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Reduced fluoxetine-induced cyto- and genotoxicity in yellowtail tetra fish *Astyanax altiparanae* (Characiformes: Acestrorhamphidae) after treatment through a constructed wetlands system

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Fluoxetine hydrochloride (FLX) is an antidepressant classified as a selective serotonin reuptake inhibitor. It is commonly found in aquatic environments due to human excretion and improper disposal practices. The presence of FLX raises concerns, as it is linked to behavioral, physiological, and genetic alterations in non-target aquatic organisms. This study investigated the toxic effects of FLX on *Astyanax altiparanae*, focusing on blood and gill responses, and evaluated the efficiency of a vertical flow constructed wetland (VFCW) as a sustainable treatment solution. Five groups were established: a control, three exposed to increasing FLX concentrations (0.01, 0.1, and 1 mg L^{-1}), and one exposed to 1 mg L^{-1} of treated effluent by VFCW. After 96 h, the *specimens* exposed to FLX showed a significant increase in the frequency of micronuclei (MN), cellular morphological changes (CMC), DNA damage, and histopathological lesions in the gills. In contrast, our results indicated that more than 99% of the FLX was removed in the VFCW, with a display of around 89% of cells without DNA damage, a significant reduction in MN, CMC, and histopathological lesions. These results demonstrate the system's efficiency in removing FLX and mitigating its adverse effects on aquatic organisms.

Keywords: Bioremediation, Ecotoxicology, Green technologies, Pharmaceuticals, Sustainable method.

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O cloridrato de fluoxetina (FLX) é um antidepressivo classificado como um inibidor seletivo da recaptação da serotonina. É comumente encontrado em ambientes aquáticos devido à excreção humana e práticas inadequadas de descarte. A presença de FLX levanta preocupações, pois está ligada a alterações comportamentais, fisiológicas e genéticas em organismos aquáticos não alvos. Este estudo investigou os efeitos tóxicos do FLX em *Astyanax altiparanae*, com foco nas respostas sanguíneas e branquiais, e avaliou a eficiência de um wetland construído de fluxo vertical (WCFV) como uma solução de tratamento sustentável. Cinco grupos foram estabelecidos: um controle, três expostos a concentrações crescentes de FLX (0.01, 0.1 e 1 mg L^{-1}) e um exposto a 1 mg L^{-1} de efluente tratado por WCFV. Após 96 horas, os espécimes expostos ao FLX mostraram um aumento significativo na frequência de micronúcleos (MN), alterações morfológicas celulares (AMC), danos ao DNA e lesões histopatológicas nas brânquias. Em contraste, nossos resultados indicaram que mais de 99% do FLX foi removido no WCFV, com cerca de 89% de células sem danos ao DNA, uma redução significativa em MN, AMC e lesões histopatológicas. Esses resultados demonstram a eficiência do sistema na remoção do FLX e na mitigação de seus efeitos adversos em organismos aquáticos.

Palavras-chave: Biorremediação, Ecotoxicologia, Fármacos, Método sustentável, Tecnologias verdes.

INTRODUCTION

Pharmaceutical compounds have consistently been released into aquatic environments (Correia *et al*., 2023). Among these, antidepressants have gained significant attention due to their presence in various ecosystems, including soil surfaces and near wastewater treatment facilities. Such contaminants often infiltrate water resources (Duarte *et al*., 2019), primarily through sewage from various sources, including hospitals, wastewater treatment plants, and pharmaceutical manufacturing sites. Consequently, this leads to the contamination and toxicity of natural water sources (Nałęcz-Jawecki *et al*., 2020).

Fluoxetine hydrochloride (FLX), an antidepressant of the selective serotonin reuptake inhibitor class, is widely used in the treatment of psychiatric disorders but can cause significant environmental impacts. The FLX is primarily excreted from the human body through the urinary system, with approximately 10% being eliminated as the parent compound (FLX) and the remainder as norfluoxetine (Hiemke, Härtter, 2000; Brooks *et al*., 2003).

Alongside the parent compound, norfluoxetine, the primary metabolite, exhibits pharmacological activity and notable persistence, with concentrations often equal to or exceeding those of FLX in the environment (Nałęcz-Jawecki *et al*., 2020). This raises significant concerns about potential ecotoxicological effects, as both the drug and its metabolites may impact aquatic organisms. Both compounds can reach wastewater treatment facilities through human excretion and the improper disposal of unused medication (Brodin *et al*., 2013).

The FLX is hydrolytically and photolytically stable, with an environmental half-life of 14–28 days depending on light and temperature, which hinders its removal during conventional wastewater treatment (Kwon, Armbrust, 2006). However, its stability is highly dependent on environmental conditions, and Pan *et al.* (2022) demonstrated that FLX can be almost completely degraded within five days under UV irradiation, underscoring the variability of its environmental persistence. Consequently, environmental concentrations of this drug are often detected at levels ranging from 0.012 to 1.4µg/L^{-1} (Calisto, Esteves, 2009; Togunde *et al*., 2012; Gomes, Gomes, 2024), raising concerns about its potential effects on aquatic biota (Daughton, Ternes, 1999).

The FLX has the potential to act as an endocrine disruptor in aquatic ecosystems, interfering with the hormonal regulation and reproductive capacities of aquatic organisms (Fong, 1998). It also impacts fish behavior, altering feeding activity, locomotion, and escape responses, which may ultimately compromise survival (Orozco-Hernández *et al*., 2022). Prolonged exposure has been linked to stress, hematological changes, and damage to vital organs, including the liver and kidneys, as well as the nervous system (Correia *et al*., 2023). Moreover, exposure to FLX can hinder feeding, growth, reproduction, and overall population success in fish, as evidenced by studies on *Cichlasoma dimerus* subjected to intraperitoneal injection (Dorelle *et al*., 2020), highlighting the compound's significant ecotoxicological implications.

Although FLX is water-soluble, solubility alone does not dictate its potential for bioaccumulation. Its moderate lipophilicity (log Kow ≈ 4.05) promotes interactions with biological membranes and plasma proteins, resulting in accumulation in tissues such as the liver, gills, and brain (Pan *et al*., 2018; Duarte *et al*., 2020). Additionally, FLX has a relatively long half-life in organisms (4–6 days in humans and up to 16 days for norfluoxetine), which further enhances its bioaccumulative potential. Furthermore, the physiological characteristics of the organisms exposed, along with various environmental factors, can significantly influence absorption, distribution, and metabolism (Yamindago *et al*., 2021).

