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Ivermectin: a multilevel approach to evaluate effects in *Prochilodus lineatus* (Valenciennes, 1836) (Characiformes, Prochilodontidae), an inland fishery species

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Abstract

Ivermectin (IVM) is one of the most widely used antiparasitics worldwide. It is a potent and effective drug for treatment and prevention of internal and external parasitic infections of livestock and humans. IVM is excreted unchanged in manure of treated animals. Thus, residues of IVM may reach aquatic systems, affecting non-target organisms such as fish. Although the presence of IVM in aquatic environments has been reported, a multilevel approach (from cellular to behavioral responses) is necessary to determine the health of exposed organisms and the environmental risks associated. The aim of the present study was to investigate the response of the Neotropical fish *Prochilodus lineatus*, one of the main target species of South American freshwater fisheries, exposed to environmental concentrations of IVM: low (0.5 μg L^{-1}) and high (1.5 μg L^{-1}). Behavioral responses were assessed in juvenile fish and included water column use, routine swimming, total distance travelled, total activity time and Maximum swimming speed achieved during the escape response. Biochemical/oxidative stress responses assessed included brain acetylcholinesterase (AChE), catalase (CAT) and glutathione S-transferase (GST) activities; total antioxidant competence against peroxyl radicals (ACAP) and lipid oxidative damage (TBARs). Hematological biomarker responses included blood glucose levels, hematocrit, hemoglobin concentration, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and mean corpuscular volume. Condition factor and hepatosomatic index were also calculated. The lowest IVM concentration caused a significant decrease in GST activity and maximum swimming speed during the escape response. Multivariate analysis with biochemical/stress and behavioral data revealed overall effects of IVM treatments. This multilevel analysis shows detrimental effects related to swimming behavior and predator avoidance which could affect population size and size-structure of *P. lineatus*. To our knowledge this is the first attempt to assess the effects of IVM on Neotropical fishes using an integrative approach based on biomarkers from different levels of biological organization.

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Keywords: antiparasitic, avermectins, biomarkers, fish behavior, neotropical fishes, oxidative stress

1. Introduction

Avermectins are a class of macrocyclic lactone drugs with anthelmintic and insecticidal properties that have been developed for the protection of animals, humans, and crops (Mougin et al. 2003). The most well-studied avermectin is ivermectin (IVM), a synthetic derivative of the naturally occurring avermectin B1 (Liebig et al., 2010). Ivermectin binds selectively and with high affinity to the ligand glutamate on the ligand-gated chloride ion channels that occur in invertebrate nerve and muscle cells, causing hyperpolarization of the cells and irreversible opening of these channels (Wolstenholme and Rogers, 2005; Ōmura, 2008). Recently, IVM was pointed out to be a substance of high concern and of high priority for further environmental monitoring and risk assessment (Nunes et al., 2021; Mesa et al., 2020; Vokřál et al., 2019; Boxall, 2018). In Argentina, the intensification of agriculture, soybean cropping in particular, has forced the relocation of livestock to more marginal grazing lands such as floodplain wetlands (PROSAP, 2009; Mesa et al., 2020). To become efficient with grazeable resources, local ranchers have implemented several new practices which include the systematic and frequent injection of cattle with IVM. This practice, in the absence of a strict veterinarian prescription, followed by contact of cattle with wetlands immediately after injection, has raised concerns about the presence and effects of the drug in these floodplain environments (Mesa et al., 2020). Concern regarding IVM has arisen in the 80s because of its high toxicity for nonparasitic invertebrates (Campbell et al., 1983; Edwards et al., 2001), and the mode of action also indicates that its toxic potential is not restricted to parasites (Brinke et al., 2010).

Non-target aquatic organisms are exposed indirectly, through runoff incidents or transport of eroded soil from pasture and arable land, or the possibility that treated animals directly excrete it into water, as large quantities (up to 80-90%) of this pharmaceutical are excreted by treated animals (Alvinerie et al., 1999; Boxall et al., 2004). Several authors have studied the effects of avermectins on fish species at different

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levels of biological organization. Fish species have been widely used in studies for assessing the biological and biochemical impact of environmental contaminants (van der Oost et al., 2013).

Prochilodus lineatus (Valenciennes, 1836) is an illiophagus (mud feeder) and migratory characiform fish. This species constitutes the main inland fishery species in the Lower Río de la Plata Basin (Baigún et al., 2016; Espinach Ros & Fuentes, 2000), and represents more than 60% of the total registered biomass (Baigún et al., 2013). The life cycle of this species involves extensive upstream spawning migrations (Lozano et al., 2019). Once the reproductive areas are reached, these fish spawn as the water levels increase in spring and summer (Lozano et al., 2019; Stassen et al., 2010). After 2 weeks, larvae enter marginal areas of the floodplain, where they find food and refuge (Lozano et al., 2019; Fuentes, 1998). It is in this floodplain environment where the juvenile fish meet the intensive livestock activity. This limited environment would maximize adverse effects of pollutants, such as IVM, and compromise the ability to escape natural predators and feed (Weis and Candelmo, 2012; Weis et al., 2001). This species was shown to be sensitive to pollutants (Santillán Deiú et al., 2021; Alé et al., 2018; Almeida et al., 2005; Martinez and Souza, 2002; Martinez et al., 2004) and is considered a potential bioindicator for environmental monitoring (Cerqueira and Fernandes, 2002). However, few studies have provided comparison of multiple endpoints under a common set of experimental conditions. This multilevel approach, wherein different biological responses ranging from molecular to behavioral are evaluated, is essential to determine the general health status of the organism. Moreover, it permits extrapolation of the relationship between responses at different levels of biological organization (Lee and Choi, 2006).

