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Experimentally exposed toxic effects of long-term exposure to environmentally relevant concentrations of CIP in males and females of the silver catfish *Rhamdia quelen*

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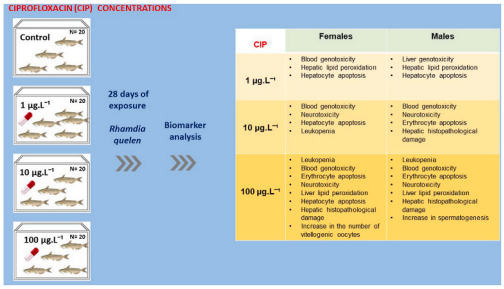
HIGHLIGHTS

- Ciprofloxacin caused neurotoxicity in *R. quelen* fish.
- Ciprofloxacin caused leukopenia, genotoxicity and apoptosis of blood cells.
- In the liver was observed oxidative stress and apoptosis.
- Females were more sensitive to blood genetic damage than males.

GRAPHICAL ABSTRACT



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ABSTRACT

Ciprofloxacin (CIP) is an antibiotic commonly used in human and veterinary medicine. It is present in the aquatic environment, but we still know very little about its effect on non-targeted organisms. This study aimed to evaluate the effects of long-term exposure to environmental CIP concentrations (1, 10, and 100 µg.L^{-1}) in males and females of *Rhamdia quelen*. After 28 days of exposure, we collected the blood for the analysis of hematological and genotoxic biomarkers. Additionally, we measured 17 β-estradiol and 11 keto-testosterone levels. After the euthanasia, we collected the brain and the hypothalamus to analyze acetylcholinesterase (AChE) activity and neurotransmitters, respectively. The liver and gonads were assessed for biochemical, genotoxic, and

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histopathological biomarkers. At 100 μg.L^{-1} CIP, we observed genotoxicity in the blood, nuclear morphological changes, apoptosis, leukopenia, and a reduction of AChE in the brain. In the liver was observed oxidative stress and apoptosis. At 10 μg.L^{-1} CIP, leukopenia, morphological changes, and apoptosis were presented in the blood and a reduction of AChE in the brain. Apoptosis, leukocyte infiltration, steatosis, and necrosis occurred in the liver. Even at the lowest concentration (1 μg.L^{-1}), adverse effects such as erythrocyte and liver genotoxicity, hepatocyte apoptosis, oxidative stress, and a decrease in somatic indexes were observed. The results showed the importance of monitoring CIP concentrations in the aquatic environment that cause sublethal effects on fish.

1. Introduction

Residues of pharmaceuticals, such as antibiotics, have been found in aquatic environments worldwide (Miller et al., 2018; Pashaei et al., 2022) and have increasingly attracted public concern (Madikizela and Ncube, 2022; Zhou et al., 2022). They can directly lead to adverse effects on nontargeted organisms (Yang et al., 2020) and pose indirect risks to human health (Guo et al., 2022; Liu et al., 2022). Antimicrobial resistance (AMR) is a significant public health problem worldwide (WHO, 2022) and has been considered a severe threat to global health (WHO, 2022). On the other hand, we still have limited knowledge with regards to the adverse effects on aquatic biota.

Ciprofloxacin (CIP) is one of the most widely used antibiotics worldwide. It is present in surface water, groundwater, wastewater, sewage, sediments, soils, and even drinking water at concentrations ranging from ng.L^{-1} to μg. L^{-1} (Kelly and Brooks, 2018; Sahlin et al., 2018; Xie et al., 2017). It is a second-generation fluoroquinolone allowing for a broad spectrum of activity against aerobic gram-negative and gram-positive bacteria (Sahlin et al., 2018). Studies have already shown that CIP is not biodegradable and can resist environmental degradation (Flach et al., 2018; Sahlin et al., 2018; Xie et al., 2017). In addition, studies have also detected CIP in fish tissues (Sehonova et al., 2019; Zhang et al., 2016). However, the effects of CIP exposure on aquatic biota are still unknown, and few studies have reported the effects of this antibiotic on fish (Kitamura et al., 2022; Plhalova et al., 2014; Ramesh et al., 2021).

This study aimed to evaluate the experimentally exposed toxic effects of long-term exposure to environmentally relevant concentrations of CIP in males and females of the South American silver catfish (*Rhamdia quelen)*. The hypotheses underlying this research are that long-term exposure of neotropical fish to CIP causes hematological damage, neurotoxicity, and hepatotoxicity and is a potential endocrine disruptor. We also investigated whether the sex of the fish can influence biomarker responses.

2. Material and methods

2.1. Sampling

Rhamdia quelen juveniles were obtained collected from the State University Toledo campus fish farm in western Paraná, Brazil, and they were acclimatized for 30 days in a glass aquarium (15 L capacity; four Fish per aquarium) at 27 ± 1 °C. The water for the experiment was filtered and chlorine-free. The study followed a strict simulated natural 12 h dark:12 h light cycle. We followed the fish density per aquarium recommended by OECD (2000). The fish were fed ad libitum once daily during the experiment with a balanced diet suitable for this species (Laguna®, Brazilian Fish, 32% protein). The study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Paraná, under article number 1227.

2.2. Chemical analysis

Initially, we performed the first chemical analysis to study the degradation of CIP in the water during the bioassay, so it was possible to know the time required for water exchange to remain with the previously established experimental concentrations of CIP. We divided twenty-four fish into a control group (without CIP) and a treatment group (with 100 μg.L^{-1} CIP, D6899 from Sigma Aldrich®), four fish per aquarium (15 L), and thee aquarium per treatment. We chose the highest concentration of CIP to facilitate the standardization of antibiotic determination during the bioassay. We added CIP to the aquarium only once at the beginning of the experiment and collected water samples at 0, 24, and 48 h time markers. They were stored in 50 mL tubes (Falcon ®) at −20 °C until CIP quantification.

During the long-term experiment, water samples (50 mL) were collected (in tubes Falcon ®) from the fish bioassay aquariums (one sample per aquarium, five samples per treatment). The samples were taken at specific times of the day and on specific days beginning at day 0, then progressing to day 1, 2, 7, 14, 21, and 28, and stored at −20 °C until the analysis. We used High-performance liquid chromatography (HPLC) (Waters Alliance®2695) coupled with a multi & fluorescence detector (Waters 2475) and software Empower Pro 2002 to quantify CIP in water, in accordance to Kitamura et al. (2022). We used a mobile phase triethylamine solution 0.4% (v/v) pH 3, acetonitrile and methanol 75:10:15 with a flow rate of 1 mL min^{-1} and a volume of 20 μL sample injection. For excitation and emission, we used Wavelengths of 278 and 453 nm, and a Supelco Analytical Ascentis C18 column (250 × 4.6 mm, 5 μm) at 35 °C.

2.3. Fish exposure

After the period of laboratory acclimatization, and the CIP degradation study, we randomly divided the fish (weight: 13.66 ± 2.82 g; length: 12.04 ± 1.02 cm; mean ± standard deviation), into four groups (Control, 1, 10 e 100 μg.L^{-1} CIP). Each group consisted of 20 fish per group (four fish per aquarium in five aquaria). As visually differentiating the sexes was not possible, at the end of the experiment, sex determination was performed by histological analysis of the gonadal tissues.

For the experiment, fish were kept in a glass aquarium (15 L), following the recommended biomass with an approximate density of 1 g. L^{-1} (OECD, 2000), and placed under the same conditions as the CIP degradation study.

The authors selected the particular concentrations from a literature search of the concentrations present in surface and groundwater, which ranged from 0.018 to 10 μg.L^{-1} (Frade et al., 2014; Janecko et al., 2016; Mutiyar and Mittal, 2014; O'Flaherty and Cummins, 2016; Quadra et al., 2017). In water treatment plant effluents and in wastewater, CIP ranged from 0.036 to 82.8 μg.L^{-1} (Frade et al., 2014; Riaz et al., 2017), while in hospital and pharmaceutical industry effluents the average concentrations were of 104.43 μg.L^{-1} (Frade et al., 2014; Mutiyar and Mittal, 2014; O'Flaherty and Cummins, 2016; Riaz et al., 2017).

The bioassay proceeded semi-statically, and every 24 h, one-third of the water was replaced with the appropriate CIP addition to the aquarium water to obtain the final test concentrations, which corresponded to the result of the CIP degradation study described previously (Supplementary material 1). We conducted the same water replacement schedule without adding CIP to the control group.

