BIOSCIENCE JOURNAL

EXPOSURE TO THE HERBICIDE ATRAZINE INDUCES OXIDATIVE IMBALANCE, MORPHOLOGICAL DAMAGE AND DECREASED SURVIVAL IN JUVENILE FISH

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How to cite: SILVA, S.B., et al. Exposure to the herbicide atrazine induces oxidative imbalance, morphological damage and decreased survival in juvenile fish. *Bioscience Journal*. 2023, **39**, e39034. https://doi.org/10.14393/BJ-v39n0a2023-65784

Abstract

Synthetic herbicides have been intensively used in weed control, although often involved in environmental contamination, critically affecting non-target species. However, never was investigated the effect of commercial formulation using atrazine on developing juvenile fish exposed for 35 days. Juveniles (*Astyanax altiparanae*) (n = 600) were assigned to the following ATZ-exposed groups: 0 (CTR-control), 0.56 (ATZ0.56), 1.00 (ATZ1.00), 1.66 (ATZ1.66) and 11.66 (ATZ11.66) µg/L. We found a 36.6% decrease in juvenile survival rate in the ATZ11.66 group compared to control and other groups. Juveniles from ATZ11.66 also showed hyperglycemia and increased cortisol levels. Increased the imbalance oxidative with an increase in malondialdehyde (MDA) and Carbonylated proteins levels markers in muscle, gills, and liver. We also found increased activity of the antioxidant enzymes superoxide dismutase (SOD) in gills and SOD and catalase (CAT) in muscles from ATZ11.66 fish, and increased glutathione S-transferase (GST) activities in the liver from all exposed groups compared to control. The morphological consequences of this were loss of secondary lamella integrity, increased mucus-secreting cells, hyperplasia, and lamellar fusion, as well as increased aneurysms percentage. The liver showed vascular congestion associated with endothelial hyperplasia, steatosis, and a decrease in the nuclei percentage. Our results showed that exposure to a commercial formulation of ATZ at 11.66 µg/L can be causing an imbalance in the oxidative markers and morphological damages and decreased survival in a juvenile Neotropical species of great ecological relevance and commercial interest.

Keywords: Ecotoxicology. Oxidative stress. Survival rate. Water pollutants.

1. Introduction

The accelerated agricultural development, aiming at optimizing the production of essential foods, added to the emergence of plants resistant to pests, are factors that contribute to the increase in the consumption of pesticides worldwide (Carvalho 2017). Annually, this consumption is higher in Neotropical regions, with herbicides accounting for 47.5% of the total world consumption of pesticides. Although they optimize agricultural production, pesticides have been impacting animal and human health, releasing residues that contaminate water resources and the ecosystem as a whole (Grewal et al. 2017).

Atrazine (ATZ) is a triazine-like herbicide introduced worldwide in 1958, widely used in soybean and sugarcane crops (Shelley et al. 2012). This herbicide has been frequently reported in water bodies due to

runoff and leaching processes, threatening aquatic fauna (Xing et al. 2012). ATZ activity is based on electrons flow interruption in photosystem II, inducing the formation of reactive oxygen species (ROS) across membranes and causing the death of weeds (Mela et al. 2013).

Among ATZ known effects in non-target organisms, endocrine alterations and oxidative stress in wildlife species have been reported, are the changes to the structure of thyroid follicles of a crocodilian species (Galoppo et al. 2020), oxidative stress in fish gills *Prochilodus lineatus* (Paulino et al. 2012a) and histopathologies in the liver of catfishes, such as steatosis, leukocyte infiltrate and vacuolization of hepatocytes (Mela et al. 2013). In rats, oral exposure to 200 mg/kg of atrazine caused an increase in testicular weight and seminiferous tubules dilatation (Martins-Santos et al. 2017). It is accepted that these alterations can be leading to the imbalance of redox metabolism. These imbalances can promote the increase of reactive oxygen species (ROS) and behavioral changes characterized by weight loss, psychomotor retardation, and cognitive dysfunction in rats (Luca and Luca 2019).

Fish are constantly exposed to pesticide residues found in the aquatic environment, which may impair the growth, development, and survival of wild species through excessive ROS production, causing an imbalance in oxidant-antioxidant status that leads to tissue damage (Paulino et al. 2012a). In the early stages of life, the integrity of metabolic-related tissues and endocrine glands is crucial for the proper regulation of the rapid growth, development, and sexual maturation involved (Dehnert et al. 2018). Considering this, the developing stage represents a highly susceptible period to environmental contamination, due to the endocrine disruption and developmental impairment that might be associated (Brander et al. 2016). Thyroid hormones are among the main chemical mediators playing important roles in early developmental stages through stimulation of cell differentiation, metabolic rate, growth, and reproduction. Regardless of the importance of this developmental window, pesticides toxicity in juvenile fish is less studied than in adults (Naidoo and Glassom 2019).

Lambaris (*Astyanax altiparanae)* is one of the most cultivated native fish in South America, is an important component of Neotropical aquaculture (Carneiro-Leite et al. 2020). Due to its small body size and easy adaptation to captivity, this species is becoming a good model in aquaculture studies, although in ecotoxicological research its use is still incipient. In the Neotropics, lambaris participate in different trophic levels and contribute to Brazilian aquifers balance (Destro et al. 2021).

Considering all these aspects, we decided to run this study aiming to investigate the toxicity of environmentally relevant concentrations of an ATZ-based commercial herbicide during subchronic exposure (35 days) on survival; blood glucose levels and plasma hormone concentrations; oxidative status; gills and liver histopathological parameters in juvenile lambaris.

2. Material and Methods

Ethics Approval

This study was performed according to the rules of the Ethics Committee on the Use of Production Animals from the Universidade Federal de Viçosa, Brazil (registration nº 043/2017).

Chemicals

The commercial formulation of the herbicide Atrazine – (ATZ, Albaugh/Argentina manufacturer) - (Atanor® 50 SC, (50,0% (w/v) atrazine and 58,4% (w/v) inert ingredients) was obtained from a specialized manufacturer. We previously prepared stock solutions of ATZ at 7.5 µg/mL weekly and stored them in the dark at 4° C. Using the stock solution, 1, 2, 4, and 20 mL were added to 15 L of water, which resulted in measured concentrations of 0.56, 1.00, 1.66 and 11.66 µg/L, respectively. Although the United States Environmental Protection Agency (EPA) has estimated the risk of chronic exposure to pesticides and their degradants (ATZ - 5 µg/L for fish) (EPA 2019), this study focused on using concentrations based on the maximum allowable limits of atrazine determined by Brazilian legislation, which are 2 µg/L for human consume, 5 µg/L for animal watering and 10 µg/L for irrigation (CONAMA 2008).

Experimental design

Juvenile fish from the species *A. altiparanae* (n = 600), 20 days post-hatching, were obtained from the fish farm PEIXECOM (Porciúncula, Rio de Janeiro, Brazil) and allocated into water tanks at the Federal University of Viçosa, Viçosa, Minas Gerais, Brazil. Juveniles were acclimated for 8 days in 500-liter tanks, containing dechlorinated aerated water under a 12:12 light: dark period (h). Fish were fed with commercial extruded feed with 28% crude protein, weighed (0.70 ± 0.076 g), counted, and randomly allocated in 20 different 60L tanks (n = 30/tank), for 8 days before the exposure period. After this period, the water tanks were prepared to have 4 replicates of the nominal concentrations of 0.5, 1, 2, and 10 µg/L of ATZ, which resulted in the measured concentrations of 0.00 (CTR, control), 0.56 (ATZ0.56), 1.00 (ATZ1.00), 1.66 (ATZ1.66) and 11.66 (ATZ11.66) µg/L. The water was renewed in tanks every 72 hours, to avoid the stress caused by handling and considering the half-life of atrazine in water at pH 7.0 - 8.5, which can reach months (Comber 1999). Water parameters were measured weekly. All fish were exposed to different concentrations of ATZ for 35 days.

