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Can exposure to Ciprofloxacin alter the biological responses of *Danio rerio*?

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Abstract

Ciprofloxacin, a widely used fluoroquinolone antibiotic, exhibits broad-spectrum efficacy against various bacterial infections in both human and veterinary medicine. However, like many other antibiotics finds its way into natural water bodies, raising concerns about environmental contamination and antimicrobial resistance. *Danio rerio*, with their genetic similarity to humans and regulatory advantages, serve as an ideal model for ecotoxicological studies. This study evaluates biochemical (acetylcholinesterase (ChE) – neurotransmission, glutathione S-transferase (GST) - phase II biotransformation, Glutathione Reductase (GR), Catalase (CAT) and Superoxide dismutase (SOD) - oxidative stress) and behaviour endpoints (swimming behaviour – distance travelled and fish angle path) on zebrafish larvae at 96 hpf after 24 hours of exposure following a dose-response experimental set up (10-50 mg/L). The results revealed that the activity of all studied biomarkers increased in all concentrations except at 50 mg/L for the GR. ChE and CAT activity showed a statistically significant increase at the concentration of 33 mg/L, GST, GR and SOD activity was the highest at 22 mg/L, 33 mg/L and 10 mg/L, respectively. The total distance travelled also showed a slight increase in all concentrations, being the highest at 33 mg/L. A general increase was observed in the percentages of Class 1 and 2 angles (erratic swimming) with increasing doses, reaching highest values at 22-50 mg/L. On the other hand, class 3 and 4 swimming angles (normal swimming pattern) were also affected in a dose-response manner, observing and increase in class 3 angles while class 4 decreased, being 50 mg/L the dose with the highest observed effect. This study found that ciprofloxacin exposure alters biochemical and behavioural responses in zebrafish larvae, highlighting potential ecological risks of pharmaceutical contaminants.

Keywords: zebrafish, *Danio rerio*, ciprofloxacin, antibiotics, biomarkers, eco-toxicology, behaviour

Introduction

Ciprofloxacin belongs to a class of antibiotics known as fluoroquinolones, extensively utilized in human and veterinary healthcare to treat bacterial infections (Ávila *et al.* 2019). These medications possess broad efficacy against a diverse array of both gram-positive and gram-negative bacteria. Their mode of action involves disrupting DNA replication in bacteria by interfering with the normal functioning of DNA topoisomerase IV. Quinolones function by binding to DNA topoisomerase IV, creating temporary complexes with enzyme-DNA cleavage activity. This interaction halts bacterial growth. Cell death occurs through various pathways, including chromosome breakage, influenced by the specific molecular structures of different quinolones. These mechanisms may involve both protein synthesis-dependent and -independent pathways. (Arnoldi *et al.* 2013; Cheng *et al.* 2013).

Antibiotics are metabolized in the body after use by humans or animals, but a large portion (70-90%) is eliminated unchanged through feces and urine. These compounds and their metabolites are released into wastewater from various sources such as hospitals, pharmaceutical companies, aquaculture, farms, and wastewater treatment plants (WWTPs). The capacity of WWTPs to remove them is limited, resulting in significant amounts of antibiotics being detected in surface water, groundwater, and even drinking water. (Maghsodian *et al.* 2022). As a result, like many other antibiotics, the concentration of ciprofloxacin in the environment has been increasing. For instance, a concentration of 0.794 μM has been observed in wastewater from livestock farms in Northern China (Li *et al.* 2018), in Switzerland, a concentration of 2.72 μM was reported in hospital wastewater (Daouk *et al.* 2016), and in Italy concentrations from 17.4 to 558.5 $ng.L^{-1}$ were discovered in rivers (Castiglioni et al. 2008). A positive association has been observed between ciprofloxacin concentrations in rivers and the incidence of fluoroquinolone-resistant *E. coli* in various countries (Kenyon 2022). Consequently, the increasing presence of antibiotics in the environment, especially in bodies of water such as rivers and lakes along with the serious concerns they raise regarding antimicrobial resistance,

ecological impact, transfer of resistance genes, and possible human health effects make antibiotics emerging contaminants (Kümmerer 2009; Bengtsson-Palme and Joakim Larsson 2016).

Recent findings discovered that after exposure to the antibiotics oxytetracycline and sulfamethoxazole, Nile tilapia's intestinal structure was altered, leading to imbalances in the gut microbiota and reducing the presence of proteins crucial for maintaining intestinal integrity. Nile tilapia exposed to low environmental concentrations experienced increased oxidative stress, heightened inflammation, and detoxification responses, and decreased natural defences. Additionally, their ability to counteract oxidative damage was hindered, resulting in increased lipid peroxidation in both intestinal and liver tissues (Limbu *et al.* 2018). Hence it is necessary and relevant to conduct studies on the toxicity of other antibiotics like ciprofloxacin for aquatic organisms. Table 1 summarizes studies that focussed on XXX. One study reported high toxicity associated to exposure to ciprofloxacin in *Cyprinus carpio* embryos, showing mortality rates of 32% when embryos were exposed to 1000 µg L^{-1} and 3000 µg L^{-1} (Zivna *et al*. 2016).