In traditional wastewater treatment facilities, the primary processes consist of coagulation/flocculation, sedimentation, filtration, and disinfection. Although these methods are effective in eliminating suspended solids and organic matter, they are less effective at removing micropollutants, such as pharmaceuticals and hormones. This limitation accounts for the persistent presence of these substances in treated effluents (Kaushik, Thomas, 2019; Reis *et al*., 2019). In response, Constructed Wetlands (CWs) have emerged as a promising and sustainable technology for the phytoremediation of contaminated wastewater. These systems typically consist of shallow ponds or canals that contain filter beds supporting aquatic vegetation. Contaminated wastewater is routed through these filter beds, where a combination of physical, chemical, and biological processes — such as filtration, sedimentation, adsorption, precipitation, and biodegradation — takes place. These processes effectively remove a wide range of contaminants from effluents (Dotro *et al*., 2021; Hassan *et al*., 2021; Kiflay *et al*., 2021). Consequently, CWs have garnered significant recognition as a viable option for treating contaminated wastewater across various contexts.

The CWs offer an ecologically sustainable solution for water remediation, presenting several advantages over traditional treatment methods, such as lower costs and simpler operation (Sezerino *et al*., 2018). These systems are effective at removing suspended solids, organic matter, and soluble nutrients, making them particularly suitable for protecting aquatic communities and enabling non-potable water reuse, like irrigation. However, their removal efficiency is influenced by factors such as the type of contaminant, hydraulic retention time, and the physicochemical characteristics of the influent. It's important to note that CWs are not recommended for direct human consumption without additional potabilization steps (Dotro *et al*., 2021; Hassan *et al*., 2021). Fish serve as effective bioindicators due to their sensitivity to contaminants and their capacity to accumulate pollutants, which leads to observable physiological, biochemical, and histological responses (Monteiro *et al*., 2010; Tincani *et al*., 2019). *Astyanax altiparanae* Garutti & Britski, 2000, commonly found in Brazilian freshwater systems, is noted for its tolerance to a variety of environmental conditions and its ecological significance, making it an ideal model for ecotoxicological research (Schulz, Martins-Junior, 2001; Ostrensky *et al*., 2016). Due to the scarcity of studies examining the cyto/genotoxic potential of FLX in aquatic environments, this research aimed to assess the toxicological effects of FLX on the blood and gills of *A. altiparanae*. Additionally, it sought to evaluate the effectiveness of a sustainable treatment approach using a vertical flow constructed wetland (VFCW) system to manage this contaminant.

MATERIAL AND METHODS

Fluoxetine hydrochloride. The pharmaceutical fluoxetine hydrochloride (TEUTO® Brazilian Laboratory S/A, FLX, batch: 2561244, CAS nº: 54910–89–3) was acquired from a local commercial supplier for experimental purposes. A stock solution was prepared by dissolving an accurate quantity of FLX in distilled water, achieving a concentration of 10 mg L^{-1}. This solution was stored at 4 °C. Experimental concentrations were derived by diluting the stock solution in specific proportions with dechlorinated water.

The drug was administered at varying concentrations for each group: Group 1 served as the negative control and received only dechlorinated water; Group 2 was exposed to a concentration of 0.01 mg L^{-1}; Group 3 to 0.1 mg L^{-1}; Group 4 to 1 mg L^{-1}; and Group 5, also at 1 mg L^{-1}, but with prior treatment through the VFCW (Vertical Flow Constructed Wetland) system utilizing phytoremediation. The concentrations were selected based on Vijitkul *et al.* (2022), who evaluated the effects of FLX on fish.

Fluoxetine hydrochloride analysis in water

Sample preparation. For the chromatographic analysis of FLX, a solution with a concentration of 1 mg L^{-1} was prepared. The sample underwent centrifugation at 10,000 rpm for 20 min at 4 °C, after which the supernatant was filtered through a 0.22 µm syringe filter. Identification of fluoxetine hydrochloride was achieved by comparing the retention time of the peak in the sample with that of a standard solution.

The samples were introduced into an Acquity UPLC® system (Milford, MA, USA) coupled with an Acquity TQDTM triple quadrupole mass spectrometer (Milford, MA, USA), which was equipped with a Waters Zspray™ (ESI) ionization source (Milford, MA, USA). The mobile phases comprised ultrapure water acidified with 0.1% formic acid (A) and acetonitrile (B). The mass spectrometer operated in positive ion mode with the following settings: capillary voltage of 3.0 kV, cone voltage adjusted based on the analyte, desolvation gas temperature of 500 °C with a gas flow rate of 400 L h^{-1}, and collision energy set at 12 V. The separation of analytes was performed on an Acquity UPLC® CSH C18 column (100 mm × 2.1 mm, 1.8 µm).

The gradient elution program utilized a flow rate of 0.20 mL min^{-1} with the following solvent ratios: 65A:35B from 0 to 2.0 min, 20A:80B from 2.0 to 4.0 min, and 65A:35B maintained until the total run time of 8.0 min. The column temperature was kept at 40 °C, and the injection volume was 2.0 µL. Data processing was carried out using MassLynx™ 4.1 software (Milford, MA, USA), and results were reported in ppm.

Experimental design. Adult individuals (both males and females) of *Astyanax altiparanae* were used, with an average weight of 9 ± 12 g and length of 8 ± 12 cm, obtained from a local fish farm. The fish were acclimated in aquaria with dechlorinated water at room temperature, under constant aeration, a natural photoperiod (12:12 h light/dark cycle), and daily feeding with specific commercial feed for small fish (Basic Alcon® Fish Food, Camboriú, SC, Brazil) for 10 days at the Sectoral Vivarium for Fish Maintenance and Experimentation at the Universidade Estadual de Maringá (UEM), in Maringá, Paraná, Brazil.