We measured the water parameters, such as the pH (7.05 ± 0.44 units), with a pH meter (HANNA Instruments®, model HI98129; ammonia (0.31 ± 0.18 ppm), and nitrite (0.06 ± 0.18 ppm) with Labcon® test kits. With a probe (HANNA Instruments®, model HI 9146) we

measured dissolved O2 (7.05 ± 0.17 ppm), and the temperature of the bioassay room (27 ± 1 °C). After 28 days of exposure, we anesthetized the animals with benzocaine and collected blood from the caudal vein with syringes containing 3% heparin. After the euthanasia of the fish by medullar section, the brain, liver, and gonads were removed and stored at −80 °C until the biomarker analysis. We measured the weight of the body, liver, and gonads to calculate the hepatosomatic and gonadosomatic indexes.

2.4. Biomarker analysis

2.4.1. Hematological biomarkers

A blood sample of 10 μL was collected from the caudal vessel with syringes coated with sodium heparin (2.500 UI/mL). The blood smears were stained with May Grünwald-Giemsa-Wright for counting the total amount of leukocytes and platelets using the Neubauer chamber under an optical microscope at 4000x (Tavares-Dias and de Moraes, 2006). We used erythrocytes to calculate the number of the leukocytes and thrombocytes: Leucocytes (μL) = counted leukocytes x counted red blood cells (RBC) x 5000, and platelets (μL) = counted thrombocytes x RBC x 5000. Then, we determined the Hematocrit levels in accordance with research by Hine (1992). We calculated the erythrocyte indices mean corpuscular volume following this formula: VCM ((Ht/Er) x 10).

2.4.2. Hepatosomatic and gonadosomatic index

For determined somatic indexes (GSI and HIS) was used the formula stated below (Tavares-Dias et al., 2000):

$$GSI = \frac{Gonad\ weight}{Whole\ body\ weight} X\ 100$$

$$HIS = \frac{Liver\ weight}{Whole\ body\ weight} X\ 100$$

2.4.3. Biochemical biomarkers

This study analyzed acetylcholinesterase activity (AChE) in the brain and muscle, through the homogenization of samples using a phosphate buffer (0.1 M; pH 7.5) at a ratio of 1:10 (m/v). We subsequently homogenized samples centrifuged at 1000×*g* for 20 min at 4 °C and stored at −80 °C until the day of analysis. The AChE activity followed Ellman et al. (1961), with modifications for microplates by Silva de Assis (1998).

We used a phosphate buffer (0.1 M; pH 7.0) at a ratio of 1:10 (m/v) to homogenize samples of the livers and gonads, which were centrifuged at 15,000×*g* for 30 min at 4 °C. We determined the superoxide dismutase (SOD) (Gao et al., 1998), catalase (CAT) (Aebi, 1984), glutathione peroxidase (GPx) (Hafeman et al., 1974), and glutathione S-transferase (GST) (Keen et al., 1976) activity from the supernatants. According to literature by Sedlak and Lindsay (1968), we determined non-protein thiol/glutathione (GSH) concentrations. Through the FOX method (Jiang et al., 1992), we determined lipoperoxidation (LPO). The quantification of the total protein concentration used bovine serum albumin as a standard according to the Bradford method (Bradford, 1976) for calculating the enzymes' specific activity.

2.4.4. Biomarkers of genotoxicity

The micronucleus piscine test was analyzed by using a blood smear obtained from a 10 μL sample of peripheral blood collected from a caudal vein. The test was analyzed counting 2000 erythrocytes per animal. One slide per fish was stained with Giemsa immediately before analysis (Leica® Microscope; model DMLS2) at 1000x magnification. We utilized the methodology by Carrasco et al. (1990) and Çava and Ergene-Gözükara (2005) for evaluating the micronucleus and the other nuclear morphological alterations such as blebbed (B), lobed (L), notched (N), and vacuolated (V). The erythrocytes comet assay was analyzed as indicated by Singh et al. (1988), with modifications by Ferraro et al. (2004) and Cestari et al. (2004). Moreover, for performing the comet assay in the liver and gonads, the methodology and adjustments described by Ramsdorf et al. (2009) was followed.

The nucleoids (N = 100) were analyzed in each animal and classified according to the damage present after the electrophoretic run (Azqueta et al., 2011). Before analysis, the samples were stained with ethidium bromide (20 mg.L^{-1} - Sigma-Aldrich®) and analyzed with a Leica epifluorescence microscope (model: DMLS2) using a rhodamine filter at 1000x magnification. We classified the nucleoids following each type of damage as follows: 0 (no apparent damage), 1 (low damage), 2 (moderate damage), 3 (severe damage), and 4 (maximum damage). We then generated the scores by using a classification and quantification of damage by multiplying the sum of the amount of damage by the value of each class (Koppen et al., 2017).

2.4.5. Assessment of ciprofloxacin-induced apoptosis

From the 10 μL blood sample and liver cells, we quantified the frequency of apoptosis using a morphometric DNA diffusion assay proposed by Singh (2005, 2000). Through this methodology, we identified the DNA with an intense fluorescent dye, YOYO-1 (Leica, model DFC 300). Apoptotic cells showed a granular DNA halo with a cloudy outer border.

2.4.6. Histopathological biomarkers

For histolopathological evaluation, we fixed a fragment of the liver and gonads in an ALFAC solution (80% alcohol, formaldehyde, and glacial acetic acid) for 16 h. After, the tissues were dehydrated in alcohols (70%, 80%, 90%, and 100%), diaphanized in xylol, and placed in Paraplast® and cut into five μm thick slices stained with hematoxylin-eosin (HE). The histopathological evaluation of lesions preceded according to the index proposed by Bernet et al. (1999) and modified by Mela et al. (2013). The essential factors considered in the evaluation were: (1) minimal pathological importance (which presents possibilities of reversal); (2) moderate importance (reversible in some cases), and (3) severe pathological importance (usually irreversible). Accordingly, under the degree of incidence of the alterations, the values assigned to the alterations were: (0) Unchanged, (2) Occasional Occurrence, (4) Moderate Occurrence, and (6) Severe Occurrence (diffuse lesion). We obtained the injury indices (II) from the mathematical equation below.

$$II = \sum rp \sum alt\ (a\ X\ w)$$

where pr = reaction pattern; *alt* = alteration; a = score value; *w* = important factor.

2.4.7. Plasma 17 β-estradiol (E2) and 11 keto-testosterone (11-KT) levels

Immediately after collection, the blood was centrifuged (2000×*g* for 5 min) and stored at −20 °C until analysis. We analyzed the plasma concentrations of the steroid testosterone and estradiol using the enzyme-linked immunosorbent assay (ELISA). From Cayman Chemical® (MI, USA; Item N. 582,751) and IBL International® (Hamburg, Germany; REF RE52041), respectively. The testosterone assay was performed in triplicate by adding a 50 μL sample per well and the 17 beta-estradiol ELISA was analyzed in triplicate using a 25 μL sample. We performed the protocols according to the manufacture's recommended procedures and expressed the results in pg. mL^{-1}.

2.4.8. Neurotransmitters concentration

We performed an analysis of the neurotransmitters; dopamine, serotonin, noradrenaline, and their metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), which was quantified using high-performance liquid chromatography with electrochemical detection (HPLC-ED), as indicated by Guiloski et al. (2017). We calculated the concentrations of neurotransmitters and their metabolites by the area under the curve (current x time) interpolated to a straight standard curve obtained from known concentrations of each

chemical species analyzed. The unit used to express these species was ng.mL^{-1} of tissue weight using a program by LC Lab Solutions.

2.5. Statistical analysis

Based on one of our hypotheses that the sex of the fish might influence the biomarker responses, we did the data analysis separately. We separated the biomarker data according to sex. We then analyzed the data's normality and homogeneity assumptions, using the Shapiro-Wilk and Levene tests, respectively. For parametric data, we applied a one-way analysis of variance (ANOVA), followed by a Tukey test, with results reported as mean ± standard error. For nonparametric data, we applied the Kruskal-Wallis test, followed by the Dunn and Mann-Whitney paired test, and we expressed the results as median ± standard error. We performed the analyses by Statistical Software (R.3.2.2, Team, 2015) and GraphPad Prism 5.00 (GraphPad Software, Inc.) for graphs.

3. Results

During the experiments, no unusual behavior or death of the collected specimens were observed.

3.1. Chemical analysis

After 24 h, the initial concentration of 100 μg.L^{-1} decreased to about 50% (49.35 ± 12.45). Based on this result, replacement every 24 h was chosen to maintain the final CIP concentrations (1, 10, and 100 μg.L^{-1}) during the 28 days of exposure by adding the volume necessary to maintain these concentrations.

CIP was not present in the control treatment group. The concentration of CIP in the water of the experiment decreased in all groups during the first 48 h. However, after this period, the concentration remained constant and close to the desired concentrations, 1 μg.L^{-1} (0.96 ± 0.02), 10 μg.L^{-1} (9.34 ± 1.15), and 100 μg.L^{-1} (93.85 ± 7.9) (Supplementary material 2).