Table 1 - Effects of ciprofloxacin exposure on zebrafish.

Model Organism/Life cycle	**Design**	**Endpoints**	**Results**	**References**
D. rerio juveniles (30 days)	Exposure for 28 days at the concentrations: 0.7 µg.L^{-1} (environmental concentration); 100; 650; 1100; and 3000 µg.L^{-1} .	Biochemical Biomarkers: Glutathione S-transferase (GST); Glutathione Reductase (GR); Glutathione Peroxidase (GPx); Lipid Peroxidation (TBARS). Histopathological examination.	GST: Increased at 0.7 and 100 µg.L^{-1}. GR: Decreased at 1100 and 3000 µg.L^{-1}. GPx: Decreased in all concentrations except at 100 µg.L^{-1}. TBARS: Decreased only at 100 µg.L^{-1}. No histopathological changes were observed, and no changes in fish growth and development.	Plhalova *et al.* 2014
	Exposure for 24 hours at the concentrations: 156; 469; 1407;	Morphological examination. Function effect:	No morphological abnormality was observed in the embryos from all ciprofloxacin concentrations.	

48 hpf *D. rerio* embryos	and 1949 mg/L.	Heart rate and atria/ventricular ratio; Cardiac output and blood flow velocity Transcriptomic alterations of genes related to calcium signaling pathway and cardiac muscle contraction: TPase-related genes (*atp2a1l*); calcium channel-related genes (*cacna1ab*); and the regulatory gene for cardiac troponin C (*tnnc1a*).	There was significant difference in the heart rate at 1407; and 1949 mg/L. Decreased cardiac output and blood flow velocity were observed at 1407; and 1949 mg/L. Results indicated that ciprofloxacin inhibited the heart rhythm and showed a dose-effect correlation. A significant down-regulation of *tnnc1a* mRNA levels at 1949 mg/L. For *cacna1ab* genes, a trending up-regulation was observed.	Shen *et al.* 2019
D. rerio adults	Exposure for 96h at the concentration 5 mg/L.	Biomarkers: Acetylcholinesterase (AChE); Lipid Peroxidation (MDA); Superoxide dismutase (SOD); and GPx. Behavioural endpoints: Total distance (m); Freezing duration (s) and Counterclockwise rotation.	No significant effect in all studied endpoints.	Jijie *et al.* 2023
D. rerio adults	Exposure for 96h at the concentrations: 6.25; 12.5; and 25 mg/L.	Behavioural tests: Exploratory behaviour Y-maze test (s) Aggression and social interaction	The distance travelled increased at 25 mg/L. Y-maze test suggested memory impairment. No social interaction deficits. Ciprofloxacin-treated animals spent more time in the segment nearest to the mirror (6.25 mg/L). Also the number of bites against the mirror was significantly increased (6.25 mg/L).	Petersen *et al.* 2021
	Exposure for 96h at the concentrations: 0.250; 0.500; and 1 µg/L.	Biochemical parameters: ALT, ALP, total bilirubin, and total protein values. Oxidative stress: SOD, CAT and GPx. Histopathological examination	Values from all biochemical parameters increased as the concentrations also increased. Occurrence of oxidative stress. SOD, CAT, and GPx: Increase in a concentration-dependent manner. Histological alterations in the liver.	

D. rerio adults		Gene expression: Genes related to oxidative stress (*nrf1* and *nrf2*); and genes related to apoptosis (*bax*, *casp3*, *casp6*, *casp8*, and *casp9*).	Ciprofloxacin induced congestion, hyperemia, and nuclear alterations in the liver. Significant increase in the gene expression of *nrf1* and *nrf2* in all treatment groups. Genes involved in the apoptosis process were significantly upregulated in all treatment groups. For all genes, the expression increased in a concentration-dependent manner.	Elizalde-Velázquez *et al*. 2022
4 hpf *D. rerio* embryos	Exposure for 92h at the concentrations: 5; 10; 15; 20; 25; 30; 35; and 40 μg L^{-1}.	Biomarkers: SOD; CAT; GPx; LPO (MDA); hydroperoxide content (HPC); and protein carbonyl content (PCC). Morphological examination.	Occurrence of oxidative stress. Increases of LPO activity at all concentrations. SOD; CAT; and GPx activities increased in all concentrations except 5 μg L^{-1}. Incresases in HPC and PCC in all concentrations except 5 μg L^{-1}. Ciprofloxacin induced craniofacial malformations; body hypopigmentation and yolk sac malformation. The concentration that showed the greatest influence on embryo development was 25 μg L^{-1}. Increase in the number of dead and malformed embryos at 40 μg L^{-1}.	Rosas-Ramírez *et al.* 2022
	Exposure for 96h at the concentrations: 0.005; 0.013; 0.031; 0.078; 0.195; and 0.488 μg/L.	Biomarkers: CAT, AChE, GST, GPx and LPO (TBARS). Behavioural parameter: Total swimming time. Epigenetic: Immunohistochemical detection of 5-methylcytidine. Morphological examination.	CAT: Statistically significant decrease at all concentrations except 0.013 μg/L. AChE: Statistically significant increase only at the highest concentration (0.488 μg/L). GST and GPx: No significant alterations at all tested concentrations. TBARS: Significant decrease in lipid peroxidation levels at concentrations of 0.078; 0.195; and 0.488 μg/L. Total swimming distance: Significant differences were observed. During the first light period, a statistical different period of hyper-activity was found at 0.013 μg/L. During	