A total of five experimental groups were tested, each consisting of four fish, and all experiments were carried out in triplicate, resulting in a total of 60 fish. The aquarium housed two fish per unit, which was determined based on the body mass-to-water volume ratio, adhering to the recommended limit of 0.5–2 g/L as outlined in the CONCEA Guide (Annex I – Fish). In the control group (Group 1), fish were maintained in dechlorinated water. In the remaining experimental groups (Groups 2 to 4), fish were exposed to FLX at concentrations of 0.01, 0.1, and 1 mg L^{-1}, respectively. In Group 5, the 1 mg L^{-1} concentration of FLX was previously subjected to phytoremediation using the VFCW system, and the fish were subsequently exposed to the resulting treated effluent. Each group was exposed to FLX dissolved in 10 L of water for 96 h, characterizing an acute test. All samples were coded and analyzed under blind conditions.

Dissection and sampling. After four days of exposure, the fish were removed from the aquarium (one at a time) and bathed in an anesthetic solution: clove oil, 5 ml, diluted with ethyl alcohol, 20 ml, as per Inoue *et al*. (2005). Then, 1 ml of this solution was added per L of water. The animal was only manipulated after it failed to respond to physical stimulus, denoting death from anesthetic overdose (Svobodová, Vykusová, 1991). After euthanasia, the blood was collected to perform the micronucleus tests, cellular morphological changes, and comet assay in erythrocytes, as well as, the gills of the animals were collected and stored for histopathological analysis, as described below. Finally, the animals were fixed in commercial absolute alcohol, recorded with number and origin, and stored in glass vials from the Universidade Estadual de Maringá – UEM/NUPELIA laboratory. Voucher specimens were deposited in the Fish Collection of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (NUPELIA), Universidade Estadual de Maringá, municipality of Maringá, Paraná State, Brazil, as *Astyanax altiparanae* (NUP 25442).

Construction of experimental built wetland treatment units with the vertical flow (VFCW). The making of the VFCW units followed some suggestions from the manual by Sezerino *et al*. (2018), with adaptations according to Silva *et al*. (2024). This study made them from two cylindrical high-density polyethylene (HDPE) containers (0.80 m in height X 0.55 m in diameter). The filter mass of the first stage was composed of 0.2 m of crushed stone (25–50 mm), 0.15 m of crushed stone (19–25 mm), and 0.45m of crushed stone (7–9.5 mm) (gravel). The second stage consisted of 0.2 m of gravel (19–25mm), 0.15m of gravel (7–9.5 mm), and 0.45 m of sand (1.2–2 mm). The adduction system consisted of 25 mm diameter polyvinyl chloride pipes and connections. The effluent was drained through an adductor system with perforations of 8.0 mm in diameter distributed along its entire extremity. The drainage pipe was positioned horizontally at the bottom of the bed, extending across the whole diameter of the units. The system contains a faucet for outputting the treated effluent at the bottom of the reservoir, installed 10 cm from the bottom (Fig. 1).

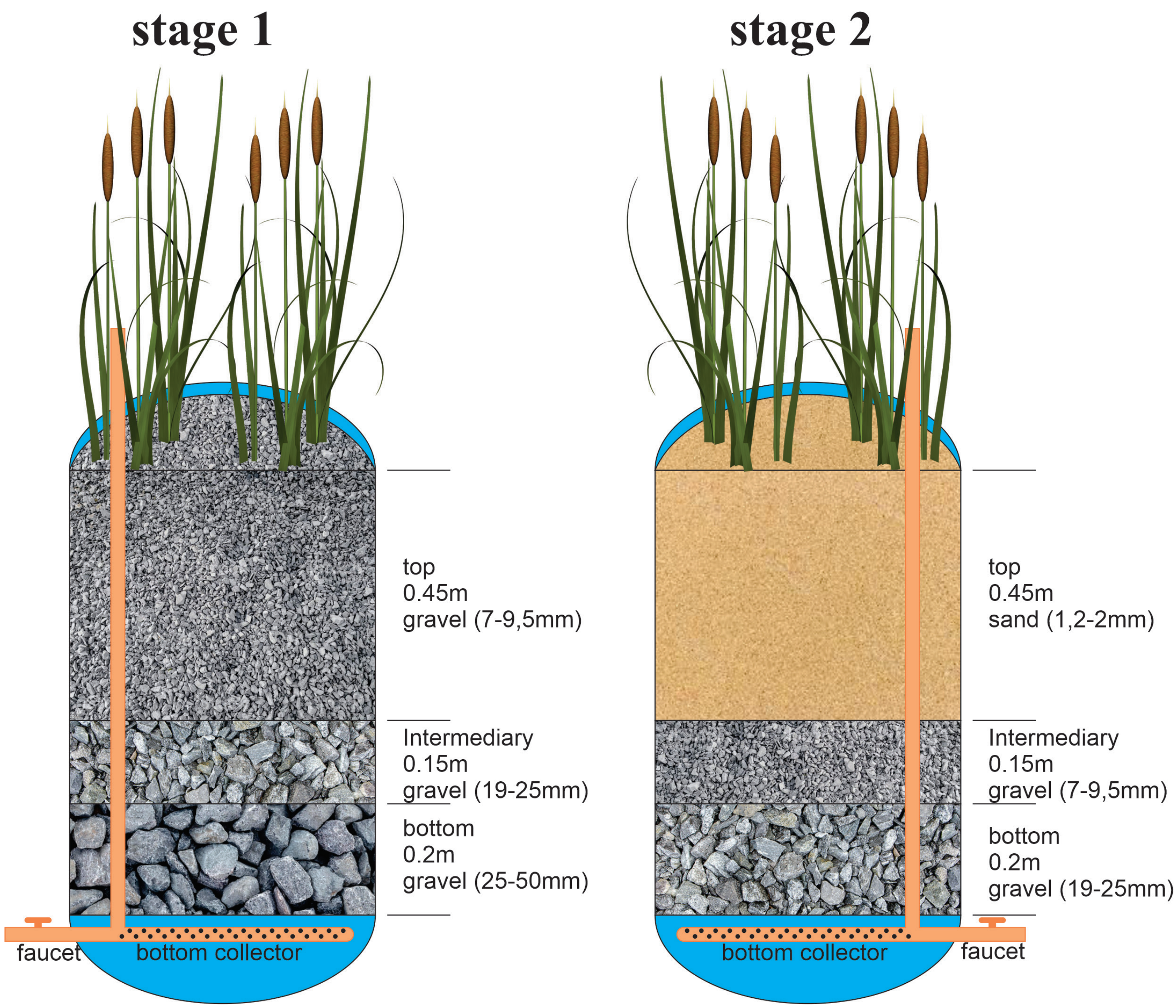

FIGURE 1 | Scheme of an experimental vertical flow constructed wetland system (VFCW) unit.