Sampling

Following the 35-day exposure period, fish in all tanks were counted, weighed, and euthanized using 500 mg/L of Ethyl 3-aminobenzoate methanesulfonate (MS-222). According to Popovic et al. (2012), concentrations between 400-500 mg/L are used for euthanasia of salmonids. Immediately after euthanasia, blood samples were collected to measure the blood glucose. Known portions of muscle, gills, and liver were collected for oxidative status determinations (n = 8/treatment). For hormone concentration determinations, the caudal peduncle (n = 8/treatment) was removed according to Guest et al. (2016) and Destro et al. (2021). The samples were flash-frozen in liquid nitrogen and stored at - 80ºC until further analysis. Another portion from the gills and the liver (n = 8/treatment) was fixed in Karnovisky for 24 hours and then immersed in 70% alcohol.

Atrazine determination

Every 15 days, water samples from 2 randomly selected tanks (tn = 4/treatment) were collected to determine ATZ levels. Collected samples were obtained after 1 h of water renewal, to ensure that they were not contaminated with biological material. The samples were stored at -20ºC and had ATZ levels measured through an enzyme immunoassay kit (ELISA) (Abnova, Taiwan).

Survival rate

At the end of the exposure period, the fish were visually counted and the survival rate was determined using the following calculation: Survival rate = (final number of fish / initial number of fish) × 100.

Blood glucose determination

Blood sampling was collected with an incision along the caudal peduncle (n = 16 samples/treatment). Blood glucose was evaluated using glucose test strips in a digital glucometer (Accu-Chek Active®, Roche, Mannheim, Germany).

Hormone extraction and determination

Hormonal extraction was performed following the protocol proposed by Guest et al. (2016) and Destro et al. (2021) and involved the removal of the caudal peduncle (100 mg) through caudal excision. We then added 2 mL of ice-cold PBS buffer (pH 7.4) with 1 mM 6-propyl-2thiouracil (PTU) to the samples. After the addition of ethyl ether for hormone extraction through a few more steps, the supernatant was

collected and dried under a hot water bath. All samples were then reconstituted in assay buffer and Cortisol, Triiodothyronine (T_3), and Thyroxine (T_4) concentrations were measured through enzyme immunoassay commercial kits (USA diagnostics).

Oxidative status

Sample preparation

Samples of frozen muscle, gills, and liver tissues (100 mg) were homogenized using a tissue homogenizer (OMNI) in 1 mL buffer (0.2 mol/L of phosphate buffer (pH 7.4) with 1 mM EDTA) and were centrifuged (15.000 g) for 10 min at 4°C. Supernatants were collected for superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), malondialdehyde (MDA), and nitric oxide (NO), and total protein assays. Pellets were collected for protein oxidation determination. All enzymatic activities were determined in duplicates using a spectrophotometer (UV-Mini 1240, Shimadzu, Japan) or a microplate reader (Thermo Scientific, Waltham, MA, USA) according to Destro et al. (2021).

Malondialdehyde determination

Tissue supernatant was homogenized in trichloroacetic acid (15%)/thiobarbituric acid (0.375%)/hydrochloric acid (0.6%) solution. The solution was kept in a water bath at 90°C for 40 min. After cooling on ice, butyl alcohol was added, and tubes were vortexed vigorously for 2 min. The samples were then centrifuged (10 min at 9.000 g) at room temperature, and the upper phase was used to measure MDA levels (λ = 540 nm) in a microplate reader. Total MDA levels in each sample were determined according to Buege and Aust (1978).

Protein oxidation

Carbonyl groups in proteins were determined according to Levine et al. (1990). Protein damage was determined based on the carbonyl groups of the 2, 4-dinitrophenylhydrazine (DNPH) reaction and measured in a microplate reader (λ = 370 nm). Carbonylated protein (PC) was calculated based on the molar extinction coefficient of $\epsilon 370$ = 22 mmol/L × cm.

Nitric oxide production

NO production was quantified by the standard Griess reaction. Briefly, 50 µL of supernatants were incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-Naphthyl) ethylenediamine, and 2.5% H_3PO_4) at room temperature for 10 min (Tsikas 2007). The absorbance was measured at 570 nm in a microplate reader. The conversion of absorbance into micromolar concentrations of NO was obtained from a sodium nitrite (0–100 µM) standard curve and expressed as NO concentrations (µmol/L).

Superoxide dismutase activity

SOD activity was measured following the method of Dieterich et al. (2000). The reaction solution had potassium phosphate buffer (99 µL, 5 mmol/L, pH 8.0) and the sample (30 µL). We then added 15 µL of pyrogallol (100 µmol/L) to start the reaction, read at 570 nm. SOD activity was calculated as units per milligram of protein, with one U of SOD defined as the amount capable of inhibiting the pyrogallol autoxidation rate by 50%.

Catalase activity

CAT activity was determined according to Aebi (1984), using hydrogen peroxide as substrate. We added 1 mL of H_2O_2 to the reaction mixture containing 10 µL of the sample in 1 mL of potassium phosphate buffer (50 mmol/L, pH 7.0). After the addition, the reaction mixture was monitored at 240 nm for 1 min at an interval of 30 s. An extinction coefficient of ε240 = 0.036 mmol/L cm was used for calculations. One unit of CAT activity was defined as the amount of enzyme that decomposes one mmol H_2O_2 for 1 min. Catalase activity was calculated as U per milligram of protein.

Glutathione S-transferase activity

The activity of GST was determined by glutathione-conjugated 2,4-dinitrochlorobenzene (CDNB) production. We added 1 mmol/L of CDNB to the buffer containing 1 mmol/L of GSH and a sample aliquot of 10 µL. After that, we performed serial readings at 340 nm, checking for alterations for 90 s. The molar extinction coefficient used for CDNB was ϵ340 = 9.6 mmol/L × cm. One unit of GST activity was set as the amount of enzyme that catalyzed the one µmole of product/min/mL formation. GST activity was expressed in µmol/min/mL, according to Habig et al. (1974).

Total protein

Total protein was measured with a standard (bovine serum albumin) to normalize the data according to the protein levels of the supernatant, using the protocol described by Lowry et al. (1951).

Histological analysis

Gills and liver portions were fixed in Karnovsky's solution (paraformaldehyde 4%, glutaraldehyde 4%, 1:1 in phosphate buffer 0.2 M, pH 7.3) for 24 hours, dehydrated in increasing series of ethanol, and immersed in glycol methacrylate (Historesin®, Leica, Germany). Then, semi-serial sections (3 µm thick) were made in the tissues using a rotating microtome (RM2265 - Leica), obtaining 12 cuts per fragment. Gills sections were stained with toluidine blue and alcian blue conjugated to PAS and the liver sections were stained with hematoxylin and eosin (HE). Digital images (10 images/animal) were taken using a photomicroscope (Olympus AX70 TRF). The images of the gills were analyzed by counting the intersection of points on mitochondria-rich cells (specialized cells in osmotic regulation), mucus-secreting cells (epidermal cells specialized in mucus secretion), aneurysms (disturbance of blood flow in the gill), lamellar fusion, secondary lamella deformation (deletion of secondary lamellar epithelium), and hyperplasia (increase in the number of gill tissue cells). Liver images were analyzed morphometrically by counting the intersection of points on nuclei and cytoplasm and blood vessels. For histopathological analyzes, leukocyte infiltration counts, vascular congestion (red cells inside the vessels), cell degeneration (cytoplasmatic alterations), and necrosis (cell death) were counted. In addition, the total diameter of hepatocytes and nuclei was measured (150 per animal). The analyzes were performed with a grid displaying 266 intersections from the Image-Pro Plus 4.5® software system (Media Cybernetics, Silver Spring, MD, EUA), which accounted for 2660 points per animal.