D. rerio embryos and larvae			the second light period, a significant period of hypo-activity was observed at 0.005 µg/L and 0.031 µg/L. During the first dark period, a statistical significant decrease was found at: 0.005 µg/L; 0.013 µg/L; 0.078 µg/L; and 0.488 µg/L. During the second dark period, a significant increase in the distance moved was observed at: 0.013 µg/L; 0.031 µg/L; 0.078 µg/L; and 0.488 µg/L. No evidence of methylation alterations was observed. No morphological deformations were observed.	Nogueira *et al*. 2019
Cyprinus carpio embryos	Exposure to the concentrations: 1; 100; 500; 1000; and 3000 µg L^{-1}.	Biomarkers: GST, CAT, GPx, GR and LPO (TBARS). Morphological examination.	GST: Significant decrease at 500 µg L^{-1} and 3000 µg L^{-1}. CAT and GPx: Increased activity in most of the tested concentrations. GR: Decreased activity at 500 µg L^{-1} and 3000 µg L^{-1}. TBARS: Concentration significantly lower in all experimental groups. Significant growth reduction only at the highest concentration (3000 µg L^{-1}). The highest numbers of macroscopic morphological anomalies at all concentrations except the lowest.	Zivna *et al*. 2016

As seen in Table 1, ciprofloxacin exposure resulted in various effects across different endpoints in fish embryos, juveniles and adults. These results indicate that ciprofloxacin induces oxidative stress across a wide range of concentrations, evidenced by increases in antioxidant enzyme activities. Morphological abnormalities were concentration-dependent, becoming more prominent at higher levels. Behavioural and cardiovascular effects were also significant, with clear dose-dependent relationships. While some endpoints did not show significant alterations (Jijie *et al.* 2023), the majority suggest that ciprofloxacin can induce multiple adverse effects in

zebrafish, highlighting the importance of understanding and mitigating its environmental impact.

The easy maintenance, rapid embryonic development, and the fact that they have 70% homology to human genes (Howe *et al.* 2013) make zebrafish a species of growing interest for ecotoxicology studies. Additionally, according to European legislation, experiments involving fish embryos until the external feeding stage are not considered "animal models", but rather alternative experimental models, and their use promoted under the 3 R's principle. This includes zebrafish embryos and larvae up to 5 days post-fertilization (dpf). (Bauer *et al.* 2021).

Analyzing and understanding the responses observed in zebrafish can help elucidate the impact of antibiotics like ciprofloxacin and inform strategies for resolving and preventing future environmental and health issues. A multiparametric analysis is essential to correctly evaluate a compound's effect. This analysis should cover various levels of biological organization, from the individual (e.g. analyzing behaviour and assessing mortality and effects on embryonic development) to the biochemical (examining biomarkers) and molecular levels (examining different endogenous molecules). This will ultimately contribute to the establishment of an Adverse Outcome Pathways (AOPs) illustrating the mode of action of the compound from the molecular-level event that triggers a cascade of effects, leading to observable phenotypic outcomes at the individual level (Vinken *et al.* 2017). By integrating these multi-parametric analyses, we aim to contribute in the definition of the AOP of ciprofloxacin, mapping its impact from the initial molecular trigger to the resulting adverse phenotypic effects.

Environmental toxicology research seeks to identify if harmful substances are impacting organism health and to evaluate the condition of ecosystems. Effective evaluation methods should detect early signs of exposure, allowing intervention before environmental damage becomes irreversible. Behaviour, an organism-level response characterized by actions, reactions, or functions of a system under particular conditions (Hellou 2011), directly influences the well-being, ability to reproduce, and survival of organisms within natural ecosystems (Petersen *et al.* 2021). By exposing larvae to ciprofloxacin solutions, we can use their behaviour as a neurotoxicity marker to determine if there are any changes resulting from

alterations in the nervous system. Behavioural endpoints thus serve as effective early warning indicator and should be used alongside biochemical analysis (biomarkers) for comprehensive risk assessment (Hellou 2011). Furthermore, behaviour is a non-invasive technique that can add ecological relevance to studies done in a laboratory environment (Hellou, 2011) and behavioural endpoints can sometimes be more sensitive than traditional biomarkers (Sanchez-Hernandez 2011). Locomotor activity and thigmotaxis, for instance, are two essential measures used to assess anxiety levels in zebrafish. Generally, zebrafish with heightened anxiety show greater locomotion and thigmotaxis (Peng *et al*. 2016).