The beds were populated with *Typha domingensis* at 16 plants per square meter density. The propagules were collected manually in a naturally flooded area located on a rural property in the municipality of Cianorte at the beginning of December 2021. The collection was carried out so that the rhizomes were preserved, carefully transported, and transplanted in the experimental units. The residence time of the effluent in the treatment system was 96 h, 48 h in the first stage, and 48 h in the second stage.

Micronucleus test and cellular morphological changes in erythrocytes. The micronucleus (MN) and cellular morphological changes (CMC) in the erythrocytes test were performed based on the description by Hooftman, Raat (1982). After the anesthesia of the animals, blood was extracted from the caudal vein with a heparinized syringe. A sample (approximately 10 µl) was dripped onto a sanitized glass slide, and the smear was performed with the aid of another slide. The dripped slide was kept at room temperature, drying for at least 12 h. Afterward, they were fixed with absolute ethanol for 20 min. Staining was performed for 10 min with 5% Giemsa solution diluted in phosphate buffer (pH 6.8). Then, the slides were washed in distilled water, left to dry naturally, and kept in closed boxes until analysis under microscopy. The slides were analyzed by optical microscopy under 1000 × magnification. The MN count and the study of CMC were performed on 2,000 erythrocytes per fish.

Comet assay. The alkaline Comet assay was performed according to Speit, Hartmann (1999), without species-specific modifications. Analyses were conducted in a blinded manner to assess conditions under an epifluorescence microscope, with images captured at 400× magnification. DNA damage was evaluated by visually classifying 100 nucleoids per fish into five categories (0–4), where class 0 indicates no damage and class 4 represents maximum damage. This assessment allowed for the derivation of two complementary parameters (Collins *et al*., 2023): (i) the DNA Damage Index (DI), representing the intensity of damage, calculated as the sum of the nucleoids in each class multiplied by the corresponding class value, with a range from 0 (no damage) to 400 arbitrary units (maximum damage); and (ii) the DNA Damage Frequency (DF), which reflects the proportion of nucleoids exhibiting some degree of damage (classes 1–4), expressed as a percentage of the total analyzed. The DI provides a measure of the average severity of DNA strand breaks, whereas the DF indicates the percentage of affected cells within each experimental group.

Histological analysis. After their gills were removed and washed in 0.9% saline solution and fixed in Bouin's aqueous solution for 12 h (Behmer *et al*., 1976), subsequently stored in 70% alcohol. For histological processing, the material was dehydrated in an ascending series of alcohol (80%, 90% and 100%), clarified in xylene and embedded in paraffin. Semi-serial cross-sections of 5 µm thickness were obtained using a LEICA rotary microtome at the Animal Histotechnology Laboratory of the Department of Morphological Sciences at the Universidade Estadual de Maringá. The slides were stained by the Hematoxylin-Eosin (H.E.) and Periodic Acid-Schiff (PAS) methods (Behmer *et al*., 1976). An optical microscope (Olympus CX31RBSFA) was used to analyze the changes.

Quantitative analysis of gill changes. For morphological analysis and changes in the gills, 30 random fields per animal were evaluated under an optical microscope with a total magnification of 40x (Olympus CX31RBSFA) according to the semiquantitative method proposed by Schwaiger *et al.* (1997). Graduated Histological Change Index (HAI) scale, depending on the severity of the lesions, as described by Mallat (1985) with adaptation: Damage 0 = no histological changes; Damage 1: small changes; Damage 2: moderate and specific changes; Damage 3: moderate and extensive changes; Damage 4: severe, extensive and irreparable changes, due to secondary lamellar fusion, hypertrophy and hyperplasia of lamellar epithelial cells. For telangiectasia and aneurysm, 30 random fields per animal were evaluated under an optical microscope at 40x total magnification (Olympus CX31RBSFA), and the quantity per field was counted.

Statistical analysis. Statistical analyses were performed separately using the Kolmogorov-Smirnov normality test. Micronucleus (MN) and cellular morphological changes (CMC) data were obtained by the One-way ANOVA test followed by Tukey's post-test. Data were received using the one-way ANOVA test for the comet assay. For analyses Gills histopathological, statistical analyses of the collected data were conducted to assess normality using the Kolmogorov-Smirnov test. Levene's test was used to assess the homogeneity of variances between the experimental groups. The results indicated that the variances between the groups were comprehensive ($p > 0.05$), allowing the application of ANOVA for data analysis. A one-way analysis of variance (ANOVA) was performed, accompanied by Tukey's post-test. A significance level of 5% was set, and the results were reported as mean ± standard error.

For the analysis of the frequency distributions of damage in the alterations of secondary lamellar fusion, hyperplasia, and hypertrophy, between the experimental groups and the control group, two statistical tests were performed: the Chi-square test and the Fisher's exact test ($p > 0.05$).

RESULTS

Analysis of MN and CMC in erythrocytes. The exposure of *Astyanax altiparanae* specimens to FLX resulted in various morphological alterations in erythrocytes. The sublethal concentrations of the compound used in this study induced a significantly higher number of micronuclei (MN) and other nuclear abnormalities (CMC) in erythrocytes compared to the control group.

A total of 120,000 erythrocytes from 60 *A. altiparanae* specimens were analyzed, revealing 13 micronucleated cells and 1,428 morphological cellular changes (Tab. 1). The observed alterations included: micronucleus (12), notched nucleus (143), binucleated cell (57), macronucleus (63), fragmented nucleus (217), ellipsoid erythrocyte (160), blebbed nucleus (30), lobed nucleus (30), nuclear constriction (65), crenated erythrocyte (197), cytoplasmic vacuole (311), and tear-shaped erythrocyte (143). Some of these alterations can be observed in Fig. 2.

No significant differences in the frequency of MN and CMC were observed between the control group and the FLX group treated via the VFCW system ($p > 0.05$ in Tab. 1).