Statistical analysis

Data distribution was determined by Shapiro–Wilk test. Normally distributed data were analyzed using unifactorial one-way analysis of variance (ANOVA) followed by the Tukey test. Data that did not meet the normal distribution criteria were submitted to Kruskal–Wallis, followed by Dunn's test for comparisons amongst groups. Results are expressed as means and standard error of the mean (mean ± SEM). Statistical significance was set at $P < 0.05$. All data were statistically analyzed using the software GraphPad Prism (version 6.0, San Diego, CA, USA).

3. Results

Water parameters

Water parameters were measured weekly and showed the following results: temperature 27 ± 1ºC; pH: 7.0–7.1, ammonia: <2.0 µg/mL, dissolved oxygen: 8 ± 1 µg/mL.

Atrazine determination in water tanks

No trace of ATZ was detected in water samples from tanks from the control groups. Measured atrazine concentrations are shown in Table 1 and were used to name the treatment groups.

Table 1. Measured atrazine concentrations in tanks from different treatments (n=4 samples/treatment).

Treatment	Measured atrazine concentration, µg/dL
CTR	ND
ATZ0.56	0.56 ± 0.04
ATZ1.00	1.00 ± 0.06
ATZ1.66	1.66 ± 0.15
ATZ11.66	11.66 ± 1.87

CTR = control group; ND = non detected.

Survival rate

We observed a 36.6% decrease in the survival rate of fish from ATZ11.66 group compared to control, ATZ0.56, ATZ1.00, and ATZ1.66 groups ($F_{(4.15)} = 10.16$; $p < 0.0001$, $P = 0.0108$, $p = 0.0277$, and $p = 0.0377$, respectively) (Figure 1).

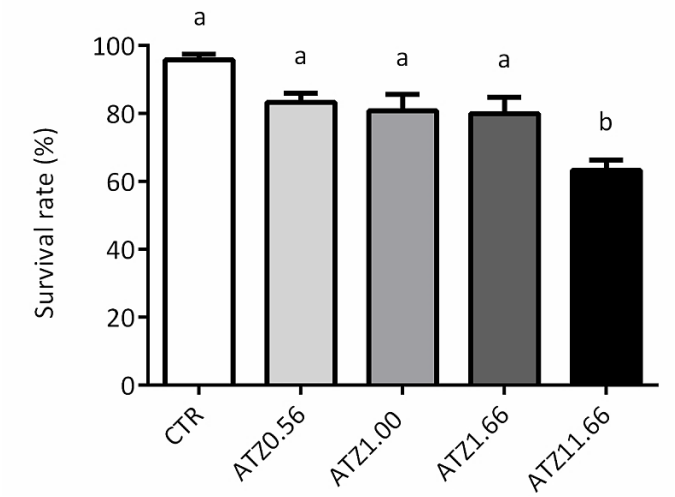

Figure 1. Survival rate (%) of juvenile lambaris exposed to atrazine for 35 days. CTR = control group, ATZ0.56 = 0.56 µg/L, ATZ1.00 = 1.00 µg/L, ATZ1.66 = 1.66 µg/L, ATZ11.66 = 11.66 µg/L. Data are shown as mean ± SEM. [a, b] Different letters indicate statistical differences among groups ($p \leq 0.05$).

Blood glucose and hormone determination

Blood glucose concentration increased in ATZ11.66 compared to control ($F_{(4,70)} = 3.482$; $p = 0.0064$) (Table 2).

Oxidative status

In the muscle, we observed an increase in MDA levels in ATZ11.66 compared to control ($F_{(4.34)} = 8.197$; $p = 0.0001$) and ATZ0.56 ($p = 0.0003$), ATZ1.00 ($p = 0.0114$), ATZ1.66 ($p = 0.0031$) groups (Figure 2a).

There was an increase in carbonylated proteins in ATZ11.66 compared to control and ATZ0.56 ($F_{(4.30)}$ = 3.50; p = 0.0185 and p = 0.0407) (Figure 2b). The levels of NO remained unaltered after ATZ exposure (Figure 2c). There was an increase in SOD activity inATZ11.66 compared to control ($F_{(4.35)}$ = 2.551; p = 0.0278) (Figure 2d). CAT activity increased in groups ATZ1.00, ATZ1.66 and ATZ11.66 compared to control ($F_{(4.35)}$ = 28.44; p = 0.0072; p = 0.0052 and p < 0.0001) and ATZ11.66 values were higher than ATZ1.00 (p < 0.0001) and ATZ1.66 (p < 0.0001) (Figure 2e). GST activity increased in all groups compared to control ($F_{(4.35)}$ = 87.16; P<0.0001), being evidently high in ATZ11.66 compared to ATZ0.56, ATZ1.00 and ATZ1.66 groups ($F_{(4.35)}$ = 87.16; p < 0.0001, p < 0.0001 and p < 0.0001) (Figure 2f).

Table 2. Blood glucose (mg/dL), cortisol (ng/g), triiodothyronine (T_3) (ng/g) and thyroxine (T_4) (µg/g) concentrations in juveniles lambari exposed to atrazine for 35 days.

	CTR	ATZ0.56	ATZ1.00	ATZ1.66	ATZ11.66
Blood glucose (mg/dL)	71.40 ± 3.26^{a}	76.20 ± 3.78^{ab}	77.47 ± 4.09^{ab}	79.27 ± 3.84^{ab}	92.20 ± 5.50^{b}
Cortisol (ng/g)	4.01 ± 0.56^{a}	5.88 ± 0.38^{ab}	5.13 ± 0.44^{ab}	5.99 ± 0.71^{ab}	6.33 ± 0.36^{b}
Triiodothyronine (T_3) (ng/g)	2.90 ± 0.28^{a}	2.85 ± 0.31^{a}	2.79 ± 0.26^{a}	2.82 ± 0.26^{a}	3.12 ± 0.18^{a}
Thyroxine (T_4) (µg/g)	11.83 ± 0.60^{a}	11.29 ± 0.60^{a}	10.95 ± 0.69^{a}	11.04 ± 0.68^{a}	11.10 ± 0.58^{a}

CTR = control group, ATZ0.56 = 0.56 µg/L, ATZ1.00 = 1.00 µg/L, ATZ1.66 = 1.66 µg/L, ATZ11.66 = 11.66 µg/L. Data are shown as mean ± SEM. a, b Different letters indicate statistical differences among groups (p ≤ 0.05).

Cortisol levels were increased in ATZ11.66 compared to control ($F_{(4.31)}$ = 3.403; p = 0.0219) (Table 2). Hormone analysis did not reveal any alterations following ATZ exposure at the concentrations tested for T_3 and T_4 (Figure 3a).