3.2. Biomarker analysis

3.2.1. Hematological biomarkers

In male fish, CIP did not alter the erythrocyte count, hematocrit, and mean corpuscular volume (MCV). On the other hand, 100 μg.L^{-1} increased the number of thrombocytes (59.82%; P = 0.0288) and decreased the number of leukocytes (63.43%; P = 0.0270; Table 1). In female fish, CIP did not change the number of erythrocytes, hematocrit, or mean corpuscular volume (MCV). However, at 10 and 100 μg.L^{-1} there was a decrease in the number of leukocytes to 48.70% and 51.28%, respectively (P = 0.0486 and P = 0.0252; Table 1).

3.2.2. Hepatosomatic and gonadosomatic indexes

Compared with the control group, the hepatosomatic index (HSI) of *R. quelen* males changed after 1 μg.L^{-1} CIP (decrease of 34.30%; P = 0.0075). The HSI of females did not change (P = 0.4817). CIP changed the male gonadosomatic index (GSI) after 1 μg.L^{-1} CIP (64.32%; P = 0.0094), compared with the control group. The GSI of females did not change (P = 0.8092). (Supplementary material 3).

3.2.3. Biochemical biomarkers

In the brain, the activity of the AChE decreased by 73.99% and 86.69% in male fish at 10 and 100 μg.L^{-1} CIP, respectively (P = 0.0108 and P < 0.001, Fig. 1A), compared with the control group. In females, a reduction was also observed in the same treatments, 76.23% and 87%, respectively (P < 0.001 in both treatments; Fig. 1A). No change in AChE activity was observed in the muscle in both sexes (Fig. 1B).

In the male fish, the increased hepatic CAT activity (60.88%; P = 0.0105; Fig. 2A) and GSH level (208.13%; P = 0.0020; Fig. 2E) and an increase in LPO of approximately 295.54% (P = 0.0245; Fig. 2F) was observed at 1 μg.L^{-1}CIP. Exposure to 10 μg.L^{-1} decreased GST activity (48.67%; P = 0.0031; Fig. 2A), increased CAT activity (77.94%; P = 0.0026; Fig. 2C), increased GSH content (190.97%; P = 0.0039; Fig. 2E), but no lipid peroxidation was observed (P = 0.9907; Fig. 2F). In contrast, after exposure to 100 μg.L^{-1}, an increase in LPO was observed (262.52%; Fig. 2F), with a decrease in SOD activity (77.14%; P < 0.001; Fig. 2B) and an increase in CAT activity (98.02%; P < 0.001; Fig. 2C).

In the liver of female fish, exposure to 1 μg.L^{-1} did not alter antioxidant system enzymes but caused oxidative stress (LPO increase of approximately 214.30%; P = 0.0119; Fig. 2F). Oxidative stress was not observed at 10 μg.L^{-1}. In contrast, exposure to 100 μg.L^{-1}CIP decreased SOD activity (70.95%; P = 0.0088; Fig. 2B) and caused oxidative stress (increased LPO by 103.76%; P = 0.0051; Fig. 2F).

In the gonads of male fish, CIP at a concentration of 10 μg.L^{-1} increased GPX activity (221.20%, P = 0.0121, Fig. 3C) and GSH content (139.58%, P = 0.0300, Fig. 3D), but no lipid peroxidation was observed. (P = 0.4351, Fig. 3E), compared with the control group. Exposure to the highest concentration (100 μg.L^{-1}) increased GPx by 41.34% (P = 0.0337, Fig. 3C). The lower concentration of CIP did not cause changes in the antioxidant system.

In females, CIP only caused an increase in GPX in the gonads by approximately 82.35% (at 100 μg.L^{-1}, P = 0.0135, Fig. 3C).

3.2.4. Biomarkers of genotoxicity

After exposure to CIP, the micronuclei were not observed in male or female fish. However, some morphological changes were observed, such as blebbed (P = 0.0059), vacuoles (P = 0.0097), and changes in the erythrocyte nuclei (P = 0.0106) at 100 μg.L^{-1}. In females, the vacuolated type was observed at 10 μg.L^{-1} (P = 0.0212) and 100 μg.L^{-1} (P = 0.0171), and nucleated erythrocyte changes were observed at 100 μg.L^{-1} (P = 0.0129) (Supplementary material 4).

DNA damage was observed in the erythrocytes of male fish at 10 μg.L^{-1} CIP (120.37%, P = 0.0059) and at 100 μg.L^{-1} (247.07%, P < 0.001) Fig. 4A. In the females at all concentrations tested caused DNA damage, at 1 μg.L^{-1} (139.89%, P = 0.0303), 10 μg.L^{-1} (81.35%, P = 0.0373) and

Table 1
Hematological biomarkers of *R. quelen* male after exposure to ciprofloxacin for 28 days.

		Environmentally relevant concentrations of ciprofloxacin			
		Control	1 μg.L^{-1}	10 μg.L^{-1}	100 μg.L^{-1}
MALE	Erythrocytes (10^6/μL)	1.93 ± 0.19^{a}	1.98 ± 0.21^{a}	2.34 ± 0.41^{a}	1,99 ± 0.23^{a}
	Hematocrit (%)	22.74 ± 1.26^{a}	23.78 ± 2.15^{a}	21.79 ± 2.17^{a}	24.26 ± 0.93^{a}
	VCM (*f*L)	107.41 ± 7.70 ^{a}	152.17 ± 43.27 ^{a}	101.51 ± 17.76 ^{a}	130.06 ± 25.51 ^{a}
	Thrombocytes (10^3/μL)	36.06 ± 4.02 ^{a}	28.69 ± 5.28 ^{a}	59.76 ± 5.44 ^{ab}	69.00 ± 12.65 ^{b}
	Leukocytes (10^3/μL)	25.24 ± 1.71 ^{a}	14.46 ± 3.56 ^{ab}	29.39 ± 8.23 ^{a}	9.23 ± 2,18^{b}
FEMALE	Erythrocytes (10^6/μL)	1.71 ± 0.08^{a}	2.01 ± 0.13^{a}	1.75 ± 0.16^{a}	1,92 ± 0.11^{a}
	Hematocrit (%)	19.48 ± 0.77^{a}	20.84 ± 0.86^{a}	21.48 ± 1.10^{a}	22.29 ± 1.59^{a}
	VCM (*f*L)	113.49 ± 5.35 ^{a}	105.92 ± 11.09 ^{a}	131.95 ± 12.57 ^{a}	115.20 ± 11.37 ^{a}
	Thrombocytes (10^3/μL)	48.05 ± 13.73 ^{a}	59.93 ± 6.67 ^{a}	45.58 ± 6.84 ^{a}	47.49 ± 4.76 ^{a}
	Leukocytes (10^3/μL)	26.26 ± 2.75 ^{a}	22.72 ± 2.89 ^{ab}	13.47 ± 1.76 ^{b}	12.79 ± 2.99^{b}

The results are expressed as mean ± standard error. Different letters indicate significant differences (P < 0.05). N = 11 males and 09 females (Control); N = 10 males and 10 females (1 μg.L^{-1}CIP); N = 6 males and 14 females (10 μg.L^{-1}CIP); N = 8 males and 12 females (100 μg.L^{-1} CIP).

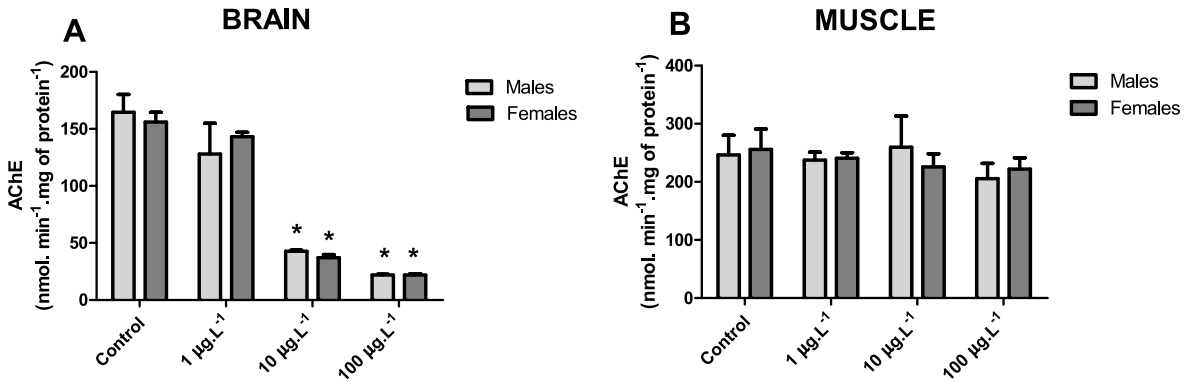

Fig. 1. Acetylcholinesterase activity in the brain (A) and muscle (B) of *R. quelen* after exposure to ciprofloxacin for 28 days. Mean ± standard error. * = Statistical differences compared to the control group ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1 µg.L^{-1}CIP); N = 6 males and 14 females (10 µg.L^{-1}CIP); N = 8 males and 12 females (100 µg.L^{-1}CIP).

at 100 µg.L^{-1} (204.12%, P = 0.0142) Fig. 4B.