However, the other exposure concentrations showed statistically significant differences when compared to both the control and the VFCW-treated fluoxetine group ($p < 0.05$ in Tab. 1).

Comet assay. DNA damage classes 1, 2, 3 and 4 were detected through the comet assay only in the groups exposed to 0.1 and 1 mg L^{-1} (Fig. 3). An apparent increase in DNA damage index (DI) was observed, with the highest values recorded at concentration 1 mg L^{-1} of FLX. In contrast, the group treated by VCFW showed a DI close to that of the control group (Fig. 4).

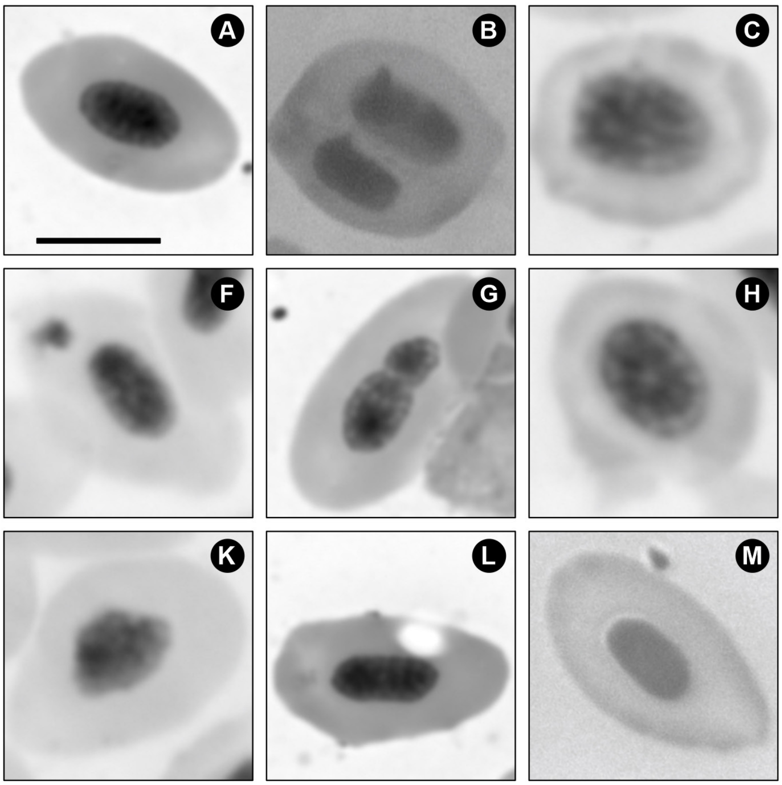

FIGURE 2 | Photomicrograph of *Astyanax altiparanae* erythrocytes exposed to fluoxetine hydrochloride and stained with Giemsa. **A.** Normal Erythrocyte; **B.** Binucleate; **C.** Crenated Erythrocyte; **D.** Macronucleus; **E.** Blebbed; **F.** Micronuclei; **G.** Nuclear Constriction; **H.** Fragmented Nucleus; **I.** Lobed; **J.** Elliptocyte; **K.** Notched; **L.** Cytoplasmic Vacuole; **M.** Tear-Drop. Scale bar = 5 µm.

TABLE 1 | Absolute numbers for alteration in *Astyanax altiparanae* erythrocytes. For each fish, 2000 cells were analyzed. *Significantly different to control group < 0.05; MN = micronuclei; CMC = Cellular morphological changes.

Group	MN	Erythrocytes with CMC	Mean ± standard deviation of MN and CMC numbers
Control	0	46	3.7 ± 2.1
0.01 mg/L^{-1}	1	282	23.5 ± 4.6*
0.1 mg/L^{-1}	2	339	27.5 ± 9.5*
1 mg/L^{-1}	8	663	56.0 ± 14.8*
Treated VFCW	1	86	7.2 ± 2.2

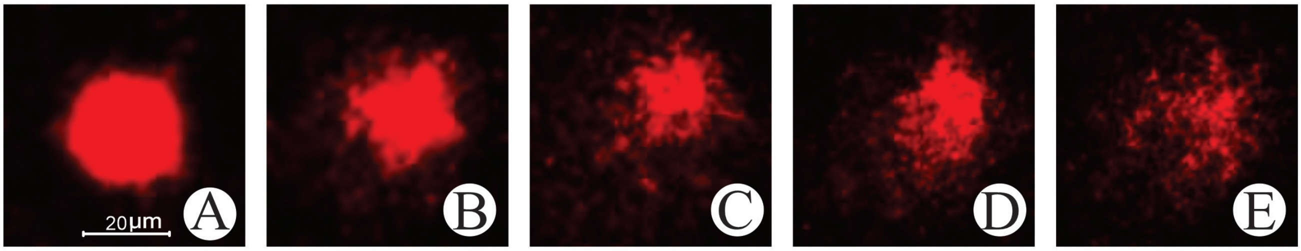

FIGURE 3 | Damage to erythrocytes from *Astyanax altiparanae* specimens from the control group and following exposure to FLX was measured by the comet assay (Magnification 400x). **A.** Normal cell – damage 0; **B.** Damage 1; **C.** Damage 2; **D.** Damage 3; **E.** Damage 4. Scale bar = 20 µm.

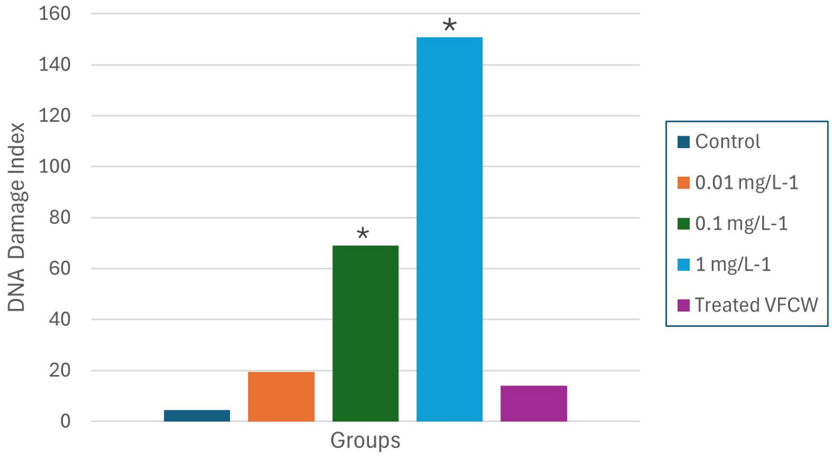

FIGURE 4 | DNA Damage Index (DI, mean ± SE) in erythrocytes of *Astyanax altiparanae* after 96 h of exposure to FLX (0.01, 0.1, 1 mg L^{-1}) and VFCW-treated effluent, compared to the control group. *Significantly different to control group < 0.05.