Promoting the well-being of aquatic environments and pinpointing vulnerable species affected by environmental toxins can be enhanced by merging chemical analysis with specific biological indicators, or biomarkers, measured in tissues (Hook *et al.* 2014). After exposure to pollutants, the levels of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), oxygen (O_2), or hydroxyl radicals (·OH) may be increased (Mansour *et al.* 2020). These ROS can damage DNA, proteins, and lipids (Juan *et al.* 2021). Cells have developed antioxidant defence mechanisms to mitigate ROS damage, comprising both enzymatic antioxidants like Superoxide Dismutase (SOD), catalase (CAT), or Glutathione S-transferase (GST) and nonenzymatic antioxidants like Vitamin A, C and E (Birben *et al.* 2012). Under stress conditions, antioxidant systems can either be upregulated or downregulated, making the measurement of these enzymes a useful biomarker for assessing the health status of aquatic organisms and their response to pollutant-induced oxidative stress (Mansour *et al*. 2020). Key biomarkers include SOD, accountable for the conversion of superoxide anion radicals into hydrogen peroxide (Birben *et al.* 2012), Glutathione Reductase (GR) which is a key enzyme in the antioxidant defense system that catalyzes the reduction of oxidized glutathione (GSSG) to its reduced form (GSH) (Robbins *et al*. 2021). GST that is important in the process of detoxification because it binds to contaminants and produces compounds that are easier to eliminate from the body. CAT, is a peroxisomal enzyme that breaks down hydrogen peroxide (H_2O_2) into water and oxygen, serving as a reliable early biomarker of contamination (Ribeiro *et al*. 2020). Acetylcholinesterase (AChE), essential for nerve function, is responsible for catalyzing the hydrolysis of acetylcholine, a neurotransmitter. The

inhibition of its activity has increasingly been utilized as a biomarker for assessing effects on the nervous system. (Lionetto *et al*. 2013).

This study aims to comprehensively assess the impact of ciprofloxacin on the biological responses of zebrafish through a multiparametric approach, evaluating mortality, behaviour, embryonic development, biomarkers, and molecular analyses. The integration of these analyses will help define the Adverse Outcome Pathways (AOPs) of ciprofloxacin, linking molecular events to adverse phenotypic outcomes (Vinken et al., 2017). By examining behavioural changes as early indicators of neurotoxicity and conducting biochemical and molecular assessments, this study seeks to elucidate the ecological and health impacts of ciprofloxacin contamination.

Material and Methods

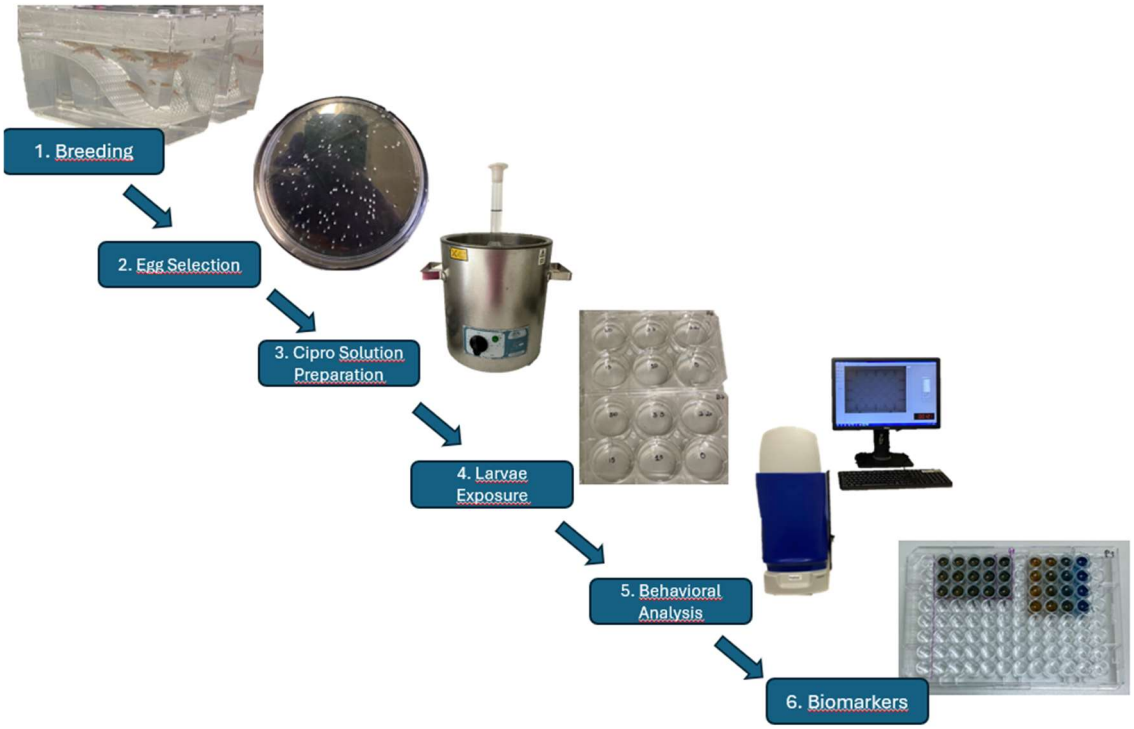

Figure 1 – Visual representation of the steps taken throughout the study.