In the liver, there was DNA damage in males at exposure to 1 µg.L^{-1} (50.69%, P < 0.001) compared to the control group. There were no changes in females (P = 0.8890). There was no genotoxicity in male gonads (P = 0.5734) and female gonads (P = 0.6805) (Supplementary material 5).

3.2.5. Assessment of ciprofloxacin-induced apoptosis

In the blood of male fish, an increase in the frequency of apoptosis was observed after exposure to 10 µg.L^{-1} (900%, P = 0.0376) and 100 µg.L^{-1} (10.233%, P = 0.0261), Fig. 5A. In females, there was an increase in the frequency of apoptosis after exposure to 100 µg.L^{-1} (12.971%, P = 0.0046), Fig. 5A.

In the liver of male fish there was an increase in the frequency of apoptosis after exposure to 1 µg.L^{-1} CIP (554.31%, P = 0.0417, Fig. 5B). In females, exposure to the three concentrations of CIP caused an increase in apoptosis frequency (602.78% at 1 µg.L^{-1}, P = 0.0029; 367.86% at 10 µg.L^{-1}, P = 0.0302; 554.17% at 100 µg.L^{-1}, P = 0.0030) (Fig. 5B).

3.2.6. Histopathological biomarkers

The liver tissue of *R. quelen* has a similar description to that of other teleost fishes. The parenchyma is composed of hepatocytes, sinusoides, and blood vessels (Fig. 6A). The histopathological index showed higher values at 10 and 100 µg.L^{-1} in a dependent concentration manner (Fig. 6). The histopathological damage observed was leukocyte infiltration (Fig. 6B), steatosis (Fig. 6C), and necrotic foci (Fig. 6D).

Evaluation of histopathological biomarkers separately by sex showed that exposure at 10 and 100 µg.L^{-1} caused leukocyte infiltration (P < 0.001), steatosis (P = 0.0409) and necrosis (P = 0.0034) in the liver tissue in male fish compared to the control group. In females, this damage was observed only in the group exposed at 100 µg.L^{-1} (leukocyte infiltration, P < 0.001; steatosis, P < 0.001; necrosis, P = 0.0413). At 10 and 100 µg.L^{-1}, CIP caused a significant increase in the Bernet index (in a dose-dependent manner) in both sexes (males, P = 0.0561; females, P = 0.0081) (Table 2).

The testis from the control fish showed spermatogenic tubules. At the margin of the lobules, spermatogonia were observed. Large numbers of spermatocytes were observed (Fig. 7A). In the group exposed to 100 µg.L^{-1} CIP, an increase in spermatogenesis was presented. In this treatment, sperm increased significantly compared to the control (P < 0.001, Fig. 7B). Interstitial tissue was observed around the lobules in this treatment (Fig. 7B).

In the ovaries of the control group, the development of oocytes was observed, which was present in the perinuclear oocytes within the cytoplasm including a large rounded nucleus, characteristic of female oocytes at the immature stage of development (Fig. 7C). At a dosage of 100 µg.L^{-1}, CIP impaired the oogenesis process. An increase in gonadal development was observed with an increase in the number of vitellogenic oocytes (P < 0.001, Fig. 7D). Vitellogenic oocytes had a larger diameter and hypertrophic follicular cells (at 100 µg.L^{-1}, Fig. 7D). Histopathological injury index in testes and ovaries showed higher values in individuals exposed to 100 µg.L^{-1} (Supplementary material 6).

3.2.7. Plasma 17 β-estradiol (E2) and 11 keto-testosterone (11–KT) levels

No changes were observed in the estradiol levels in of males (P = 0.3806) and females (*P* = 0.1579) and 11-KT levels in males (P = 0.1900) and females (P = 0.1574) (Supplementary material 7).

3.2.8. Concentration of neurotransmitters

In male fish, exposure to 10 µg.L^{-1} CIP decreased DA concentrations (52.30%, P = 0.0384) and increased the 5-HIIA/5-HT ratio (81.35%, P = 0.0471). The values of 5–HT (p = 0.3135), DOPAC (P = 0.1782), 5–HIIA (P = 0.1660), NA (p = 6148), and the DOPAC/AD ratio (P = 0.2751) did not change. In females, CIP increased at 1 µg.L^{-1}NA (62.65%, P = 0.0405). However, CIP did not change the levels of DA (P = 0.1349), 5-HT (P = 0.6534), DOPAC (P = 0.2128), 5-HIAA (P = 0.0852), and the ratio of DOPAC/DA (P = 0.2023) and 5-HIAA/5-HT (P = 0.0568) (Supplementary material 8).

4. Discussion

4.1. Experimental design

In this study, the experimental design was developed with both sexes in the same aquarium due to the difficulty of differentiating the gender of the fish before the experiment. After the histological results, the relation of males and females are quite similar, only the concentrations of 10 and 100 µg.L^{-1} showed much more females than males. We took this into account in the statistical analyses and with relation to the discussion of the results. Therefore, the aim of this study was to evaluate CIP exposure effects in both sexes. By developing experiments with random fish and both sexes in the same aquarium, we can try to reproduce similar results as observed in a natural habitat. This can be considered as a positive aspect in testing. No abnormal behavior or death was observed during the experiments.

4.2. Hematological biomarkers

Some hematological biomarkers changed in males and females. At 100 µg.L^{-1}, CIP increased thrombocyte counts and decreased leukocyte counts. In females, 10 and 100 µg.L^{-1} caused a decrease in leukocyte counts. Thrombocytes in fish are analogs of thrombocytes in mammals. In Osteichthyes, these cell fragments are involved in homeostatic