The distribution of damage classes (DF) indicated that the control group demonstrated approximately 96% of erythrocytes without alterations, with only 4% exhibiting some degree of damage. In contrast, the group exposed to 1 mg L^{-1} showed a significant decrease in intact cells (36%) and an increase in damaged cells to 64%. Intermediate concentrations revealed about 15% of damaged cells at 0.01 mg L^{-1} and 33% at 0.1 mg L^{-1}. Conversely, the group exposed to VFCW-treated effluent displayed around 89% of cells without damage, a result comparable to the control group ($p > 0.05$) (Fig. 5). Statistically significant differences ($p < 0.05$) were observed between the groups exposed to concentrations of 0.1 and 1 mg L^{-1} of FLX and both the control and VFCW-treated groups (Fig. 4).

Histological analysis of gills. Normal gills exhibited the expected structure, with well-defined and organized lamellae, and no signs of damage or abnormalities. However, several other pathological alterations were observed in samples exposed to FLX (Fig. 6), with severity and frequency increasing proportionally to the concentration.

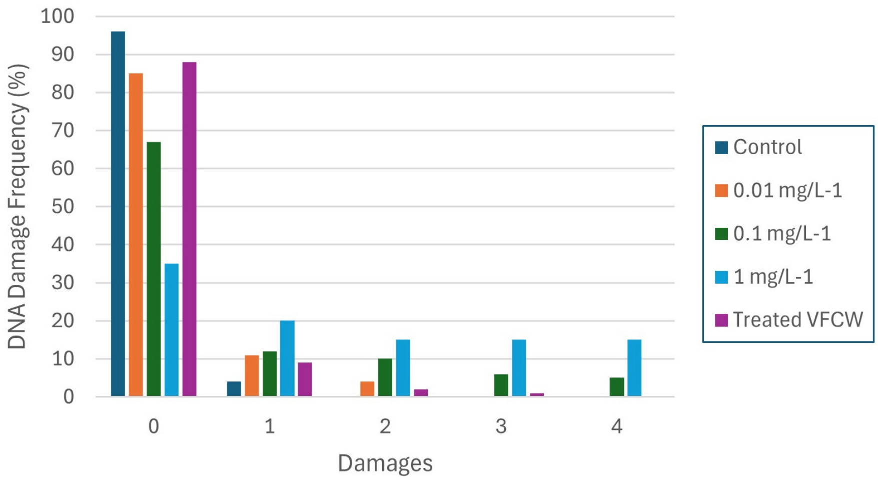

FIGURE 5 | DNA Damage Frequency (DF, %, mean ± SE) in erythrocytes of *Astyanax altiparanae* after 96 h exposure to FLX (0.01, 0.1, 1 mg L^{-1}) and VFCW-treated effluent. Class 0 = no damage; Classes 1–4 = increasing DNA damage.

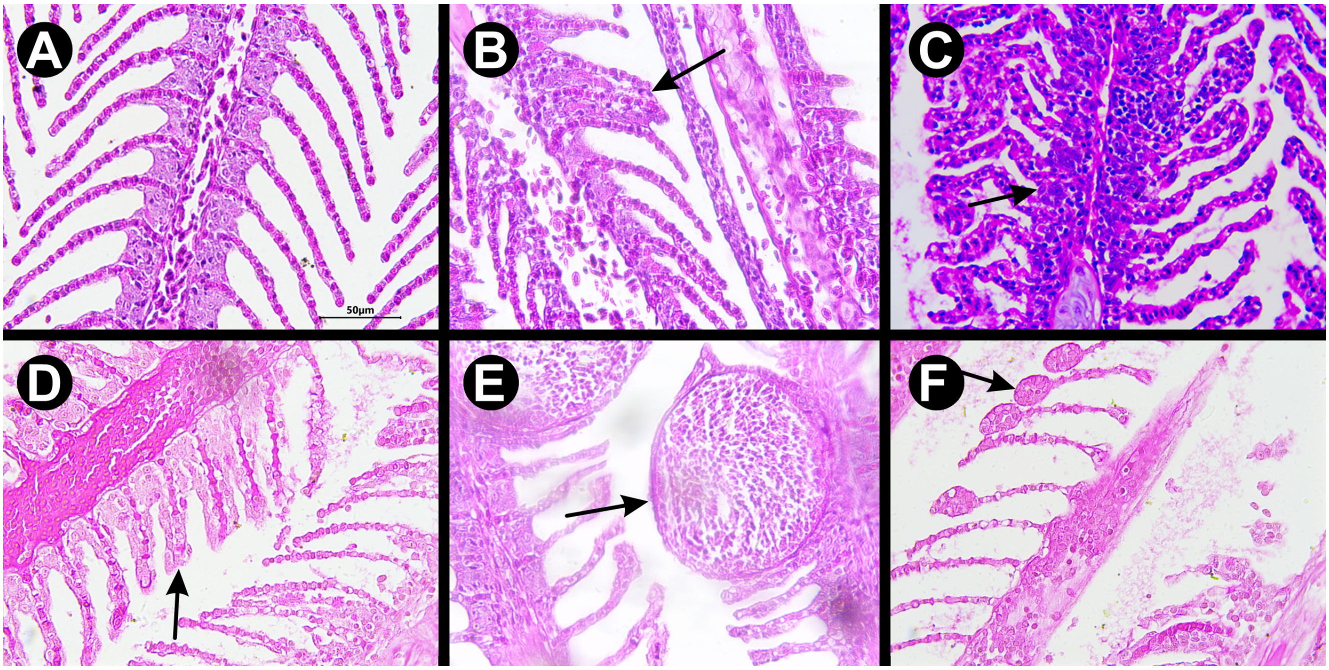

FIGURE 6 | Photomicrograph of *Astyanax altiparanae* gills exposed to different concentrations of fluoxetine hydrochloride and stained with hematoxylin and eosin (HE). **A.** Control; **B.** Secondary lamellar fusion; **C.** Hypertrophy; **D.** Hyperplasia; **E.** Telangiectasia; **F.** Aneurysm. Scale bar = 50 µm.