1. Ciprofloxacin solution preparation

To prepare the ciprofloxacin (CAS 86393-32-0, Sigma Aldrich) stock solution, 50 milligrams of ciprofloxacin were added to a volumetric flask containing 1 L of

water. The flask was then placed in an ultrasound bath for an hour to ensure complete dilution. A series of successive dilutions were performed to obtain the following concentrations: 50, 33, 22, 15, and 10 mg/L. These concentrations were below lethal levels, meaning they were not anticipated to cause death but could influence vital physiological functions (that can be passed through generations) and ecological dynamics (Blahova *et al*. 2021).

2. Test Organisms

Adult zebrafish (wild type AB) were housed in a ZebTEC (Tecniplast) recirculating system at the University of Aveiro. The culture water was created by applying reverse osmosis to tap water and adding Instant Ocean Synthetic Sea Salt (Spectrum Brands). The system automatically regulated the pH to 7.5 ± 0.5 and maintained conductivity at 800 ± 50 μS. Water temperature was consistently held at 26 ± 1°C, and dissolved oxygen levels were kept at or above 95% saturation (7.6 mg/L). The zebrafish experienced a 12h:12h (light: dark) photoperiod cycle. They were fed daily with GEMMA Micro 500 (Skretting®, Spain), a commercial artificial diet.

To obtain the necessary eggs to perform the tests, 4 aquariums containing 24 adult fishes were put to breeding. Three hours post fertilization (hpf), the eggs were collected with a sieve, gently washed with culture water, and checked under a stereomicroscope (Nikon Stereoscopic Zoom Microscope-SMZ 1500). Eggs in the blastula stage (Kimmel *et al*. 1995) were selected for the assays.

3. Assay for apical and biochemical endpoints

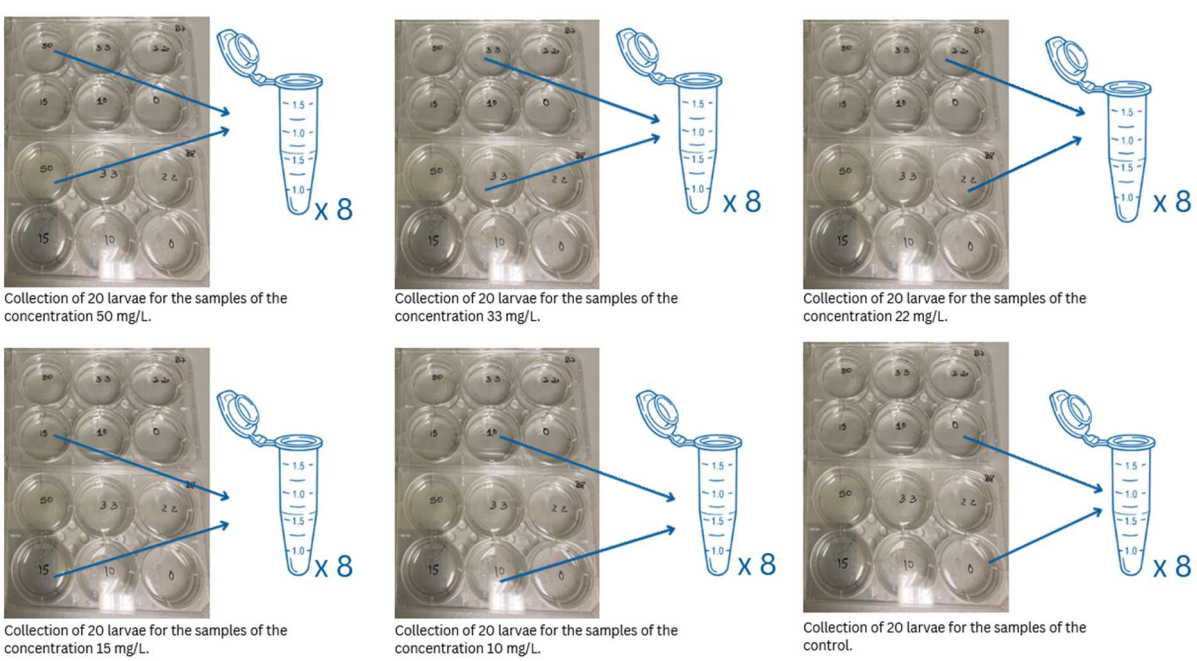

Figure 2 – Preparation of the samples for biochemical endpoints.

The test was conducted according to the guidelines of the Organization for Economic Co-operation and Development (OECD), specifically Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013 with some adaptations. Zebrafish eggs at blastula stage were distributed among 21 petri dishes (~100 eggs per Petri dish) containing 45 mL of water from the zebrafish system and left to grow until 96 hpf. At 96 hpf, the larvae were exposed to the test solutions in 6-well culture plates. The experiment was conducted using 6-well plates. Each well contained 11 larvae and 10 mL of the previously prepared solution with varying concentrations (50, 33, 22, 15, 10 and 0 mg/L). At 120hpf mortality was assessed by using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon).