In fact, animal behavior appears to be among the most sensitive indicators of environmental alterations. Behavior arises from the cumulative interaction of a variety of biotic and abiotic factors and represents the animal's response to internal (physiological) and external (e.g. environmental) stressors, and is a visible reaction of an organism to a stimulus on the whole-organism organization level (Gerhardt, 2007). In fishes, behavioral endpoints can be as sensitive as biochemical and physiological biomarkers (Weis, 2014; Peterson et al., 2017). Juvenile and adult *Danio rerio* (Hamilton-Buchanan, 1822) exposed to a

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range of IVM concentrations in water (10 and 200 $\mu g.L^{-1}$) showed behavioral alterations including slow movement and erratic swimming with periods of paralysis (Oliveira et al., 2016). Domingues et al. (2016) reported changes in feeding activity and water column use for this species after a sub-chronic exposure to lower IVM concentrations (0.25 and 25 $\mu g.L^{-1}$). Thiripurasundari et al. (2014) observed changes in swimming behavior (swimming and loss of equilibrium) in *D. rerio* when the concentration of IVM was maintained at 5 $\mu g.L^{-1}$ or higher; moreover even at lower concentrations (1 $\mu g.L^{-1}$), similar behavioral alterations on *Catla catla* were reported (Hamilton-Buchanan, 1822).

On the other hand, it has been clearly established that stress is usually accompanied by oxidative stress (Lushchak, 2011). Many xenobiotics can produce reactive oxygen species (ROS) via several mechanisms, such as interference in electron transport in the mitochondrial membrane and subsequent accumulation of reactive intermediates, inactivation of antioxidant enzymes, depletion of non-enzymatic antioxidants and membrane lipid peroxidation (Winston and Di Giulio, 1991). Ogueji et al. (2020) showed that a short-term exposure (4 days) to IVM (9 to 25 $\mu g.L^{-1}$) induced oxidative stress (lipid peroxidation) and altered the activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase) in *Clarias gariepinus* (Burchell, 1822). *D. rerio* exposed to up to 25 $\mu g.L^{-1}$ of IVM showed reduction of catalase and glutathione S- transferase activity (Domingues et al., 2016).

In addition, stress response involves various physiological changes including alteration in blood composition and immune mechanisms (Rey Vázquez et al., 2020). Hematological parameters are closely related to the response of the animal to the environment (Gabriel et al., 2004). These indices have been employed in effectively monitoring the response of fishes to stressors and thus their health status under adverse conditions (Rey Vázquez and Lo Nostro, 2014). Ogueji et al. (2020) reported an increase in glucose serum concentrations in *C. gariepinus* treated with 9 to 21-$\mu g.L^{-1}$ IVM concentrations after a 24 h exposure. These authors found that glucose response varied with exposure duration.

Despite the cited literature, just a few studies have simultaneously measured an array of different biomarkers and linked their responses to different biological organization levels. In addition, considering

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the high potential risk of IVM in floodplain and wetlands environments (Mesa et al., 2020; Liebig et al., 2010), there is scarce information on the effects of this drug in neotropical fishes. Therefore, the aim of the present study was to investigate the response on the Neotropical fish *Prochilodus lineatus* -one of the main target species of South American freshwater fisheries- exposed to environmental and sublethal concentrations of IVM, and to analyze biomarkers at different levels of biological organization. We hypothesize that low environmental concentrations of IVM are capable of causing adverse effects on *P. lineatus* biomarkers at different levels of biological organization.

2. Materials and methods

2.1. Test model species: *P. lineatus*

The test animal used in the present study was the freshwater fish *P. lineatus* (Characiformes, Prochilodontidae), at the juvenile stage. Fish were obtained from the stock culture at the laboratory of the Instituto de Ictiología del Nordeste (INICN), Facultad de Veterinaria, Universidad Nacional del Nordeste (UNNE, Argentina).

Prior to the start of the exposure, fish were individually placed randomly in 6 L glass aquaria and allowed to acclimate to bioassay conditions (24 ± 2°C, 12:12 photoperiod, dechlorinated water and continuously aerated) for 20 days. Each aquarium was previously covered with white walls using an adhesive sheet -except on the top surface and the front- to eliminate social interactions between animals. Fish were fed with Otohime B1 (Crude Protein 51%, Crude Fat 11%, Crude Fiber 3%, Marubeni Nisshin Feed) once a day. Assays were run under the same temperature and photoperiod conditions as those described in section 2.2.