For instance, secondary lamellar fusion was observed with 30 mild alterations in the control group, whereas the group exposed to 1 mg L^{-1} presented 64 severe, extensive, and/or irreversible alterations (Tab. 2). Regarding lamellar epithelial hyperplasia, the control group showed only 18 mild changes, while the 1 mg L^{-1} group exhibited 61 severe, extensive, and/or irreversible changes. Similarly, in epithelial cell hypertrophy, the control group showed only 14 mild alterations, while the group exposed to 1 mg L^{-1} showed 53 severe alterations (Tab. 2).

The results for telangiectasia at the 0.01 mg L^{-1} concentration ($P = 0.1861$) and in the group treated with WCFV-treated fluoxetine showed no significant differences compared to the control group ($p > 0.05$ in Tab. 3). In the aneurysm analysis, only the group exposed to VFCW-treated fluoxetine did not differ significantly from the control ($p > 0.05$ in Tab. 3), while all other concentrations presented statistically significant differences from the control. Both telangiectasia and aneurysms showed dose-dependent responses.

Fluoxetine hydrochloride analysis in water. Water analysis was performed using the UPLC-MS/MS technique, which yielded a calibration curve for FLX with an R^2 value of 0.9948, represented by the equation $y = 125547x - 4320$ (Fig. **S1**). The water sample containing 1 mg L^{-1} of FLX, after being treated through the WCFV system, presented a concentration below the detection limit, indicating that the fluoxetine concentration in the sample was less than 0.01 mg L^{-1}.

TABLE 2 | Levels of histopathological damage in the gills of *Astyanax altiparanae*, consolidating lamellar fusion, hyperplasia, and hypertrophy. Damage 0 = no histological alteration; Damage 1 = mild alterations; Damage 2 = moderate and specific alterations; Damage 3 = moderate and extensive alterations; Damage 4 = severe, extensive, and/or irreversible alterations. *Significantly different to control group < 0.05.

Group	Secondary Lamellar Fusion					Hyperplasia					Hypertrophy				
Damage	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
Control	120	30	0	0	0	122	18	10	0	0	130	14	6	0	0
0.01 mg/L^{-1} *	62	42	30	11	5	52	68	30	0	0	86	32	21	11	0
0.1 mg/L^{-1} *	21	30	38	44	17	0	18	61	57	14	49	77	11	13	0
1 mg/L^{-1} *	3	20	31	32	64	0	21	33	35	61	0	19	38	40	53
Treated (VFCW)	72	52	16	10	0	68	46	27	9	0	110	22	10	8	0

TABLE 3 | Whole values representing the total number of aneurysms and telangiectasias observed in the gills of *Astyanax altiparanae*. Data correspond to the sum of lesions recorded in five individuals per experimental group. *Significantly different to control group < 0.05.

FLX Concentration	Total Aneurysms	Total Telangiectasia
Control	41	24
0.01 mg/L^1	132*	53
0.1 mg/L^1	268*	111*
1 mg/L^1	387*	143*
Treated (VFCW)	81	48*

DISCUSSION

The cyto/genotoxic effects of FLX in *A. altiparanae* were observed starting from the lowest dose, with significant changes evident from a concentration of 0.1 mg L^{-1}. Although the toxic effects tended to increase with FLX concentration, no statistically significant differences were observed between the intermediate doses (0.01 and 0.1 mg L^{-1}). Thus, our results demonstrate the occurrence of adverse effects starting from a threshold concentration, but not a strictly dose-dependent response. However, the increase in damage related to increasing concentrations is in line with the findings of other studies conducted in several organisms and tissues, including *Schmidtea mediterranea* (Ofoegbu *et al*., 2019), *Drosophila melanogaster* (Öz *et al*., 2024), rat C6 glioma cells (Slamon *et al*., 2001), and hamster ovary cells (Lemos *et al*., 2005).

The findings of this study suggest that exposure to FLX can lead to CMC in the erythrocytes of *A. altiparanae*. Data analysis indicated a notable increase in the frequency of erythrocyte alterations among the group exposed to the highest concentration of FLX (1 mg L^{-1}) when compared to the control group. A similar pattern was observed in the study by Vijitkul *et al*. (2022), which reported that after 96 hours of exposure to FLX, concentrations exceeding 100 µg L^{-1} resulted in alterations in the erythrocytes of *Oreochromis niloticus*.

An apparent increase in DNA damage was observed, with the highest values recorded at a concentration of 1 mg L^{-1} of FLX, as measured by the DNA Damage Index (DI) and DNA Damage Frequency (DF). These parameters facilitated a comprehensive evaluation, revealing a significant intensity and extent of DNA damage in this group compared to those with lower concentrations of FLX. Specifically, group 2 (0.01 mg L^{-1}) exhibited up to approximately 10% of cells showing DNA damage classified as classes 1 and 2, with no cells categorized in classes 3 and 4. In contrast, group 3 (0.1 mg L^{-1}) showed up to around 10% of cells with DNA damage across all classes from 1 to 4.

Significant results were observed in the comparison between groups 4 and 5, both of which were treated at the same concentration (1 mg L^{-1}). Notably, group 5, which underwent treatment in VFCW, achieved average outcomes comparable to those of the control group, while the untreated group displayed a substantially higher average in changes. This highlights the effectiveness of the VFWC treatment in removing FLX compounds. Previous studies have already established the efficacy of VFCW in eliminating contaminants, as evidenced by its cytotoxic effects after treatment with various xenobiotics, along with similar findings concerning textile effluent (Silva *et al*., 2023), herbicides (Andrade *et al*., 2024), and textile dyes (Silva *et al*., 2024). Utilizing the ULPC-MS/MS technique, our study revealed that the VFCW system achieved an impressive contaminant removal rate of 99.99% at a dosage of 1 mg L^{-1}. This finding aligns with the research conducted by Ilyas *et al*. (2020), which examined 34 widely studied pharmaceuticals and reported an average removal efficiency of up to 93% in constructed wetlands.

