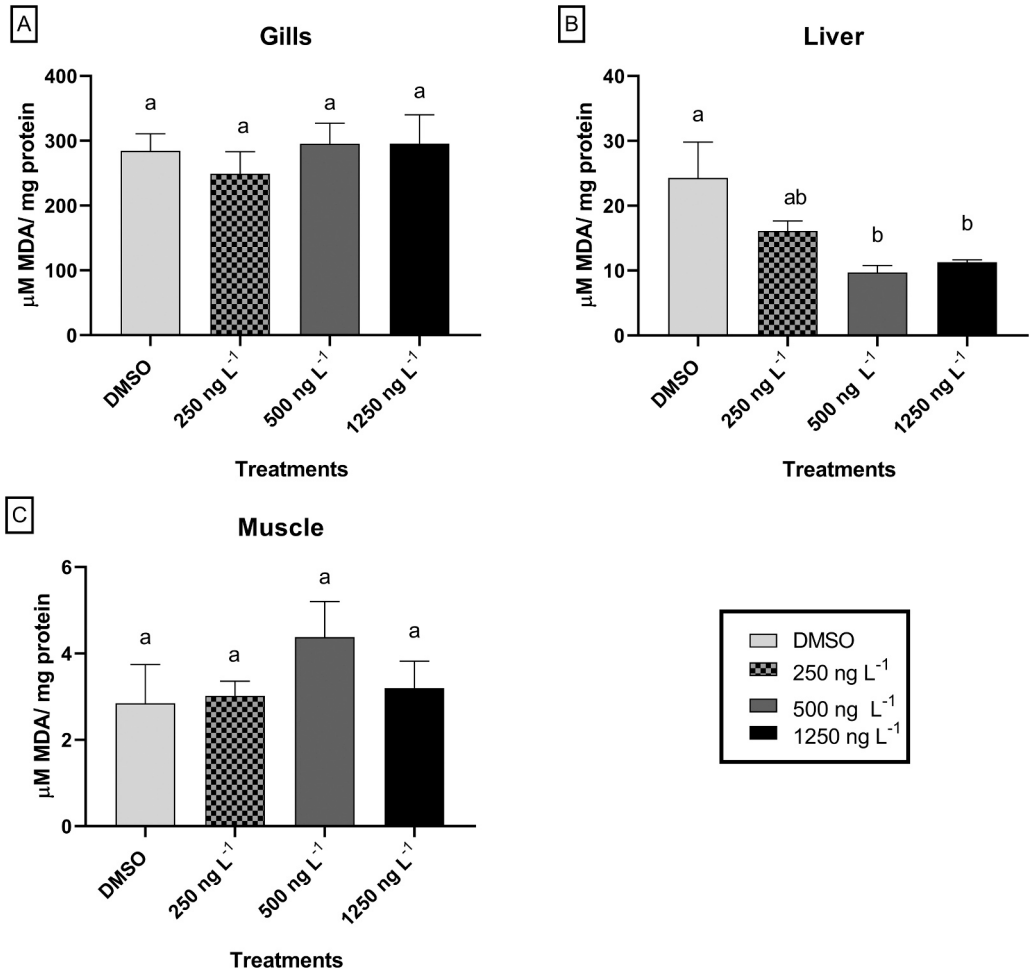

Fig. 7. Lipid Peroxidation in gills (A), liver (B) and muscle (C) of *Astyanax lacustris* females exposed for 7 days to CBZ. Different letters represent statistical differences ($p \leq 0.05$).

present study when in comparison to the studies of Shi et al. (2019) and Yan et al. (2018).