In the gills, MDA levels increased in ATZ11.66 compared to control ($F_{(4.35)}$ = 3.258; p = 0.0151) (Figure 3a). PC and NO levels and SOD activity remained unaltered after ATZ exposure (Figure 3b; c; d). CAT activity increased in ATZ11.66 compared to control and other groups ($F_{(4.35)}$ = 15.64; p < 0.0001, p < 0.0001, p < 0.0001 and p = 0.0002, respectively) (Figure 3e). GST activity increased in ATZ0.56, ATZ1.00, ATZ1.66 and ATZ11.66 groups compared to control ($F_{(4.35)}$ = 35.88; p < 0.0001, p < 0.0001, p < 0.0001 and p < 0.0001, respectively). GST activity was also shown to be high in ATZ11.66 compared to ATZ0.56, ATZ1.00, and ATZ1.66 groups ($F_{(4.35)}$ = 35.88; p <0.0001, p < 0.0001, and p = 0.0004) (Figure 3f).

In the liver, MDA levels increased in ATZ11.66 compared to control ($F_{(4.35)}$ = 2.638; p = 0.0284) (Figure 4a). PC and NO levels remained unaltered after ATZ exposure (Figure 4b; c). SOD activity increased in ATZ11.66 compared to control ($F_{(4.33)}$ = 4.470; p = 0.0017) (Figure 4d). CAT activity increased in ATZ11.66 compared to control group ($F_{(4.35)}$ = 3.273; p = 0.0131) (Figure 4e). GST activity increased in ATZ11.66 compared to control and ATZ0.56 groups ($F_{(4.35)}$ = 5.116; p = 0.0009 and p = 0.0399) (Figure 4f).

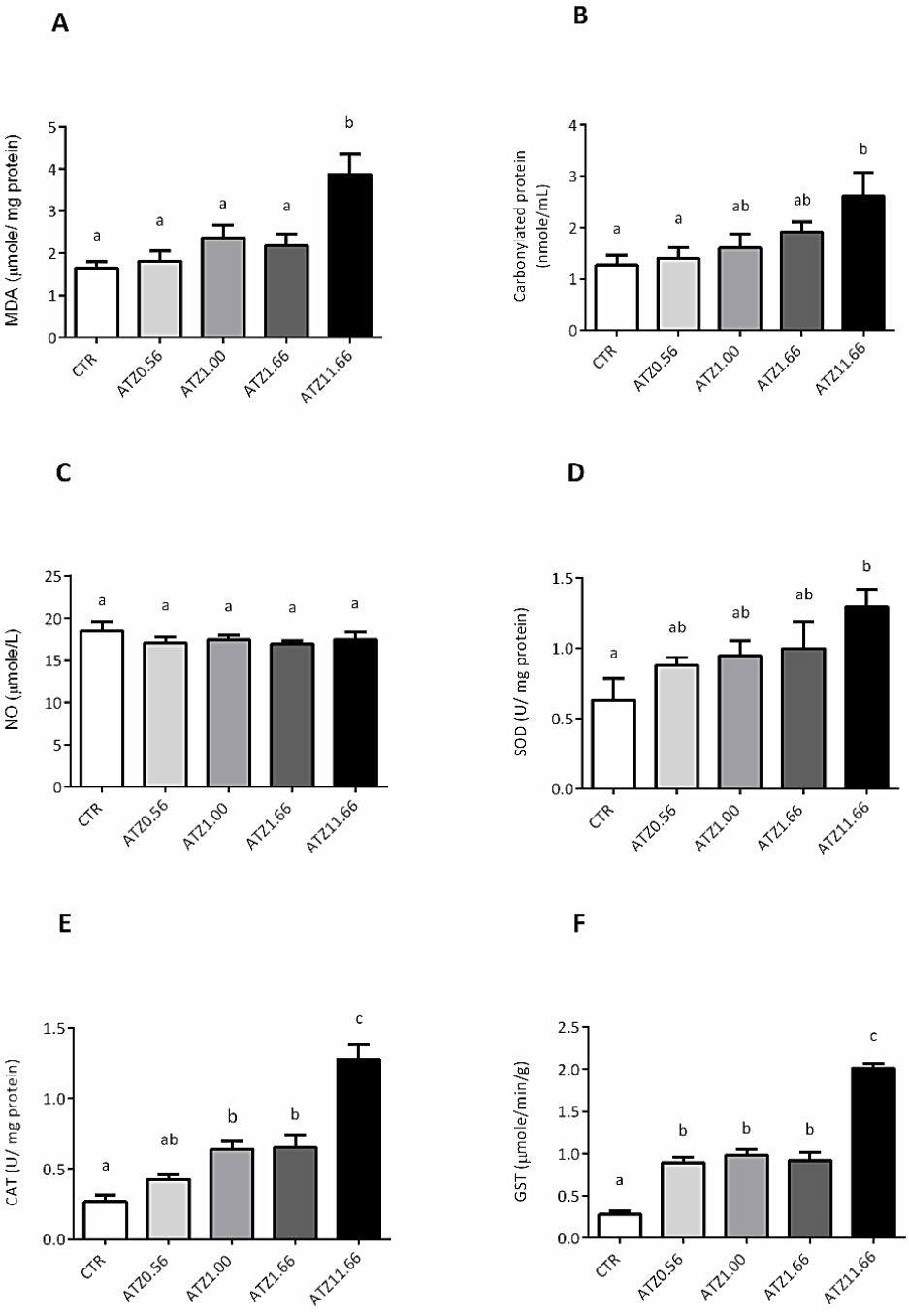

Figure 2. Oxidative stress markers and antioxidant enzymes activity in muscles of juvenile lambari exposed to different atrazine concentrations for 35 days. Oxidative stress biomarkers: (A) malondialdehyde – MDA, (B) carbonylated proteins – PC, (C) nitric oxide - NO. Antioxidant enzymes: (D) superoxide dismutase - SOD, (E) = catalase - CAT, F = glutathione S-transferase – GST. Data are shown as mean ± SEM. [a, b, c] Different letters indicate statistical differences among groups ($p \leq 0.05$).