The significant reduction of FLX in VFCW (>99%) resulted in the display of around 89% of cells without DNA damage, a significant reduction in MN, CMC, and histopathological lesions in *A. altiparanae,* a result comparable to the control ($p > 0.05$), reinforcing the efficiency of the system in mitigating the cyto/genotoxic effects of FLX. Similar high efficiencies for drugs have been reported in other CWs

(Ilyas *et al*., 2020; Chen *et al*., 2016). However, previous studies indicate that removal performance may be less consistent at environmentally very low concentrations (<1 μg L^{-1}) or in the presence of complex mixtures of micropollutants, which represents an important limitation to be considered in practical applications (Matamoros, Bayona, 2006; Verlicchi, Zambello, 2014).

Similarly, the histopathological changes observed in this study were significant following exposure to FLX, particularly at the highest concentration. However, the alterations in the gills were notably less pronounced in group 5, which was treated with the VFCW system. These changes are frequently linked to adaptive responses to environmental stressors in aquatic ecosystems (Pramanik, Biswas, 2024). Similar findings were reported by Rezaeipour *et al*. (2024), who studied *Danio rerio* exposed to FLX concentrations ranging from 0.1 to 100 μg L^{-1} over 96 h. Their observations included histopathological alterations such as epithelial hyperplasia, lamellar fusion, aneurysm formation, and lamellar clubbing. These results support our findings in *A. altiparanae*, emphasizing that even environmentally relevant concentrations, significantly lower than those tested here, can lead to notable gill lesions. This underscores the ecological necessity of removing FLX from aquatic systems.

While some of these changes may be reversible in the short term, prolonged exposure results in detrimental effects such as lamellae obstruction, epithelial thickening, and a reduced water contact area, as previously observed in fish exposed to antibiotic oxytetracycline and Metformin hydrochloride (Rodrigues *et al*., 2017; Barbieri *et al*., 2022). Therefore, despite the initial protective role of gill modifications, this process ultimately impairs the efficiency of gas exchange, jeopardizing tissue oxygenation and, as a result, the overall health of the organism (Kumar *et al*., 2019).

The vascular lesions identified in this study, including telangiectasias and aneurysms, may be linked to hemodynamic changes in the branchial lamellae due to increased blood flow, which can lead to the rupture of pillar cells and capillaries (Ahmed *et al*., 2013). These lesions are indicative of significant damage, potentially resulting in internal hemorrhages and respiratory dysfunction (Azadbakht *et al*., 2019; Hasan *et al*., 2022). The presence of telangiectasias further underscores the impact of FLX on the branchial microvasculature, as noted by Strzyżewska-Worotyńska *et al*. (2017).

Fish gills serve as the primary organs for respiration, excretion, gas exchange, and osmoregulation, making them vital indicators of population health and valuable tools for aquatic biomonitoring. In this study, the gill histology of *Astyanax altiparanae* exposed to FLX revealed several alterations, including lamellar fusion, epithelial hyperplasia, hypertrophy, aneurysms, and telangiectasia. Changes in lamellar structure and epithelial tissue are recognized as primary pathological indicators in fish (Thophon *et al*., 2003) that can reduce respiratory efficiency and may become irreversible with prolonged exposure (Hesni *et al*., 2011; Mauryaa *et al*., 2019). Additionally, vascular lesions can lead to hemorrhage and significant impairment (Ahmed *et al*., 2013).

Numerous studies support these findings, indicating that fish exposed to various contaminants, including pesticides, pharmaceuticals, and industrial effluents, exhibit similar patterns of gill damage, irrespective of the pollutant's chemical nature (Kumar *et al*., 2016; Nowakowska *et al*., 2020; Ogunwole *et al*., 2021; Rezaeipour *et al*., 2024). Overall, the observed changes reflect the fish's defense mechanisms against contaminant entry but underscore the ecological risk posed by FLX as it compromises respiratory and osmoregulatory functions in fish.

Moreover, existing literature suggests that FLX is not metabolized extensively in fish, which contributes to its bioaccumulation in various tissues, such as the liver, brain, muscles, and gills (Pan *et al*., 2018; Duarte *et al*., 2020). Yan *et al*. (2020) found that both FLX and its active metabolite, norfluoxetine, were present in all tissues of Carassius auratus, with concentrations increasing over the duration of exposure. Likewise, Vaclavik *et al*. (2022) note that the gills serve as a primary entry route for FLX into the body and are among the most structurally impacted tissues.

We emphasize that the VFCW effectively removed FLX to non-detectable levels and substantially reduced cytogenotoxic and histopathological outcomes in *A. altiparanae*. By reducing dissolved FLX through substrate sorption, microbial degradation, and phytoremediation, the VFCW decreases branchial exposure, which in turn mitigates epithelial and vascular lesions. These results, consistent with reports of high pharmaceutical removal in constructed wetlands (Chen *et al*., 2016; Ilyas *et al*., 2020; Dotro *et al*., 2021), validate previous evidence and reinforce that FLX exposure, even at environmentally relevant concentrations, can seriously compromise gill integrity and fish homeostasis. This underscores the ecological relevance of prioritizing FLX monitoring and implementing sustainable treatment technologies, such as VFCWs, to mitigate the impact of pharmaceutical effluents on aquatic systems.

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AUTHORS' CONTRIBUTION

Josiane Rodrigues Rocha da Silva: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing-original draft.

Camila Oliveira de Andrade: Visualization, Writing-original draft, Writing-review and editing.

Patrícia Daniele Silva dos Santos: Formal analysis, Investigation, Methodology, Software.

Oscar Oliveira Santos: Formal analysis, Investigation, Methodology.

Roney Peterson Pereira: Formal analysis, Software, Writing-review and editing.

Carlos Alexandre Fernandes: Project administration, Resources, Supervision, Validation, Visualization, Writing-review and editing.

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ETHICAL STATEMENT

All procedures involving the use of animals in research were approved by the Animal Ethics Committee (CEUA – UEM), under license number 3359040723.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

AI STATEMENT

The authors did not use any AI-assisted technologies in the creation of this manuscript or its figures.

COMPETING INTERESTS

The authors declare no competing interests.

SUPPLEMENTARY MATERIAL

Supplementary material **S1**

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