Another important physiological endpoint to screen when animals are exposed to contaminants is the antioxidant defense system, represented mainly by SOD, CAT, GST and the glutathione (GSH) content. As a consequence of the aerobic metabolism, organisms produce reactive oxygen species (ROS) which can, in low levels, aid the immune system in combating pathogens (Li et al., 2021) or, otherwise, in higher levels, damage proteins, lipids and carbohydrates, disrupt signalization cascades, alter DNA and disbalance the antioxidant defenses mentioned (Jones, 2008; Lushchak, 2016). Many xenobiotics, pharmaceuticals included, are able to induce damages to those macromolecules and impair the activity of several of those enzymes (Aguirre-Martínez et al., 2016; Guiloski et al., 2015; Iftikhar et al., 2022; Li et al., 2010b; Muñoz-Peñuela et al., 2021). For instance, the 28 days-exposure of *C. carpio* to the antibiotic sulfamethoxazole increased ROS levels both in gills and brain (Iftikhar et al., 2022) and the 45 days-exposure of *Oreochromis niloticus* to diclofenac induced the higher activity of CAT, SOD, GST and GSH in the gills (Ajima et al., 2021). Moreover, diclofenac and caffeine, the later, an anti-inflammatory drug and a nervous system stimulant, inhibited hepatic antioxidant enzymes (SOD, CAT, GPx and GST) and induced lipid peroxidation when evaluated in isolated or combined forms (Muñoz-Peñuela et al., 2021).

In regards to CBZ, Li et al. (2011) reported that the activities of SOD, CAT, GR and GPx decreased in the gills and brain of juvenile *O. mykiss* exposed for 96 h to 19.9 mg L^{-1}. Moreover, authors observed that, in CBZ-exposed fish, the levels of LPO and protein carbonyls (PCO) were higher in those tissues, suggesting the possible role of the pharmaceutical in the induction of oxidative stress. Likewise, this pattern of response was not only observed in juveniles, but also in adults of *O. mykiss* exposed to 0.2 mg L^{-1} and 2 mg L^{-1} of CBZ for 21 and 42 days. Fish reduced the levels of SOD, CAT, GR and GPx, concomitantly with the increase in lipid peroxidation in the brain (Li et al., 2010c) and in the muscle (Li et al., 2010d). SOD, CAT and GPx are known scavengers of radical anion superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroperoxides, respectively, and are considered the first line of defense against oxidative damage (Lushchak, 2016; Storey, 1996). Thus, CBZ might be related to oxidative stress in some species of fish.

In other teleost species, such as *C. carpio* (Gasca-Pérez et al., 2019), *D. rerio* (da Silva Santos et al., 2018) and *Lepomis gibbosus* (Brandão et al., 2013), the antioxidant defenses were also modulated, but in a different manner: while in *C. carpio*, the responses were similar to the previous studies where SOD, CAT and GPx were decreased in the gills and brain of fish exposed to 2 mg L^{-1} of CBZ for up to 96 h, in the zebrafish and in the sunfish, different concentrations, such as 10 µg L^{-1} and 1000 µg L^{-1} and 62.5 µg L^{-1}, 125 µg L^{-1}, 250 µg L^{-1}, 500 µg L^{-1} and 1000 µg L^{-1} of CBZ, respectively, did not change the activity of GST or the levels of LPO, for example, in the gills, but increased the activity of GR (Brandão et al., 2013). In *A. lacustris* females, those enzymes (SOD, CAT and GST) as well as LPO, also evaluated in the muscle and gills of the animals did not change after the 7 day-exposure to any of the concentrations tested (250, 500 or 1250 ng L^{-1} of CBZ).

Considering the liver, the organ with the main role in biotransformation and detoxification processes (Livingstone, 1998; van der Oost

et al., 2003), the studies also observed different responses. Nkoom et al. (2020) observed that SOD, CAT, GST and GR activities were significantly increased in the liver of *Carassius carassius*. Authors reported a concentration (2 and 10 μg L^{-1}) and time-dependent manner (1, 4 and 7 days) of their results and suggested that the increase observed in the activity of those enzymes could be a protective mechanism from oxidative stress. Likewise, Li et al. (2010b) reported increases in SOD, CAT, GPx and GR in the liver of the rainbow trout *O. mykiss* exposed for 21 and 42 days to 0.2 and 2 mg L^{-1} of CBZ and Sibiya et al. (2023) observed increases in SOD, CAT, GPx and GST in the liver of *Oreochromis mossambicus* exposed for 14 days to 10 μg L^{-1} of CBZ. In the present study, however, none of the concentrations tested were able to alter SOD, CAT or GST in the liver of the fish. It is important to observe, though, that the basal levels of GST are higher in the liver of organisms than in any other organs. Also, LPO levels were decreased in animals exposed to 500 ng L^{-1} and 1250 ng L^{-1} of CBZ, suggesting that the liver is able to cope with this pharmaceutical exposure.