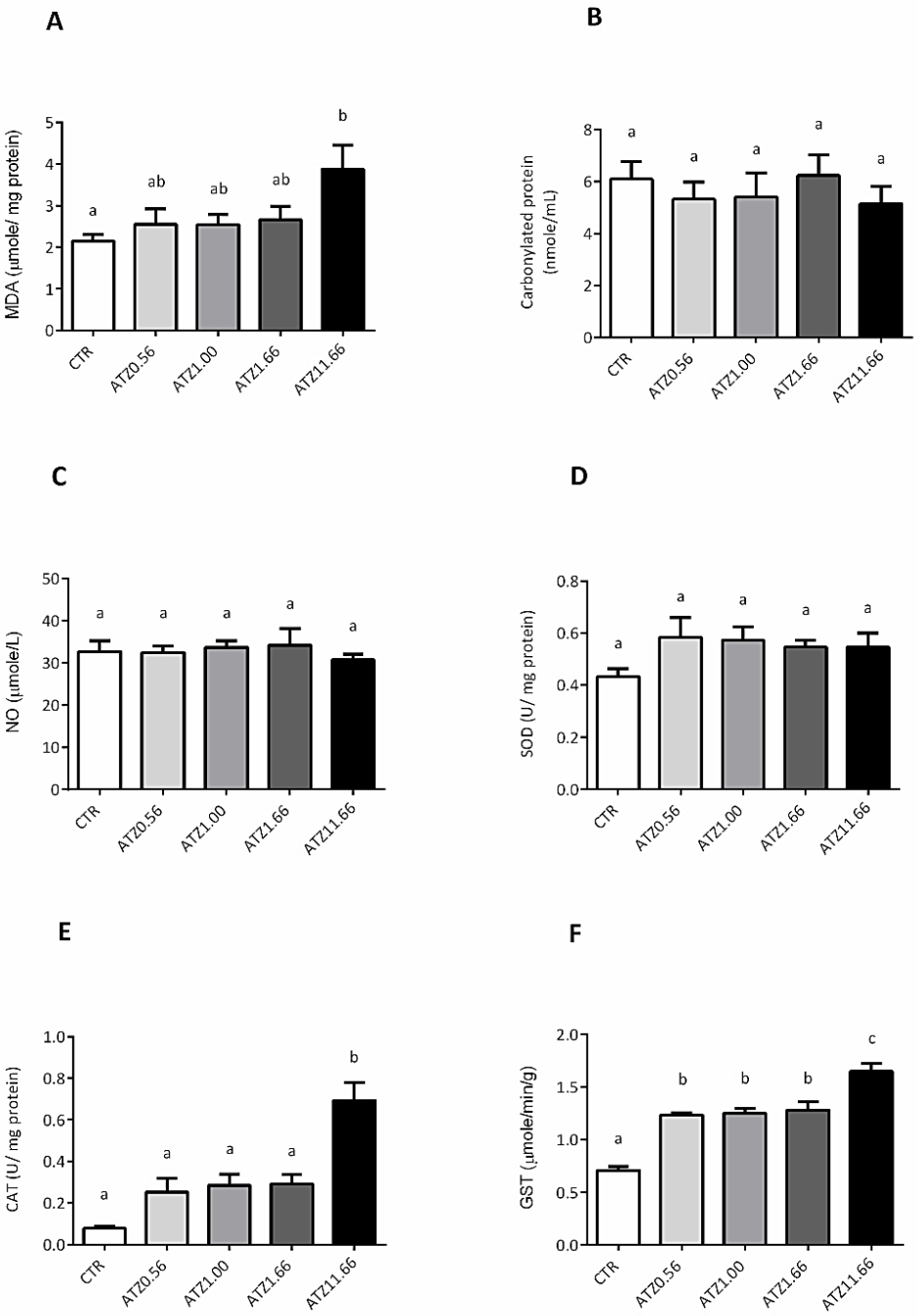

Figure 3. Oxidative stress markers and antioxidant enzymes activity in gills of juvenile lambari exposed to different atrazine concentrations for 35 days. Oxidative stress biomarkers: (A) malondialdehyde – MDA, (B) carbonylated proteins – PC, (C) nitric oxide - NO. Antioxidant enzymes: (D) superoxide dismutase - SOD, (E) catalase - CAT, (F) glutathione S-transferase – GST. Data are shown as mean ± SEM. [a, b, c] Different letters indicate statistical differences among groups ($p \leq 0.05$).

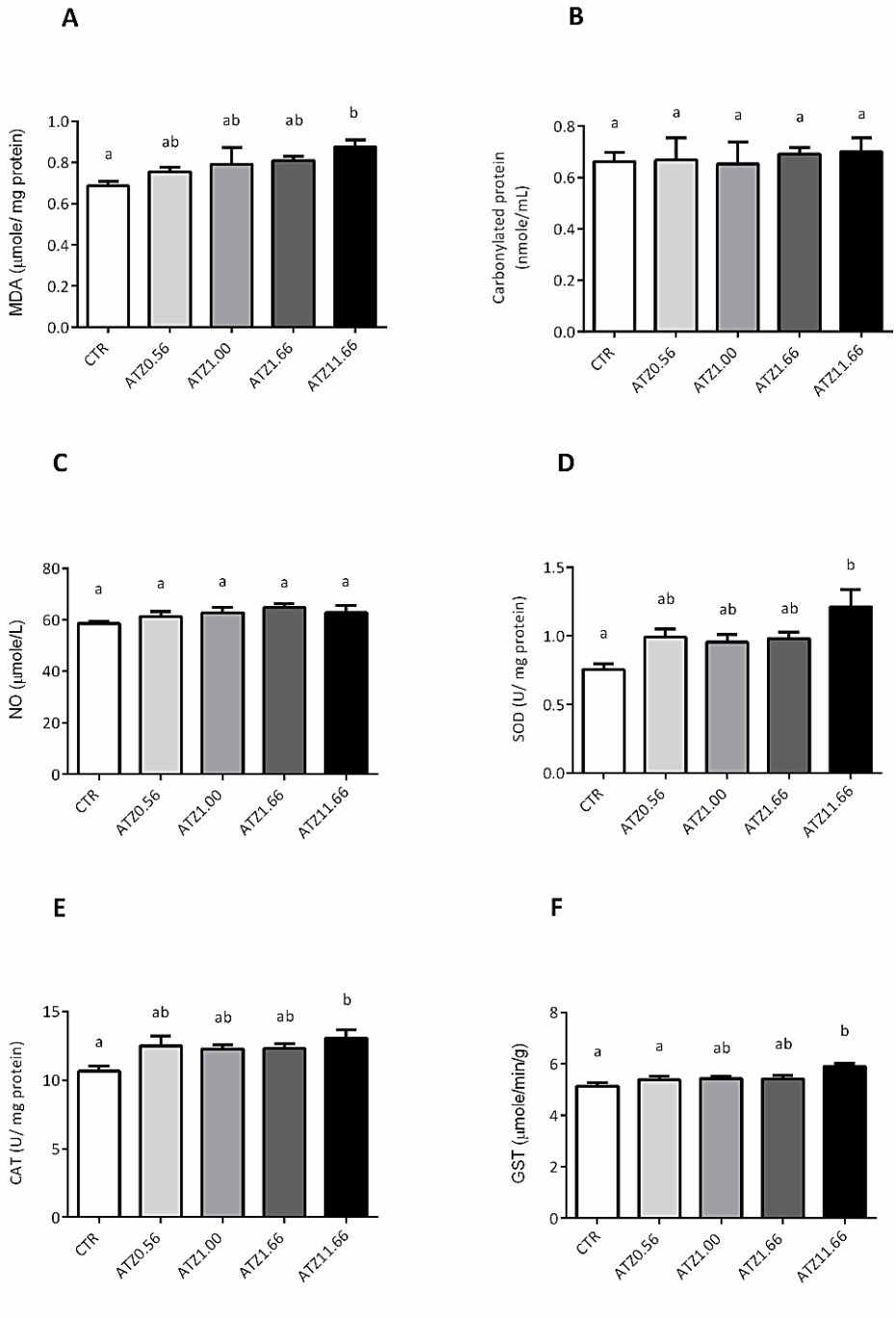

Figure 4. Oxidative stress markers and antioxidant enzymes activity in liver of juvenile lambari exposed to different atrazine concentrations for 35 days. Oxidative stress biomarkers: (A) malondialdehyde – MDA, (B) carbonylated proteins – PC, (C) nitric oxide – NO. Antioxidant enzymes: (D) superoxide dismutase - SOD, (E) catalase - CAT, (F) glutathione S-transferase – GST. Data are shown as mean ± SEM. [a, b] Different letters indicate statistical differences among groups ($p \leq 0.05$).