To prepare samples for the biomarkers analysis, at the end of the test, pools of 20 larvae (collected from 2 wells of different plates, each containing 11 larvae) were transferred to Eppendorf tubes (8 Eppendorf tubes per concentration), frozen using liquid nitrogen, and stored at - 80°C until analysis. Spectrophotometric methods adapted to 96-well microplates were followed to perform the biomarkers analysis by using a Thermo Scientific Multiskan Spectrum microplate reader. The Catalase activity was expressed as micromoles of substrate hydrolyzed per minute per milligram of protein. As for the remaining enzymatic activities, they were expressed as

nanomoles of substrate hydrolyzed per minute per milligram of protein. Protein concentrations in the samples were determined using the Bradford method (Bradford, 1976) with γ-globulin as the standard, measuring absorbance at 595 nm.

Samples were homogenized on ice with a Branson 250 Sonifier in a 0.1M potassium phosphate buffer at pH 7. The samples were divided into three aliquots, aliquot A for LPO activity, aliquot B for GR activity, and aliquot C for GST, AChE, and CAT activities determinations. For aliquot B, samples were centrifuged at 15,000g for 15 minutes at 4ºC. GR activity was measured at 340 nm by adding 0.2 mL of 0.3 mM NADPH and 0.01 mL of 22 mM GSSG to 0.03 mL of sample, following Massarsky *et al*. 2017. For aliquot C, samples were then centrifuged at 10,000g for 5 minutes at 4ºC. GST activity was assessed using 0.05 mL of homogenate and 0.250 mL of a reaction mixture containing 10 mM reduced glutathione (GSH) and 60 mM 1-chloro-2.4-dinitrobenzene, with the increase in absorbance measured at 340 nm for 5 minutes, as described by Habig *et al*. 1974. AChE activity was measured at 414 nm using 0.05 mL of homogenate and 0.250 mL of a reaction solution containing 10 mM 5,5-dithiobis-2-nitrobenzoic acid with sodium hydrogen carbonate and 0.075 M acetylcholine, following the method of Ellman *et al*. 1961. CAT activity was determined by monitoring the decrease of H_2O_2 at 240 nm over 5 minutes, using 0.02 mL of homogenate, according to Clairborne 1985.

5. Behaviour Assay

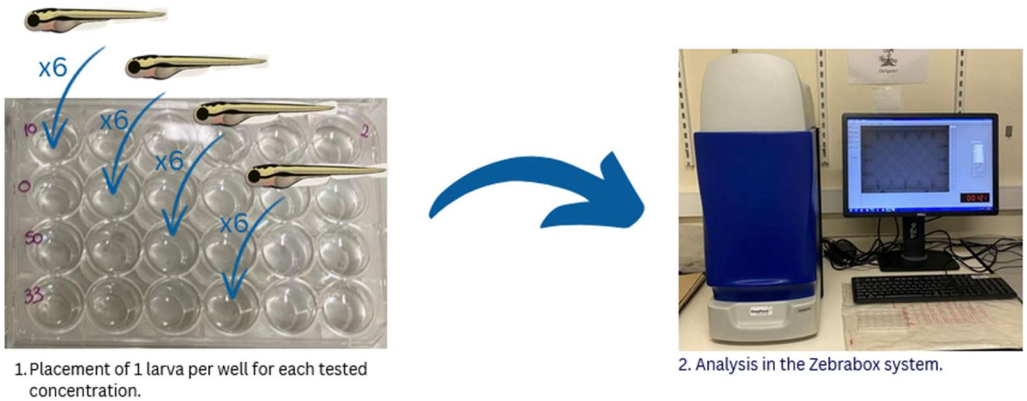

Figure 3 – Visual representation of the steps taken to perform the behavioural analysis.

To conduct a behavioural analysis, a similar test as the previously described was conducted but, using 24-well culture plates, and one larva was placed per well with 2 mL of solution. Each row of wells was assigned a different concentration, with six wells per concentration. The concentrations selected were the same as those used in the biochemical analysis. The movement of the larvae was evaluated using the Zebrabox system (ZebraLab® v3, Automated Behavioral Analysis). The larvae were allowed to acclimatize to test conditions for 6 minutes, followed by 2 minutes of movement analysis in darkness. This light design was selected because Zebrafish larvae at this age do not possess significant baseline movement in the light, however, the sudden change from light to darkness elicits a burst of activity (hyperactivity) in the organisms (startle response) (Burgess and Granato 2007). Alterations in this response is used to assess stress, anxiety-like behaviour, or neuronal disruption when embryos are exposed to chemical stress (Almeida *et al*. 2019). The following endpoints were measured: distance (percentage) covered during rapid and slow movements, angles (percentage) of fish path (Classes 1, 2, 3, and 4), and total distance travelled (mm).