In fact, the liver plays a fundamental role in the biotransformation of pharmaceuticals, including CBZ (Kerr, 1994). Certain liver enzymes, particularly the cytochrome P450 (CYP) family members, introduce or expose a functional group (-OH, -NH2, -COOH) to enhance the compound's hydrophilicity (Parkinson, 2001) and, for CBZ, both CYP2C8 and CYP3A4 isoforms are specifically involved in converting CBZ into carbamazepine-10,11-epoxide, a more stable and less toxic compound (Breton et al., 2005; Parkinson, 2001; Kerr et al., 1994). Therefore, induction of CYPs due to CBZ exposure might provide less toxicity to the liver, supporting the decrease in LPO levels observed in the present study. Additionally, studies suggested that CBZ, as many other drugs, induce some nuclear transcription factors such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), which, in turn, activate CYP3 (Ke et al., 2019; Oscarson et al., 2006). Altogether, the induction of PXR and CAR and, consequently, CYP3 offer a possible explanation to the decrease in the liver LPO levels.

Overall, three strategies could be employed to avoid oxidative damage, according to Storey (1996): 1. maintain high levels of the antioxidant defenses; 2. elevate those antioxidant defenses as soon as possible, anticipating the impact of the stressor; and 3. endure damage and dispose of the by-products of the damage. Considering that none of the enzymes were altered in *A. lacustris* and that LPO was decreased in the liver, it is possible that animals follow the third strategies, presenting a higher endurance capacity and being able to cope with the stressor by disposing the by-products of the damage or the oxidative burst rapidly after the start of the insult. Moreover, there are many aspects that can be influencing CBZ's toxicity, such as species specificity, gender, stages of development, period of exposure and concentration of the contaminant. For instance, studies observed impairment of CBZ mostly in males (Fraz et al., 2018; Galus et al., 2013) and juvenile fish (González-Mira et al., 2018; Li et al., 2011) or even with longer exposure periods (da Silva Santos et al., 2018; Li et al., 2010b), including those that observed the effects of concentrations that are not frequently present in ecosystems. In this context, the present study presented novelty in regards to the effects of CBZ in females of the teleost *A. lacustris*, which is a native species from South America, ecologically and economically important for the region.

5. Conclusion

There is insufficient data available on the effects of CBZ on aquatic organisms. However, while some reports have highlighted its potential to disrupt reproduction and other physiological processes in teleost and bivalves, these studies have predominantly focused on male specimens. In contrast, the present study demonstrated that environmentally relevant concentrations of CBZ can affect female fish in distinctive ways. *A. lacustris* females exhibited an increase in the expression of *fshβ* when exposed to 500 ng L^{-1} of CBZ, but without any turn-over in relation to hormonal production. This finding is important because it suggests that females may maintain at least one phase of reproduction unaffected, even under adverse situations. Additionally, females reduced the lipid peroxidation in the liver, indicating strong endurance capacity, antioxidant defenses and/or efficient biotransformation systems, protecting themselves from oxidative damage. At last, it is necessary to consider that, since *A. lacustris* is a native fish species capable of inhabiting both clean and polluted ecosystems, they may display potential physiological adjustments to challenging environments.

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CRediT authorship contribution statement

Amanda da Silveira Guerreiro: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Guilherme de Aguiar**: Investigation, Methodology, Visualization. **Cecilia Bertacini**: Conceptualization, Investigation, Methodology, Writing – review & editing. **Filipe Guilherme Andrade de Godoi**: Investigation, Methodology, Writing – review & editing. **Giovana Souza Branco**: Conceptualization, Investigation, Methodology, Writing – review & editing. **Renato Massaaki Honji**: Investigation, Methodology, Writing – review & editing. **Larissa Caminhas**: Investigation, Methodology, Writing – review & editing. **Susanne Rath**: Investigation, Methodology, Writing – review & editing. **Renata Guimarães Moreira Whitton**: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & 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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2025.104653.

Data availability

No data was used for the research described in the article.

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