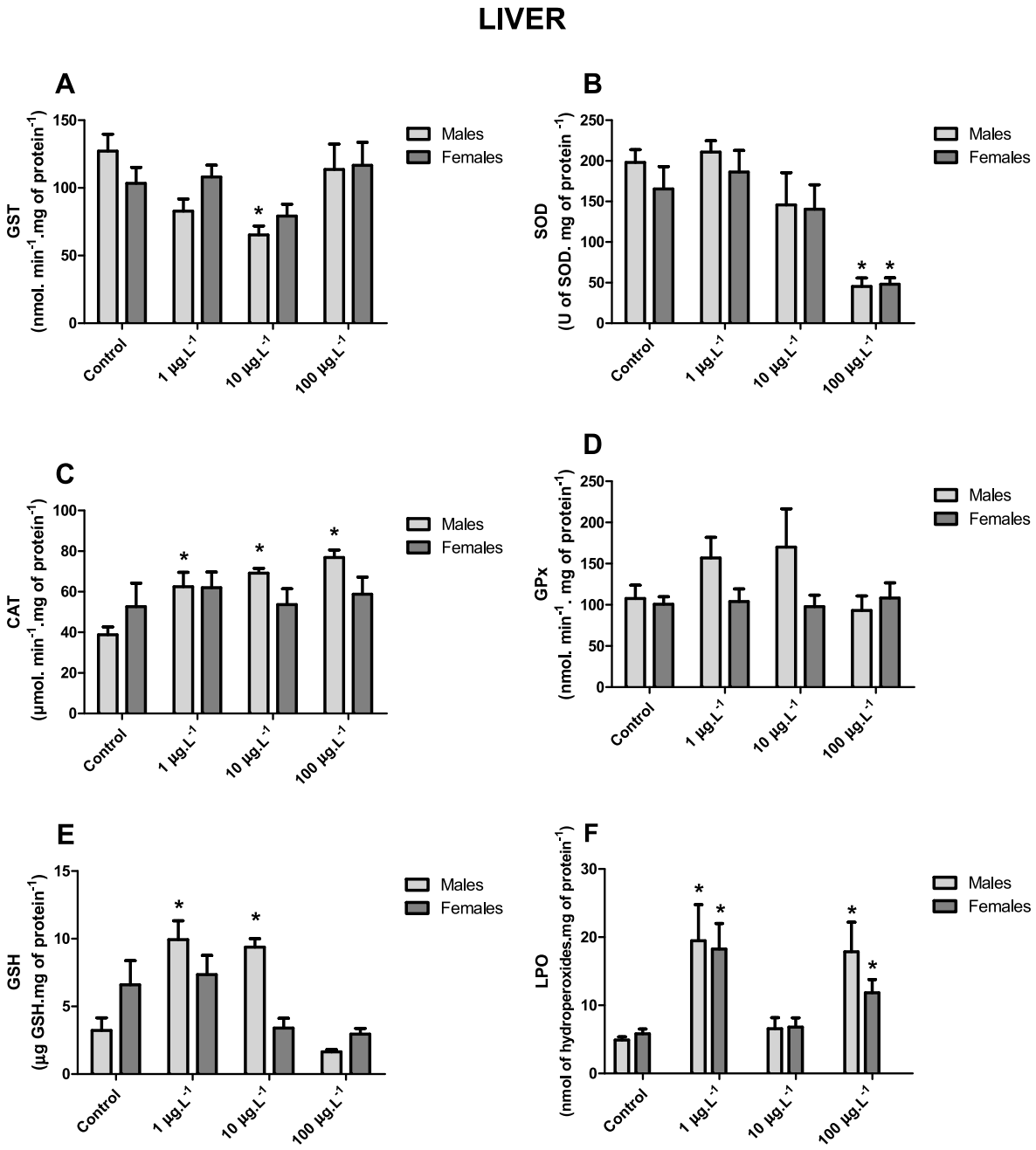

Fig. 2. Biochemical biomarkers in the liver of *R. quelen* males and females after 28-day exposure to ciprofloxacin. Glutathione S-transferase (GST) (**A**), superoxide dismutase (SOD) (**B**), catalase (CAT) (**C**), glutathione peroxidase (GPx) **(D)**, reduced glutathione (GSH) (**E**), and lipid peroxidation (LPO) (**F**). Mean ± standard error. * = Statistical differences compared to the control group ($P < 0.05$). ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1 µg.L^{-1}CIP); N = 6 males and 14 females (10 µg.L^{-1}CIP); N = 8 males and 12 females (100 µg.L^{-1}CIP).

processes and in the development of the immune response, with a phagocytosis capacity (Stosik et al., 2019; Witeska et al., 2022). Leukocytes also play an essential role in the body's defense against xenobiotics through phagocytosis (Chen et al., 2002). Leukopenia observed in our study may increase the susceptibility towards a microbial invasion and allow the spread of infections (Barton and Zwama, 1991; Sumpter, 1997). Leukopenia is known to be a crucial physiotherapeutic measure for harmful substances (Gonçalves et al., 2010). According to our results, the decrease in leukocytes in the blood may be accompanied by an increase in leukocytes in the liver, characterized by leukocyte infiltration in the tissue. Corroborating our results, Kitamura et al. (2022), reported a decrease in leukocytes in the blood and a leukocyte infiltration into the liver after 100 µg.L^{-1}CIP from a short-term exposure.

4.3. Biomarkers of genotoxicity in the blood

We observed nuclear abnormalities after CIP exposure (in males at 100 µg.L-1 and in females at 10 and 100 µg.L^{-1}) without the induction of micronuclei. Ayllon and Garcia-Vazquez (2000) found the induction of nuclear abnormalities without a presence of micronuclei. As blood is not a hematopoietic tissue, the cell multiplication index is practically non-existent and it is normal not to find micronuclei in 2000 cells. This has already been highlighted in several studies (Calado et al., 2020; Oya-Silva et al., 2021; Santos et al., 2022).

There is ongoing research and discussion about using nuclear

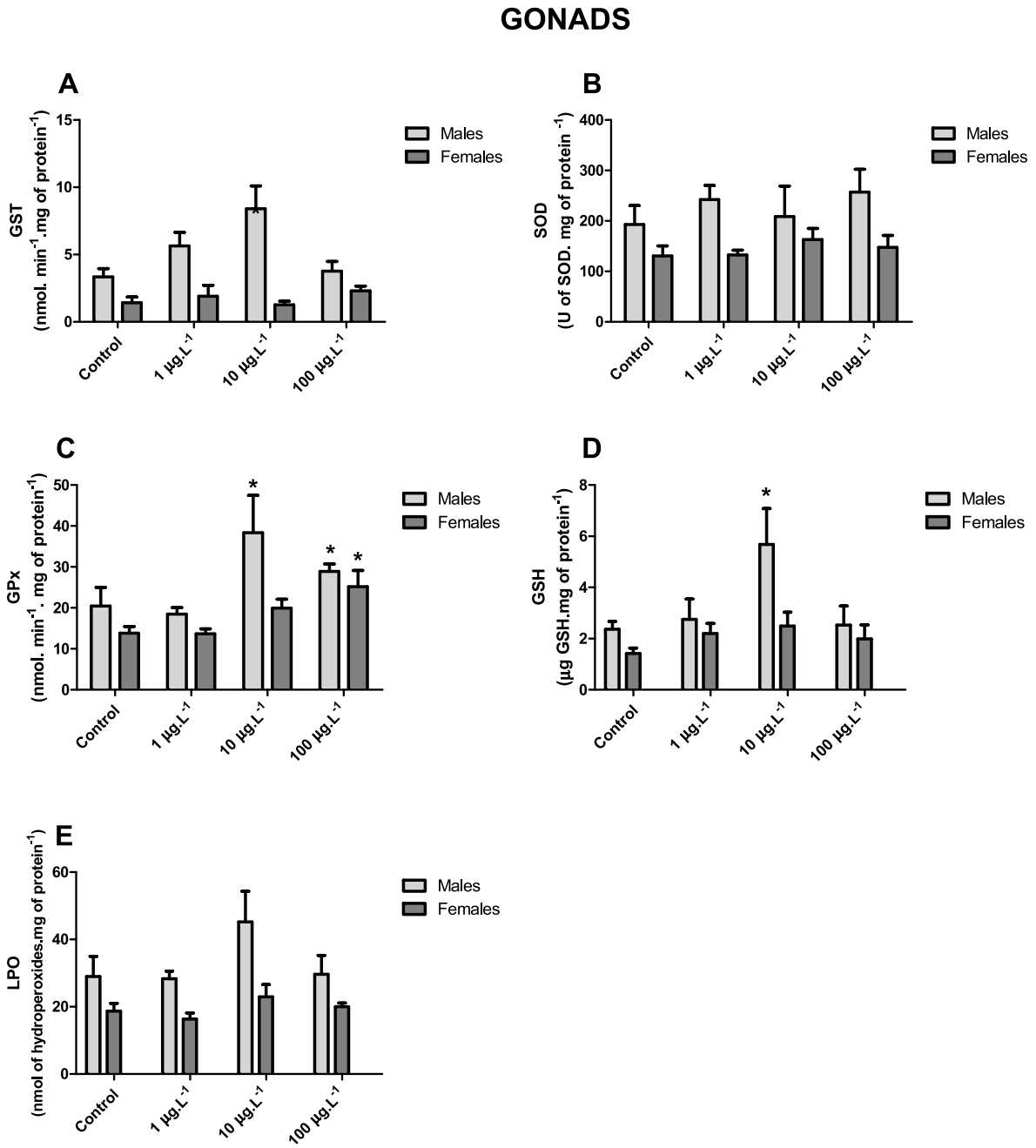

Fig. 3. Biochemical biomarkers in the gonads of male and female *R. quelen* after 28-day exposure to ciprofloxacin. Glutathione S-transferase (GST) (**A**), superoxide dismutase (SOD) (**B**), glutathione peroxidase (GPx) (**C**), reduced glutathione (GSH) **(D)**, and lipid peroxidation (LPO) (**E**). Mean ± standard error. * = Statistical differences among treatments compared to the control group ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1 µg.L^{-1}CIP); N = 6 males and 14 females (10 µg.L^{-1}CIP); N = 8 males and 12 females (100 µg.L^{-1}CIP).

erythrocyte alterations to indicate genotoxic damage in human and nonhuman models. Some studies have suggested that these alterations may be useful biomarkers for evaluating the genotoxic effects of various environmental (Hayashi, 2016) and occupational exposures (Bonassi et al., 2007). However, there are also some limitations and challenges associated with using nuclear erythrocyte alterations as a biomarker for genotoxic damage because the nuclear abnormalities and micronuclei may not have a common origin (Krupina et al., 2021).