Histopathological analysis

Histological sections of the gills showed increased mitochondria-rich cells percentage in ATZ1.00, ATZ1.66, and ATZ11.66 compared to control ($F_{(4.25)} = 11.16$; $p = 0.0300$, $p = 0.0008$ and $p < 0.0001$, respectively) and in ATZ 1.66 and ATZ11.66 compared to ATZ0.56 ($F_{(4.25)} = 11.16$; $p = 0.0442$ and $p = 0.018$). Increased in the number of mucus secreting cells in ATZ11.66 compared to control, ATZ0.56 e ATZ1.00 ($F_{(4.25)} = 7.13$; $p = 0.0003$, $p= 0.0385$, and $p = 0.0059$, respectively). We found hyperplasia and increased lamellar fusion percentage in ATZ11.66 compared to control ($F_{(4.25)} = 2.83$; $p = 0.0307$) and ($\chi^2 = 10.82$; $p = 0.0390$). We observed loss of secondary lamella integrity in ATZ1.00, ATZ1.66 and ATZ11.66 compared to

control ($F_{(4.25)}$ = 19.07; p= 0.0054, p < 0.0001 and p < 0.0001, respectively) and also observed in ATZ1.66 and ATZ11.66 compared to ATZ0.56 and ATZ1.00 ($F_{(4,25)}$ = 19.07; p= 0.0037, p < 0.0001) and ATZ11.66 compared to ATZ1.00 ($F_{(4,25)}$ = 19.07; p = 0.0062). The branchial tissue showed an increased aneurysm percentage in ATZ1.66 compared to control ($F_{(4.25)}$ = 3.67; p = 0.0164) (Table 3 and Figure 5).

Table 3. Histopathological parameters in gills of juvenile lambari exposed to atrazine for 35 days.

	CTR	ATZ0.56	ATZ1.00	ATZ1.66	ATZ11.66
Mitochondria-rich cells (%)	2.75 ± 0.15^{a}	3.28 ± 0.26^{ab}	3.77 ± 0.16^{bc}	4.25 ± 0.13^{c}	4.68 ± 0.35^{c}
Mucus secreting cells (%)	0.18 ± 0.38^{a}	0.41 ± 0.07^{a}	0.32 ± 0.11^{a}	0.51 ± 0.04^{ab}	0.77 ± 0.11^{b}
Hyperplasia (%)	2.74 ± 0.13^{a}	3.08 ± 0.48^{ab}	3.59 ± 0.32^{ab}	3.35 ± 0.34^{ab}	4.38 ± 0.42^{b}
Lamellar fusion (%)	0.26 ± 0.71^{a}	1.04 ± 0.29^{ab}	0.99 ± 0.37^{ab}	1.87 ± 0.46^{ab}	2.52 ± 0.79^{b}
Loss of secondary lamella integrity (%)	1.74 ± 0.42^{a}	4.35 ± 0.52^{ab}	6.60 ± 1.02^{b}	9.40 ± 1.10^{c}	11.38 ± 1.07^{c}
Aneurysm (%)	0.97 ± 0.33^{a}	1.79 ± 0.32^{ab}	2.59 ± 0.51^{ab}	3.45 ± 0.54^{b}	2.95 ± 0.72^{ab}

CTR = control group, ATZ0.56 = 0.56 µg/L, ATZ1.00 = 1.00 µg/L, ATZ1.66 = 1.66 µg/L, ATZ11.66 = 11.66 µg/L. Data are shown as mean ± SEM. a, b, c Different letters indicate statistical differences among groups (p ≤ 0.05).

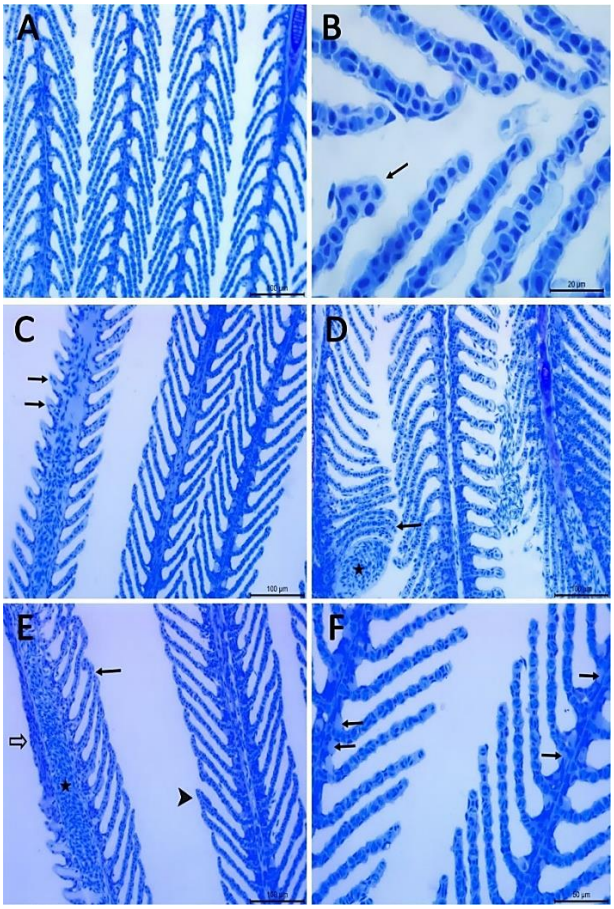

Figure 5. Photomicrographs of histological sections of juvenile lambari gills exposed to atrazine for 35 days. A) CTR; B) ATZ0.56: hyperplasia (arrow); C) ATZ1.00: loss of the integrity of the secondary lamella (arrow); D) ATZ1.66: lamellar fusion (arrow) and aneurysm (star); E) ATZ11.66: hyperplasia (arrow), lamellar fusion (arrowhead), aneurysm (star) and loss of secondary lamella integrity (empty arrow); F) ATZ11.66: Mitochondria-rich cell (arrow). Toluidine blue staining; photomicrographs A, C, D and E: scale bar = 100 µm; B = 20 µm; F = 50 µm.

Histological analysis of the liver showed decrease in the number of nuclei in ATZ1.00, ATZ1.66, and 11.66 compared to control ($F_{(4.22)}$ = 4.62; p = 0.0234, p = 0.0154, p = 0.0113, respectively) and in the diameter of the nucleus (µm) in ATZ11.66 compared to control (χ^2 = 15.85; p = 0.0009). There was an increase in the number of blood vessels in ATZ1.66 and ATZ11.66 compared to control (χ^2 = 12.27; p =

0.0293 and p = 0.0401). In addition, the fish presented an increased vascular congestion percentage and steatosis percentage in ATZ11.66 compared to control (χ^2 = 10.14; p = 0.0323), (χ^2 = 17.69; p = 0.0044) (Table 4 and Figure 6).

Table 4. Histopathology parameters in liver of juvenile lambari exposed to atrazine for 35 days.