6. Statistical Analysis

SigmaPlot V.14.0 for Windows was employed for the statistical analyses. To check for normality and homogeneity of variances, the Shapiro-Wilk test and Brown-Forsythe test were utilized, respectively. Data from ??? was analyzed using One-Way ANOVA with the Holm-Sidak method, and Dunnett's Method was applied for comparisons. For data that did not meet normality assumptions, the Kruskal-Wallis One Way Analysis was used. A significance level of 0.05 was maintained for all statistical tests.

Results

1. Apical Endpoints

Regarding apical endpoints, no mortality was registered and no alterations in the swimming bladder were observed for the tested concentrations (data not shown).

2. Biochemical Analysis

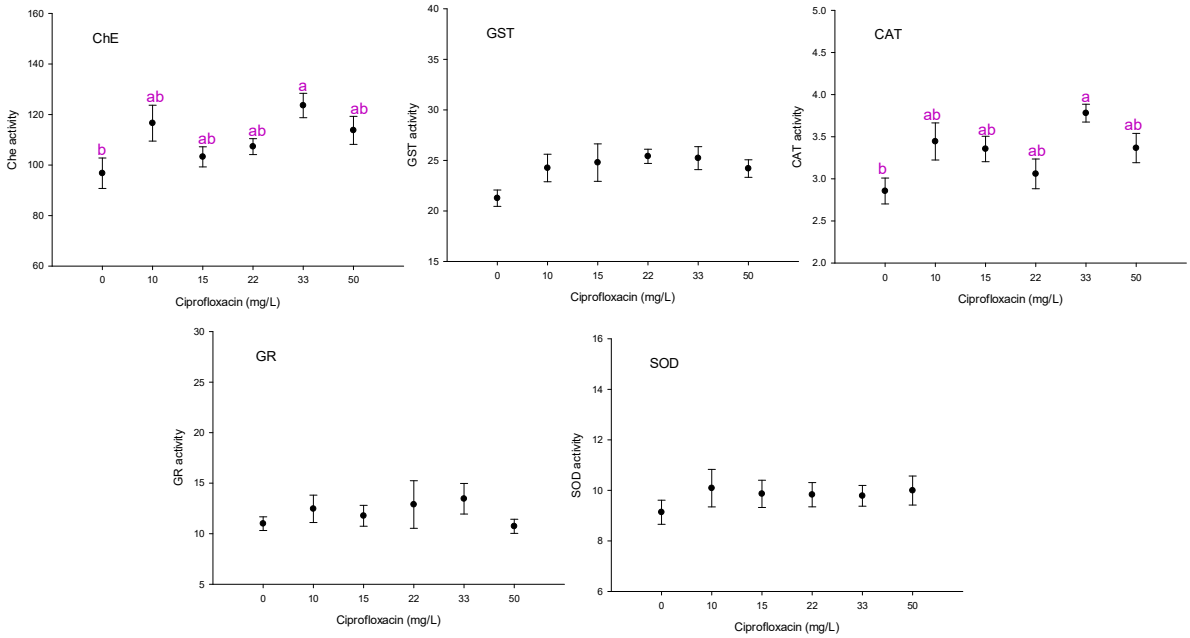

Figure 4 - Biomarkers analyzed: Acetylcholinesterase (ChE), Glutathione S-transferase (GST), Catalase (CAT), Glutathione Reductase (GR), and Superoxide dismutase (SOD). Groups with the same letter are not significantly different from each other. Groups with different letters are significantly different from each other.

Among the five biomarkers analyzed, only two—ChE and CAT—showed a significant increase in activity, both at the concentration of 33 mg/L. The other biomarkers, SOD, GST, and GR present a trend for increasing activities with concentrations, but the increment was not significant (Fig 4).

3. Behavior assay

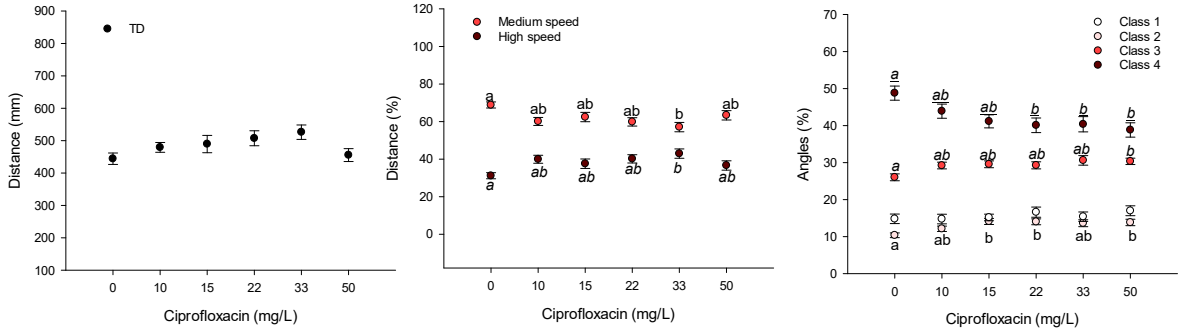

Figure 5 -Behaviour parameters evaluated: Total Distance (mm), Distance at medium and high speed (%), and Angles (%). Groups with the same letter are not significantly different from each other. Groups with different letters are significantly different from each other.