Rodrigues et al. (2016) observed nuclear abnormalities in peripheral erythrocytes of *O. mykiss* after acute and chronic exposure to oxytetracycline, another antibiotic. DNA damage occurred in the erythrocytes of males in all three CIP contractions and in females only at 10 and 100 µg. L^{-1}. DNA damage in *Danio rerio* blood cells was observed after acute exposure to CIP alone and with a mixture of acetaminophen (Elizalde-Velázquez et al., 2022). The genotoxicity caused by CIP, such as nuclear abnormalities and damage to erythrocyte DNA, can result from topoisomerase inhibition. We already know that the primary mechanism of action of fluoroquinolones is to interfere with DNA synthesis by affecting two enzymes; DNA gyrase (topoisomerase II) and DNA topoisomerase IV. In a bacterial cell, these two topoisomerases take action by unwinding the DNA double helix (Champoux, 2001). However, eukaryotic organisms also have topoisomerases that play vital roles in the metabolism of DNA. DNA topoisomerase II is essential for various nuclear processes, such as DNA replication, chromosome segregation, and the maintenance of the chromosome structure (Bakshi et al., 2001). Type II is also a cellular target for a variety of clinically relevant

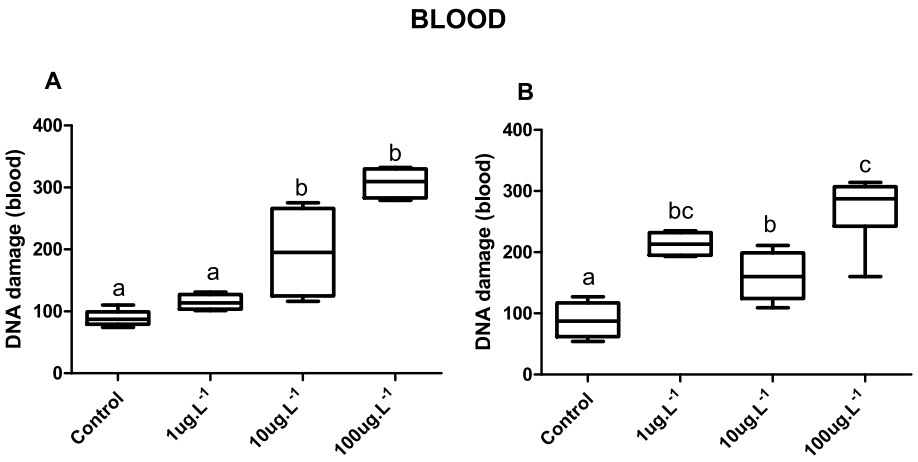

Fig. 4. DNA damage score (median ± interquartile range) in blood of *R. quelen*, (**A**) male and (**B**) female animals, exposed to three concentrations of CIP for 28-day. Different letters indicate significant differences ($P \leq 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1 μg.L^{-1}CIP); N = 6 males and 14 females (10 μg.L^{-1}CIP); N = 8 males and 12 females (100 μg.L^{-1}CIP).

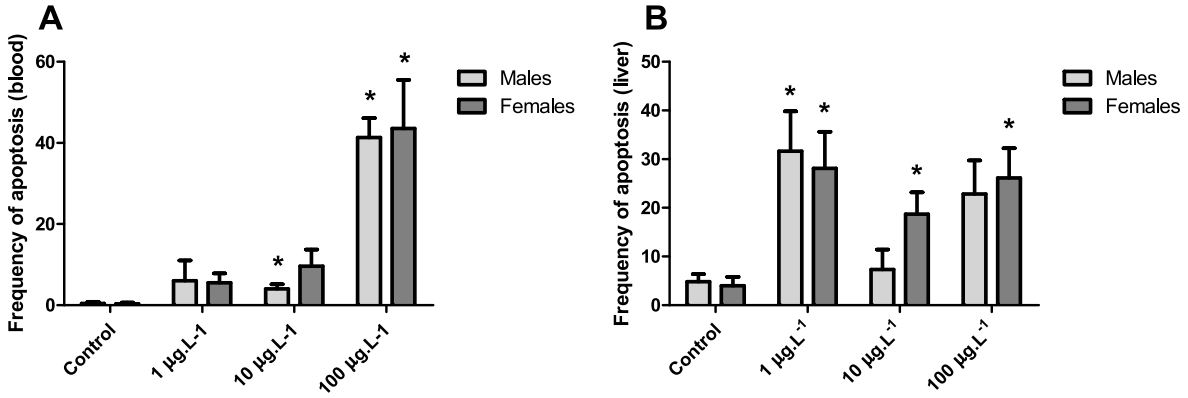

Fig. 5. Frequency of apoptosis in blood (A) and liver (B) of male and female *R. quelen* exposed to three concentrations of CIP for 28-day. * = Statistical differences among treatments compared to the control group ($P < 0.05$). N = 11 males and 09 females (Control); N = 10 males and 10 females (1 μg.L^{-1}CIP); N = 6 males and 14 females (10 μg.L^{-1}CIP); N = 8 males and 12 females (100 μg.L^{-1}CIP).

antitumor drugs (Bakshi et al., 2001) and for quinolones-class antibiotics (Champoux, 2001).

4.4. Assessment of ciprofloxacin-induced apoptosis in the blood

Diffusion assays showed that exposure to CIP at a concentration of 100 μg.L^{-1}, caused apoptosis of blood cells. Apoptosis or programmed cell death is characterized by cell shrinkage, chromatin condensation, formation of cytoplasmic opacities, and apoptotic bodies (Ziegler and Groscurth, 2004). Multiple factors are involved in apoptosis, and different apoptotic stimuli converge in a common apoptotic pathway mediated by the mitochondria (Kroemer, 2003; Wang, 2001). Apoptosis via the mitochondrial pathway may be preceded by overproduction of reactive oxygen species (ROS) (Liu et al., 2015; Spierings et al., 2005). Although we did not measure the production of ROS, exposure to CIP may have caused an overproduction of ROS by oxidative stress in hepatocytes, as demonstrated in the liver. A significant increase of CAT activity was observed at 1, 10 and 100 μg^{-1}, an indirect indicative of ROS excess in the male hepatocytes (Fig. 2C). This overproduction of ROS may have been a stressor that triggered intrinsic apoptosis (Ott et al., 2007). Herold et al. (2002) reported that CIP induced apoptosis and inhibited the proliferation of human colorectal carcinoma cells. Growth arrest was mediated by inhibiting DNA synthesis, an induction of mitochondrial damage, and thus, leading to apoptosis (Herold et al., 2002).

4.5. Neurotoxicity biomarker

In this study, we used AChE activity as a biomarker for neurotoxicity. The enzyme is essential for maintaining the nervous system, and its inhibition is considered an indicator of environmental pollution in aquatic organisms (Li et al., 2012). A reduction in the activity of this enzyme may lead to physiological and behavioral changes, which may result in an effects pertaining to survival, feeding, and reproductive behavior (Weis et al., 2001). Our data showed a significant reduction in AChE activity in the brain at 10 and 100 μg.L^{-1} CIP, in both sexes. Liu et al. (2014) found a similar result, in which the brain activity of acetylcholinesterase was significantly reduced by norfloxacin at 0.04 mg.L^{-1} after 4 and 7 days.

4.6. Biochemical biomarkers in the liver

The hepatic GST plays an essential role for protecting cellular components against various toxic effects and oxidative stress (Sen and Semiz, 2007). It has also been demonstrated that the role of GST in oxidative stress is of conjugating endogenously produced electrophiles such as membrane lipid peroxides in animals (Halliwell and Gutteridge, 1999). According to our present results, GST activity decreased only at 10 μg.L^{-1}. This can suggest a loss of protection against damage induced by CIP at this concentration. However, lipoperoxidation was not observed. There was an increased frequency of apoptosis in the

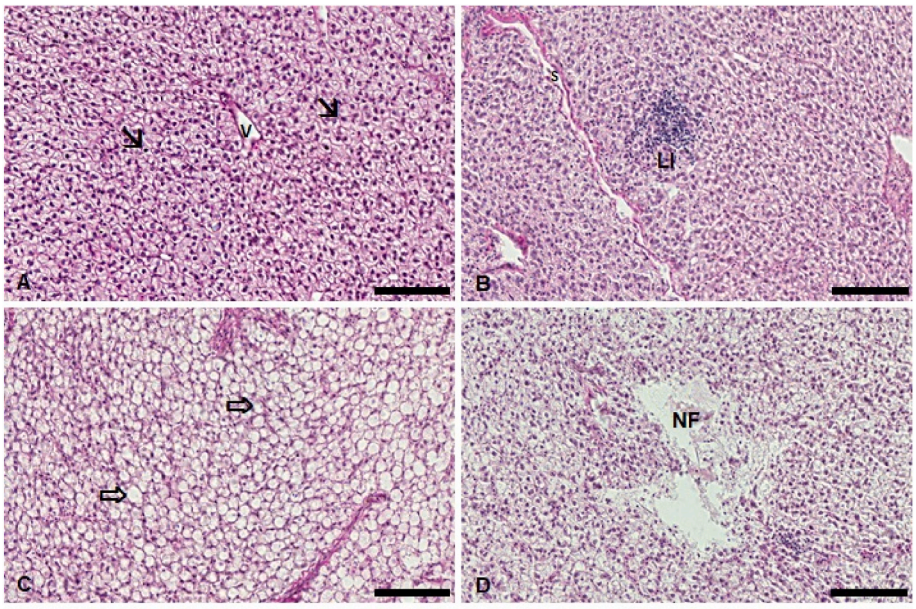

Fig. 6. Histological sections of *R. quelen,* liver counter stained with hematoxylin/eosin. **(A)** Liver of the control group. Blood vessels (**V**) and hepatocyte nuclei (↘). **(B)** Liver of the exposed group (10 μg.L^{-1}). Sinusoid (**S**) and leukocyte infiltration **(LI)**. **(C)** Liver of the exposed group (100 μg.L^{-1}). Steatosis (A) (**D**) Liver of the exposed group (100 μg.L^{-1}). Necrotic foci (**NF**). Scale bar: 100 μm.