	CTR	ATZ0.56	ATZ1.00	ATZ1.66	ATZ11.66
Nuclei (%)	22.94 ± 0.63^{a}	19.78 ± 1.06^{ab}	18.89 ± 0.65^{b}	18.46 ± 0.40^{b}	18.50 ± 1.30^{b}
Nuclei diameter (µm)	15.41 ± 0.25^{a}	14.03 ± 0.39^{ab}	13.96 ± 0.13^{ab}	13.97 ± 0.09^{ab}	13.42 ± 0.13^{b}
Hepatocyte diameter (µm)	24.43 ± 0.58^{a}	23.88 ± 0.39^{a}	23.79 ± 0.19^{a}	23.36 ± 0.21^{a}	23.40 ± 0.22^{a}
Cytoplasm (%)	47.06 ± 1.54^{a}	47.08 ± 2.94^{a}	45.47 ± 2.37^{a}	45.08 ± 2.78^{a}	45.43 ± 2.50^{a}
Blood vessel (%)	2.13 ± 0.20^{a}	6.91 ± 2.45^{ab}	8.21 ± 2.02^{ab}	8.27 ± 1.32^{b}	7.60 ± 1.65^{b}
Vascular congestion (%)	0.26 ± 0.08^{a}	1.04 ± 0.29^{ab}	0.99 ± 0.37^{ab}	1.87 ± 0.46^{ab}	2.52 ± 0.79^{b}
Leukocyte infiltrate (%)	0.02 ± 0.02^{a}	0.03 ± 0.02^{a}	0.05 ± 0.01^{a}	0.00 ± 0.00^{a}	0.07 ± 0.07^{a}
Steatosis (%)	0.00 ± 0.00^{a}	1.79 ± 0.32^{ab}	2.59 ± 0.51^{ab}	3.45 ± 0.54^{ab}	2.95 ± 0.72^{b}
Necrosis (%)	0.00 ± 0.00^{a}	0.18 ± 0.07^{a}	0.09 ± 0.04^{a}	0.69 ± 0.37^{a}	0.45 ± 0.16^{a}

CTR = control group, ATZ0.56 = 0.56 µg/L, ATZ1.00 = 1.00 µg/L, ATZ1.66 = 1.66 µg/L, ATZ11.66 = 11.66 µg/L. Data are shown as mean ± SEM. a, b Different letters indicate statistical differences among groups (p ≤ 0.05).

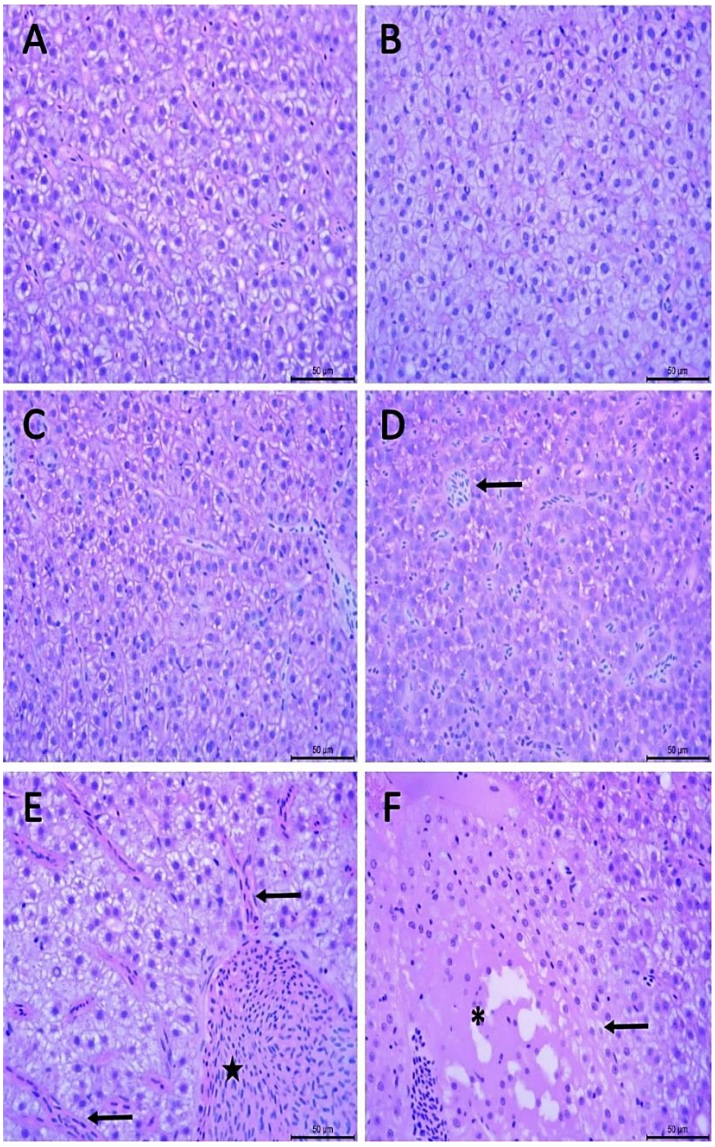

Figure 6. Photomicrographs of histological sections of juvenile lambari liver exposed to atrazine for 35 days. A) CTR; B) ATZ0.56; C) ATZ1.00; D) ATZ1.66: Blood vessel (arrow); E) ATZ11.66: Blood vessel (arrow) and vascular congestion (star); F) ATZ11.66: steatosis area (arrow) and central necrotic area (asterisk). HE staining; scale bar represents 50 µm.

4. Discussion

This is the first study to investigate the effects of long-term exposure to low concentrations of a commercial atrazine-based herbicide formulation in juveniles from a native fish species. Our results show that exposure to 11.66 µg/L of ATZ induced an imbalance in ROS production and oxidative markers and consequently promoted morphological changes such as hyperplasia and lamellar fusion in the gills, and vascular overload, with the development of degenerative processes and cell death in the liver. We evaluated the oxidative status in three different organs of juvenile lambaris, which play important roles in homeostatic processes. Gills are in constant contact with the external environment and are susceptible to contaminants exposure. Due to the high level of oxygen consumption in muscles, they are usually involved in high ROS generation. The liver participates in the metabolism of exogenous compounds and is the target of toxins. Here, we found these tissues responded differently to ATZ exposure, regarding either the oxidative stress responses and histological alterations.

Oxidative stress biomarkers, such as lipid peroxidation and protein carbonylation products, indicate cell damage and are very important in assessing tissue oxidative stress status (Gaschler and Stockwell 2017). In our study, we found an increase in lipid peroxidation in all three tissues studied following the highest ATZ concentration tested, showing the oxidative tissue damage associated with this herbicide in developing fish, mainly in the cellular membranes.

Nitric oxide is an important cell signaling and mediator, which plays different physiological and pathological roles (Garcia and Stein 2006). Although this study did not show any changes in NO levels in any of the tissues analyzed from exposed juveniles, other studies have shown an association between NO production and reproduction in adult fish (Pathak and Lal 2008; Lal and Dubey 2012). The exposure to nitric oxide donor during 15 days impaired steroidogenesis and oocyte maturation in *C. batrachus* (Singh and Lal 2017). Liver oxidative damage may have been potentiated due to vascular congestion and consequent impairment of oxygen supply to hepatocytes since changes in oxygen supply can increase ROS formation (Ozmen et al. 2009). Previous studies also reported increased lipid peroxidation in other juvenile fishes (Paulino et al. 2012a; Marins et al. 2018).

Regarding the antioxidant defenses, we observed alterations in different tissues of juvenile lambaris exposed to atrazine. In fish from ATZ11.66, all tested antioxidant enzymes were increased in muscle and liver, indicating a possible attempt to attenuate the excessive ROS production induced by the herbicide exposure (Paulino et al. 2012a). In gills, CAT and GST activities were also increased in ATZ11.66. Here, the increases in antioxidant enzymes activity can be interpreted as an attempt to minimize damage to lipids and proteins caused by herbicide exposure. However, we observed an increase in MDA and PC levels at the highest concentration tested (11.66 µg/L), suggesting that antioxidant defenses were sufficient to neutralize possible damage at ATZ concentrations only at lower concentrations. Unrepaired oxidative damage during development may be extended to other life cycles, leading to age-dependent diseases. Common carp exposed to an environmentally relevant concentration of atrazine (0.3 µg/L) for 33 days, at early life stages, also showed increased SOD, CAT, and GST and no oxidative damage (Chromcová et al. 2013).