Regarding behavioural endpoints (Fig 5), our results indicate that distance travelled by larvae did not change with the exposure to ciprofloxacin. When analyzing the distance travelled in the two categories (high speed movements and medium speed movements), we observed that distance travelled in medium speed movements tend to decrease (statistically significant at 33 mg/L) while distance travelled in high speed movements tends to increase with ciprofloxacin concentrations (statistically significant at 33 mg/L treatment)

Regarding path angles, as the concentrations increase, the frequency of the angles from classes 2 and 3 tends to increase while the frequency of angles from class 4 tends to decrease. Class 2 angles were significantly increased at 15, 22, and 50 mg/L, class 3 angles were significantly increased at 50 mg/L while class 4 angles decreased significantly at 22, 33, and 50 mg/L of ciprofloxacin.

Discussion

The chosen concentrations were below lethal levels, so as expected, no mortality was registered, however, alterations in the swimming bladder were neither observed. This indicates that these concentrations did not reach levels toxic enough to disrupt swimming bladder inflation. However, it was anticipated that all biomarkers would increase due to activation of the antioxidant system. While there was a noticeable trend of increased activity for GST, GR, and SOD, the changes were not statistically significant. This suggests that ciprofloxacin do not have the potential to activate the antioxidant system. However, the lack of significant results could be

due to the downregulation of genes related to detoxification pathways (Cunha *et al*., 2016) and data variability.

Bartoskova *et al*. 2014 described a significant increase in CAT activity at a norfloxacin concentration of 30 mg/L. Similarly, in this research, a significant increase in CAT activity was observed at a ciprofloxacin concentration of 33 mg/L. Elizalde-Velazquez *et al*. 2022 also supported these findings by observing an increase in CAT activity in a concentration-dependent manner with ciprofloxacin concentrations of 0.250, 0.500, and 1 µg/L. These results indicate that ciprofloxacin triggers an oxidative stress response.

As for the AChE activity, Jijie *et al.* 2023 found no significant effects on its activity after exposure to ciprofloxacin, however, in the present study, a significant increase in its activity was noted at the concentration of 33 mg/L. Similarly, Nogueira *et al.* 2019 reported an increase in AChE activity at the highest ciprofloxacin concentration (0.488 µg/L). Evidence suggests that reactive oxygen species (ROS) contribute to the elevation of AChE activity. For instance, Melo *et al.* 2003 indicate that the rise in AChE activity is preceded by a generation of ROS. The increase in ROS production, along with subsequent membrane lipid peroxidation, likely leads to higher AChE levels. Elevated ChE activity in the presence of high concentrations of reactive oxygen and nitrogen species (ROS/RNS) has been linked to neurodegeneration (Nunes 2011).

Exposure to 33 mg/L of ciprofloxacin appears to increase stress behaviour compared to control conditions. This is evident from the observed increase in in high-velocity movements, which is indicative of hyperactivity. This is consistent with findings reported by Petersen *et al.* 2021 which showed that larvae exposed to 25 mg/L of ciprofloxacin (the highest of 3 concentrations) travelled longer distances and Nogueira *et al.* 2019 who also described a period of hyperactivity on two of the treatments in the first light period. Changes in locomotor behaviour are frequently linked to neurological impairment caused by AChE inhibition, however, in this case, there was an increase in AChE activity, similar to Nogueira *et al*. 2019. Concerning the assessment of angles along the fish's trajectory, the low-amplitude angles (class 4) showed a dose-dependent decrease accompanied by an increase in larger amplitude angles (class 2 and 3) which shows a transition to a swimming pattern

with more changes of direction, which may be an indicator of an erratic swimming pattern (zig zag swimming), a measure of anxiety in fish.

Conclusion

In this study, the effects of various concentrations of ciprofloxacin on zebrafish larvae were evaluated, at both the biochemical and behavioural levels. To respond to the question posed initially, exposure does alter the biological responses of *Danio rerio* embryos, as supported by the results. There was a decrease in class 4 angles, which are associated with normal swimming behaviour followed by an increase in class 2 and 3 angles, indicative of a more erratic swimming pattern. Additionally, the distance travelled at high speed increased, suggesting hyperactivity. These findings highlight the potential of ciprofloxacin to disrupt typical behavioural patterns in fish larvae indicating that this antibiotic has some degree of neurotoxicity. Regarding the biomarkers, the results are somewhat unclear. While there was a significant increase in CAT and AChE activity, indicating ciprofloxacin's ability to induce oxidative stress, no significant differences were observed for GST, GR and SOD activity. These behavioural and biochemical alterations could have significant implications for the survival and fitness of fish in natural environments, potentially affecting predator-prey interactions and overall ecological balance. Future research should focus on exploring long-term effects of ciprofloxacin exposure.

Overall, the study underscores the importance of considering the potential environmental risks of pharmaceutical contaminants and the need for more stringent regulations and monitoring of such substances in aquatic habitats.

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