Table 2
Mean values (score changes) for histopathologic effects in liver tissue after 28-day exposure of *R. quelen*, males and females, to three concentrations of ciprofloxacin (1, 10, and 100 μg.L^{-1}) and the control group.

	Tissue/Treatments	Histopathology Biomarkers			
		LI	S	N	Bernet Index
MALES	Control	0.00	0.36	0.36	1.45
	1 μg.L^{-1}	0.00	0.00	0.40	1.20
	10 μg.L^{-1}	2.00*	2.40*	2.80*	14.80*
	100 μg.L^{-1}	1.75*	3.25*	2.75*	15.00*
FEMALES	Control	0.00	0.20	0.40	1.40
	1 μg.L^{-1}	0.00	0.40	0.40	1.60
	10 μg.L^{-1}	1.14	1.71	1.43	8.29*
	100 μg.L^{-1}	2.00*	2.31*	3.08*	15.54*

* = Statistical differences among treatments compared to the control group ($P < 0.05$). LI: Leukocyte infiltration; S: Steatosis; N: Necrosis. N = 11 males and 09 females (Control); N = 10 males and 10 females (1 μg.L^{-1}CIP); N = 6 males and 14 females (10 μg.L^{-1}CIP); N = 8 males and 12 females (100 μg.L^{-1}CIP).

hepatocytes in females (Fig. 5 B), and necrosis in males (Table 2). In males, leukocyte infiltration and steatosis were also observed. Results showed an increase in the Bernet's index in both sexes, and the blood genotoxicity with apoptosis in males. One other study only suggests that GST activity increased at ciprofloxacin concentrations of 0.7 and 100 μg.L^{-1} of a juvenile of *D. rerio* for 28 days (Plhalova et al., 2014).

SOD activity decreased in males and females after exposure to 100 μg.L^{-1}. SOD is an antioxidant enzyme that converts superoxide (O_2^-) to H_2O_2., and exposure of *C. mrigala* to CIP for 5, 10, and 15 days also increased SOD activity in the liver (Ramesh et al., 2021).

CIP caused an increase in CAT activity at the three concentrations in male fish; however, it was not altered in females. CAT it an enzyme specializing in splitting hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2) (Van der Oost et al., 2003). Acute exposure to CIP increased the activity of CAT in *R. quelen* at 10 μg.L^{-1} (Kitamura et al., 2022) and in *C. auratus* exposed to norfloxacin (Liu et al., 2014).

GSH levels increased after exposure to 1 and 10 μg.L^{-1} (in males), but as in CAT, there were no changes in females for this biomarker. GSH is a nonenzymatic component of great importance to the antioxidant system. It acts as a cofactor for GPx, in addition to having other functions of great importance for the detoxification process, such as in phase II of metabolization (Van der Oost et al., 2003).

CIP caused lipid peroxidation in male and female animals at the lowest and highest concentrations (1 and 100 μg.L^{-1}). We observed a non-monotonic concentration-response curve. Endogenous disruptors are known to act via non-monotonic curves, and therefore it has been argued that the effects observed at high doses cannot be used to conclude the low dose range (Carnevali et al., 2018). Non-monotonic curves for biological responses at low concentrations of contaminants have been observed for contaminants such as bisphenol-A and atrazine (Vandenberg et al., 2012). This effect has also been accepted for pharmaceutical products (Wilkinson et al., 2016). Interestingly, in our study, we observed that in the group treated with 100 μg.L^{-1} CIP demonstrated an increase in the gonadal development in both sexes.

Ramesh et al. (2021) studied the effects of CIP on C. mrigala and observed an increase in LPO in the treated groups. CIP also induced a significant oxidative stress response in the liver of *D. rerio* after acute exposure (Elizalde-Velázquez et al., 2022). Lipid peroxidation significantly contributes to the loss of cell function under oxidative stress (Storey, 1996). During oxidative stress, cytotoxic reactive oxygen species (ROS) can react with critical cellular macromolecules, thus, possibly leading to enzymatic inactivation, lipid peroxidation (LPO), DNA damage, and occasional cell death (Van der Oost et al., 2003).

Our data showed differences in enzymatic responses concerning gender, and, possibly, in the sensitivity to xenobiotics. However, the effect of lipid peroxidation on liver tissue was the same in males and females. Our study observed more changes to the antioxidant system in males than in females.

4.7. Biomarkers of genotoxicity in the liver

In the present study, damage to hepatocyte DNA was observed in the liver of male fish at an exposure of 1 μg.L^{-1}, which was possibly caused by the generation of reactive oxygen species (Yang et al., 2020) even through the mechanism of action of CIP as discussed by Bakshi et al. (2001). In the female fish, liver genotoxicity was not observed. Acute exposure of *R. quelen* to CIP has been reported to damage DNA (Kitamura et al., 2022). In another acute study with *D. rerio* (Elizalde-Velázquez et al., 2022), CIP also caused damage to hepatocyte DNA

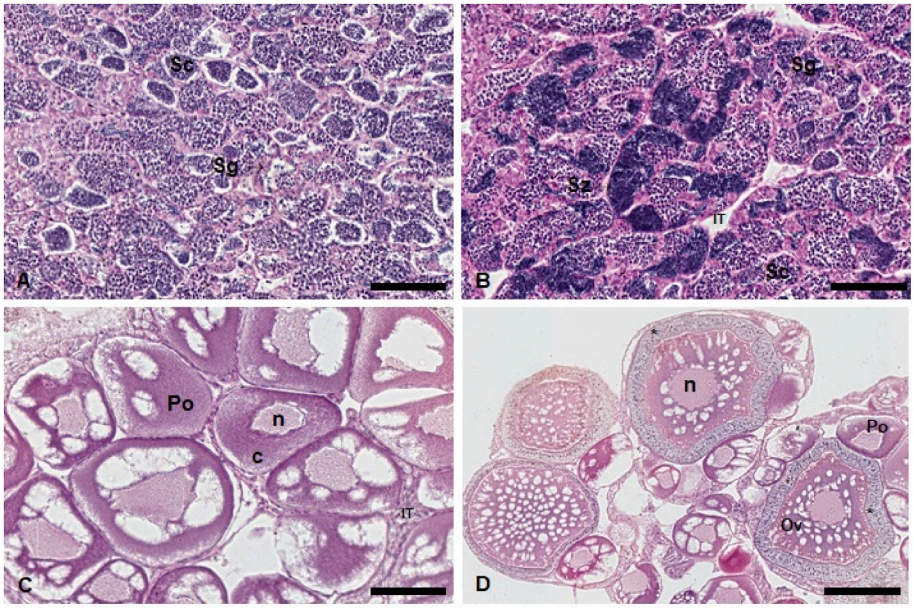

Fig. 7. Histological sections of gonads of *R. quelen*, counterstained with hematoxylin/eosin. (**A**) Male testis of the control group. Spermatocytes (**SC**) and spermatogonia (**Sg**). (**B**) Male testis of the exposed group (100 μg.L^{-1} of CIP). Spermatocytes (**SC**), spermatogonia (**Sg**), spermatozoa (**Sz**) and interstitial tissue (**IT**). (**C**) Female ovary of control group (**C**) Perinucleolar oocytes (**Po**) with different size and shape, with nucleus (**n**) and cytoplasm (**c**). (**D**) Female ovary of the exposed group (100 μg.L^{-1}). Perinucleolar oocytes (**Po**), nucleus (**n**), vitellogenic oocyte (**Ov**) and hypertrophy of follicular cells (*). Scale bar: 100 μm.

at all concentrations tested (0.250, 0.500, and 1 μg.L^{-1}). Xenobiotics-induced genotoxicity is mainly due to the inhibition of the DNA repair process (Hartwig and Schwerdtle, 2002). Genotoxicity is usually accompanied by changes in the fish's physiological and biochemical biomarkers (Yang et al., 2020).