High levels unrepaired of oxidative stress may induce changes in tissue structure and promote the emergence of inflammatory processes (Frederico et al. 2007). Our study also showed several histopathologies in atrazine exposed juveniles, including damage to the respiratory membrane. The damage to lamellar integrity in fish exposed to 1.00, 1.66, and 11.66 µg/L indicates that ATZ has the potential to induce critical damage in juveniles, since the gills are multifunctional organs with an important role in maintaining hydro electrolytic homeostasis and ammonia acid/base balance and excretion, in addition to gas exchange (Evans et al. 2005). The loss of integrity can be seen as a defensive strategy to reduce organs functionality and therefore reduce herbicide damage. The increase in mitochondria-rich cells (MRC) in the gills of juvenile fish might have occurred as part of an adjustment process in osmotic/ionic homeostasis. Juvenile fish from *P. lineatus* species, exposed to 10 and 25 µg/L of atrazine for 14 days showed an increase in osmolarity, plasmatic sodium, and chloride concentrations, as well as a reduction in carbonic anhydrase enzyme activity and morphological changes in MRCs (Paulino et al. 2012b), corroborating our results. Increased MRC represents higher energy consumption for ions

movement against the concentration gradient in order to achieve ion homeostasis (Stevens et al. 2017), which may impair ionic regulation and may be related to the decreased survival rate in fish exposed to the highest concentration tested.

The increase in mucous-secreting cells might be considered a defense mechanism against environmental chemical pollutants, such as atrazine, in order to reduce the pollutant uptake (Paulino et al. 2012a; Mela et al. 2013). However, this strategy also induces an increase in the distance for oxygen and carbon dioxide diffusion between water and blood (Paulino et al. 2012a). This effect, associated to the loss of lamellar integrity, may impair gas exchange in ATZ exposed juveniles, which could disfavor oxidative metabolic pathways involved in growth and development. A previous study also showed hypertrophy, hyperplasia and desquamation of gill epithelial cells in juvenile zebrafish subchronically exposed to low simazine concentrations, another triazine herbicide (Plhalová et al. 2011). All branchial histopathologies reported in our study involve obstruction of the area responsible for gas exchange and may lead the organism to death by asphyxiation.

Regarding the liver, we found increased blood vessels, vascular congestion, and steatosis, mainly at 11.66 µg/L, which may indicate a vascular event caused by an inflammatory process. Excessive free radical production promotes changes in cellular membranes and consequently cell redox imbalance, leading to processes associated with inflammation and cell degeneration (Gaschler and Stockwell 2017). All histopathological findings showed that ATZ induced liver damage and were supported by oxidative status results. Corroborating our findings, low ATZ concentration also induced the onset of inflammation by increasing pro-inflammatory cytokine (IL-1ß) gene expression and reducing anti-inflammatory IL-10 expression in the liver from common carps exposed for 40 days (Toughan et al. 2018). Other studies carried out in juvenile fish from different species also showed hepatoxicity such as liver lipid metabolism disorders following ATZ and other herbicides exposure (Shelley et al. 2012; Liu et al. 2021).

Besides the oxidative damage and histopathologies reported, we also found an increase in cortisol and blood glucose levels in juveniles exposed to the highest ATZ concentration tested, which also represent extra energy expended that could have been used in energy storage and growth (Sokolova 2013). Under chronic stress, fish show a reduction in immune system activity, which affects tissue healing and decreases the capacity of the reproductive and antioxidant system and may affect survival. In addition, juvenile fish exposed to pesticides were shown to be more susceptible to predation due to the neurotoxic effect of some pollutants and a higher susceptibility to parasites. In other species, exposure to low atrazine concentrations impaired the metabolic pathways that control blood glucose concentrations and regulate energy homeostasis (Akhtar et al. 2021). Metabolic effects of cortisol include an increase in blood glucose levels, which on the other hand, may impair the juvenile fish performance, diverting energy that could use in vital functions such as growth, development, and sexual maturation. Interestingly, a previous study from our laboratory with adult fish exposed to the same ATZ concentrations did not show changes in cortisol levels, suggesting that juveniles from this species are more sensitive to ATZ than their adult counterparts (Destro et al. 2021).

Despite the changes previously reported, no endocrine-disrupting effects of ATZ were found at the thyroid axis in developing fish at the concentrations tested. This finding suggests that, despite being related to growth and development regulation, the levels of these two hormones remained unaltered after the 35-day atrazine exposure. However, previous studies found an organizational dysfunction in the thyroid gland in exposed juveniles reptiles (Galoppo et al. 2020) and demasculinization in juvenile fish and reptiles, suggesting that atrazine exposure altered aromatase activity and induced excessive estrogen production (Hayes et al. 2011).

The decrease in survival rate observed in developing lambaris from the ATZ11.66 group suggests that atrazine may reduce young fish populations in natural environments and cause losses for breeders of this species. Harm to the production chain can be even greater if we consider the effect of muscle lipid peroxidation on meat quality duration since we observed an increase in MDA levels in muscles. Unlike mammals, 40% of fish lipids contain unsaturated long-chain fatty acids, highly susceptible to oxidation and degradation. Consequences of lipid oxidation include the formation of the reactive molecule, which may impair the quality, taste, and odor of meat, in addition to producing harmful substances to human health (Secci and Parisi 2016). Juvenile freshwater fish species from six families exposed to low concentrations of

2,4-D herbicide for 90 days also showed decreased survival rates, although adults were not affected (Dehnert et al. 2020). Exposure to low atrazine concentrations for 14 days also altered oocytes final maturation and reduced the total egg production of fathead minnows, impairing offspring's development (Tillitt et al. 2010). Thus, the ATZ-induced alterations we reported here in the oxidant-antioxidant balance, histopathological and physiological parameters are likely involved with the stress response that led to the decrease in the survival rate of juvenile lambaris exposed to 11.66 µg/L.

5. Conclusions

In conclusion, our results show that subchronic exposure to environmentally relevant concentrations of a commercial atrazine-based herbicide formulation, mainly at the highest concentration tested (11.66 µg/L), induced an imbalance in the oxidative markers and morphological damages in gills and liver in a juvenile Neotropical species of great ecological relevance and commercial interest. Taken together, we believe that these altered parameters may impact fish production and might be potentially harmful to growth and development processes, suggesting that other Neotropical species at early life stages need to be further evaluated to ensure that they are not threatened.

Authors' Contributions: SILVA, S.B.: acquisition of data, analysis and interpretation of data and drafting the article; GREGÓRIO, K.P.: acquisition of data and analysis and interpretation of data; DESTRO, A.L.F.: acquisition of data and analysis and interpretation of data; GONÇALVES, R.V.: analysis and interpretation of data and drafting the article; SALARO, A.L.: conception and design, analysis and interpretation of data and drafting the article; ZUANON, J.A.S.: conception and design, analysis and interpretation of data and drafting the article; FREITAS, M.B.: conception and design, analysis and interpretation of data, drafting the article and critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: This study was performed according to the rules of the Ethics Committee on the Use of Production Animals from the Federal University of Viçosa, Brazil (registration nº 043/2017).

Acknowledgments: We thank the Higher Education for Personnel Improvement Coordination (CAPES) (scholarship to S. B. Silva), the National Council for Scientific and Technological Development (CNPq) (scholarship to K. P. Gregório) and the Research Support Foundation of the State of Minas Gerais (FAPEMIG), Brazil, for the financial support received (grant numbers APQ-02341-16 and PPM-00687-17).

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Received: 24 May 2022 | **Accepted:** 12 August 2022 | **Published:** 10 March 2023