4.8. Assessment of CIP-induced apoptosis in the liver

Exposure to 1 μg.L^{-1} increased the frequency of apoptosis in male liver tissue. At a concentration of 100 μg.L^{-1}, there was an increase of 372.91%, but it was insignificant compared to the control group. In the females, the frequency of apoptosis increased at all three CIP concentrations. Oxidative stress damage can lead to a permeabilization of the outer mitochondrial membrane, resulting in the release of pro-apoptotic proteins and the activation of caspase to in order to maintain tissue homeostasis (Spierings et al., 2005). To date, only one study (Elizalde-Velázquez et al., 2022) has reported CIP-induced oxidative stress and an upregulation of genes related to apoptosis (BAX, CASP3, CASP6, CASP8, and CASP9), and oxidative stress (Nrf 1 and Nrf 2), thus, further supporting our findings on the hepatotoxicity and genotoxicity of CIP. Liu et al. (2015) investigated the effects of enrofloxacin in grass carp, and the cell damage caused by enrofloxacin was confirmed through the application of apoptosis assays.

4.9. Hepatosomatic index and histopathological biomarkers in the liver

Another biomarker used in this study was HSI, a proven marker of liver stress (Al-Ghais, 2013; Kumar et al., 2017). In males, exposure to 1 μg.L^{-1} CIP decreased the HSI, while this parameter did not change in females. The pattern of HSI changes suggest that males and females may respond differently to CIP exposure. Other studies targeting fish gender, also report significant differences in HSI after drug exposure (Flippin et al., 2007; Koporikov and Bogdanov, 2013).

Hepatic histopathological evaluation showed lesions such as leukocyte infiltration (inflammation), steatosis, and necrosis in both sexes at 10 and 100 μg.L^{-1}. Data are comparable to the short-term studies by Kitamura et al. (2022) and by Elizalde-Velázquez et al. (2022). The injury index showed a concentration-dependent relation (in both sexes). The observed steatosis may be a consequence of the disorder's actions resulting from the action of toxic products and, in several processes, precedes necrosis. The necrosis can have several causes, such as converting a contaminant to a metabolite toxic that may include free radicals or reactive oxygen. These leads to peroxidative damage to membrane lipids and hence necrosis. Regarding the leukocyte infiltrates, it is a nonspecific response produced by chemical agents or pathogens. Its function is to maintain the integrity of the tissue when physical or chemical stimuli affect the liver tissue (Rocha et al., 2010). Therefore, an extensive area of leukocyte infiltration in the liver indicates that this tissue was suffering from a significant influence of CIP (Matushima et al., 2006).

4.10. Biochemical biomarkers in the gonads

In the gonadal tissue, enzymatic changes occurred only at the intermediate concentration of 10 μg.L^{-1}. GPx activity and GSH levels increased. There is little data on evaluating the gonads of fish exposed to fluoroquinolones. We did not detect DNA damage in the gonads of males and females. In contrast, Liu et al. (2014) reported a strong and positive correlation between the extent of DNA damage in the gonads and the concentrations of norfloxacin (another fluoroquinolone) alone, and in combination with sulfamethoxazole during a 7-day exposure.

4.11. Histopathological biomarkers in the gonads

Histopathological analysis showed that both sexes had altered gonadal tissue after exposure to CIP at the highest concentration. In the males, CIP exposure impaired spermatogenesis and stimulated testicular development. Spermatozoa increased significantly compared to the control group. In female gonads, CIP impaired the process of oogenesis and stimulated gonadal development. Although we were unable to evaluate vitellogenin, Liu et al. (2014) examined the concentration of vitellogenin after exposure to norfloxacin alone, and in combination with sulfamethoxazole. The highest concentration of norfloxacin and two higher concentrations of the sulfamethoxazole mixtures increased the serum's concentration of vitellogenin. These data corroborate our results showing that CIP can increase the number of vitellogenic oocytes. These results are significant because they show that fluoroquinolones such as norfloxacin and CIP can affect the gonads of fish and potentially affect reproduction.

4.12. 17 β-estradiol (e2) and 11 keto-testosterone (11–kt) plasma levels

However, these effects did not lead to changes in IGS and in the levels of E_2 and 11-KT, possible because the samples were taken 28 days after exposure, when the gonads had already developed (Chaves-Pozo et al., 2008). Consequently, E_2 and 11 KT secretion ceases and the biosynthetic pathway turns to the secretion of progestin, which initiates the final maturation of the oocytes or testes (Hachfi et al., 2012). In goldfish exposed to bisphenol A, the gonadosomatic index (GSI), hepatosomatic index (HSI), and E2 levels were not affected, nevertheless, an increase in hepatic vitellogenin was induced.

4.13. Concentration of neurotransmitters

In the hypothalamus of male fish, 10 $\mu g.L^{-1}$ CIP caused a decrease in the levels of DA, suggesting a stimulatory effect on the HPG axis. Therefore, an increase in the 5-HIIA/5-HT ratio was observed. It should be noted that at 10 $\mu g.L^{-1}$, no significant increase in sperm frequency was observed in the males, and a no significant increase in vitellogenic oocytes were observed in females. However, this was not reflected in hormonal and histopathological changes by the end of the experiment, which may indicate the onset of the process. In females, exposure to 1 $\mu g.L^{-1}$ CIP increased NA levels (62.65%). This result could indicate an initial neuroendocrine stimulus. It is possible that the higher concentrations of CIP triggered the development of the gonads in the male and female fish at 28 days after the initial and continued exposure, while the lower concentrations did not do so until a more long-term exposure.

4.14. Integrative discussion

The results showed that the sex can influence the response of biomarkers. Exposure of fish to environmental concentrations of CIP showed that responses to biomarkers were more similar in males and females when exposed to the highest concentration (100 $\mu g.L^{-1}$). In this treatment, leukopenia, nuclear abnormalities, erythrocyte DNA damage, and an increased frequency of apoptosis were observed in both the males and females. Cerebral neurotoxicity and hepatic histopathological changes were also observed in both sexes.

Exposure to an intermediate concentration of CIP (10 $\mu g.L^{-1}$) caused different effects in the males and females. The number of leukocytes only decreased in the females. On the other hand, both the males and females presented blood genotoxicity and neurotoxicity. In addition, an increase in the frequency of apoptosis was observed in the males. At this concentration, antioxidant system changes were not observed in the females. In males a decrease in GST activity, and an increase of GSH levels were observed. However, the frequency of apoptotic hepatocytes increased in the females.

The response of genotoxicity biomarkers in the blood showed that females were more sensitive than males. In females, there were nuclear abnormalities in the red blood cells at 10 and 100 $\mu g.L^{-1}$, while in males, only at 100 $\mu g.L^{-1}$. In females, all CIP exposures caused DNA damage to the erythrocytes; including the lower concentrations found in rivers and public water supplies. However, in the males, only at 10 and 100 $\mu g.L^{-1}$ levels. The frequency of apoptosis was the only biomarker in males, which showed an increase after exposure to CIP at 10 and 100, and in females only at the highest concentration.

At 1 and 10 $\mu g.L^{-1}$ an increase in different responses were observed between the sexes. At the lowest concentration, we observed that only the females presented blood genotoxicity. Both sexes presented lipid peroxidation in the liver. However, in this treatment, hepatic genotoxicity was observed in the males but not in the females. In contrast, both sexes had an increased frequency of hepatocyte apoptosis. Interestingly, this increase was observed in females at all CIP concentrations, while in males, it was only at 1 $\mu g.L^{-1}$. It seems that the antioxidant system responses were insufficient to prevent damage at this concentration.

5. Conclusion

The present study showed that long-term environmental concentrations of CIP cause adverse effects on non-target organisms. CIP caused oxidative stress, genotoxicity, apoptosis, and histopathological changes such as the infiltration of leukocytes, steatosis, and necrotic foci in the liver. In addition, CIP caused the development of female and male gonads. Moreover, it was demonstrated that there was a difference between biomarker responses concerning the male or female variety of the fish, especially in the hematological, biochemical, and genotoxic biomarkers. Some of these alterations may affect the health of fish and indirectly influence essential behaviors such as locomotion, predation, reproduction, and feeding through the decrease in AChE. These results suggest that environmental exposure to CIP needs to be of concern in relation to risk assessment.

Author contribution statement

Lucicleide Ângelo Silva Jungles de Carvalho designed and made the experiments, performed of formal analysis, conceptualization, investigation and wrote the paper; Maiara Carolina Perussolo corroborated with methodology (hematological biomarkers) and conceptualization; Júlio César Moreira Brito performed chemical analysis, conceptualization and revise the paper; Allan Arnold Evans contributed to the English editing and revision of the manuscript; Gisele de Oliveira Guaita performed neurotransmitters analysis, conceptualization and revised the paper; Laís Fernanda Oya-Silva and Marta Margarete Cestari performed the supervision and corroborated with methodology (genotoxicity biomarkers); Maritana Mela Prodocimo performed the supervision and corroborated with methodology (Histopathological biomarkers), conceptualization and wrote the paper; Tarcio Teodoro Braga wrote the paper and gave technical support and conceptual advice, Helena Cristina Silva de Assis designed the experiments, wrote the paper and gave technical support and conceptual advice.

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.2023.139216.

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