All experiments were conducted in accordance with international standards on animal welfare (National Research Council 2011) as well as local Ethical Committee guidelines (Protocol number 103/18, CICUAL, FCEN, UBA).

2.2. Bioassay design and exposure to IVM

IVM stock solution (500 mg.L-1) was prepared by dissolving 0.1250 g of IVM (99.8 %, Saporiti, Argentina) and making up to 250.00 mL with methanol (p.a., 99.8%, Biopack). Dilutions were prepared from the stock solution by adding 24 or 36 µL to aquaria final volume of 6 L. For the lowest IVM concentration an extra volume of 12 µL of methanol was added to compensate for the concentration of the vehicle. The stock solution was stored at 3-5 °C in the dark.

The juveniles of *P. lineatus* (N=24; 5.39 ± 0.90 cm standard length (SL), 4.60 ± 2.34 g total weight) were individually exposed to nominal concentrations of IVM of 0 (control), 0.5 $\mu g.L^{-1}$ and 1.5 $\mu g.L^{-1}$ during 15 days in 6 L aquaria. A vehicle control group was also run in parallel (methanol 0.001 %v/v). Each treatment was tested 6 times (replicas). Aquarium water was completely renewed every 48 h. Fish were fed once a day. The experimental concentrations were chosen based on published data (see Mesa et al., 2020; Kövecses & Marcogliese, 2005). The LC50 48 h value estimated for the post-larval stage was 1.75 µg IVM L^{-1} (confidence interval: 1.30-2.38) (data not shown).

At the end of the exposure (day 15) fish were video recorded for behavioral analysis. They were then anesthetized with iced water (1-2 °C), measured and weighed and blood was collected by puncture of the caudal vein (1 mL syringe and previously heparinized 27G x 0.5" needle) before dissection. Gills and brain were immediately removed, frozen and stored at -80 °C until biochemical determinations.

2.3. Ivermectin decay

Prior to the exposure bioassays, the decay of IVM in the aquaria was measured. Concentrations of IVM of 0 (control), 0.05 mg.L-1 and 0.5 mg.L-1 were tested in duplicate aquaria without the presence of fish, in the experimental conditions described above. Replicate water samples (n=2) were taken upon addition of IVM (time 0) and after 24, 48 and 72 h. Samples were concentrated by solid phase extraction (SPE) on

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Hypersep C18 cartridges (Thermo Scientific, USA) followed by elution with methanol before injection in the HPLC. Concentration of IVM was measured by reverse-phase HPLC coupled to UV detector (Accela, Thermo Scientific, USA) according to Shurbaji et al. (2010). The column employed was a Kinetex C18 100A - 100 mm x 4.6 mm, 2.6 µm particle size (Phenomenex, USA). The mobile phase was composed of 56% acetonitrile / 36% methanol / 7.5% water, with 0.5% acetic acid. Elution was performed at a flow rate of 1 mL/min. Detector was set at 245 nm. For quantification, calibration curves were constructed for peak areas, from injection of standard solutions daily prepared by adding known amounts of IVM to control water and processed in the same manner as the samples. For each set of replicate samples, mean and standard deviations were calculated after interpolation of IVM chromatographic peak area in the calibration curve (R^2 =0.99). The recovery rate of the method was 85%; the limit of quantification and limit of detection were 0.01 $mg.L^{-1}$ and 0.005 $mg.L^{-1}$, respectively.

2.4. Swimming behavior endpoints

At the end of the exposure, swimming behavior was analyzed by video recording each aquarium over 10 min. The first 5 minutes were excluded from the analysis because it was considered a period of adaptation to video camera setup.

Each aquarium was externally marked in the front glass at 3 different heights (bottom, middle and upper). The time spent by each fish in each of the layers of the aquarium was used to calculate a swimming frequency per altitude (water column use). Also routine swimming (RS) ($SL.s^{-1}$), total distance travelled by each fish (TDT) (m) and total activity time (TAT) (% of total time) were obtained from video analysis using EthoVision XT software (Noldus). The routine swimming speed was measured as the mean rate of travel for individual fish during undisturbed activity in the absence of food (Fuiman et al., 1999).

To study visual startle response, a 5-second-high speed video (400 fps) following a visual stimulus was added. The dummy stimulus was a white plastic card (10 X 25 cm) with a black circle (9 cm of diameter) swinging close to the tank. The black circle was meant to mimic the frontal silhouette of an attacking

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piscine predator. Maximum swimming speed (MSS in $SL.s^{-1}$) developed during the escape response was registered. The high-speed video analysis was performed with Tracker 5.1.5 software (Brown, 2013).

2.5. Morphometric and hematological biomarkers

The condition factor (CF) of the experimental fish was estimated from the relationship: $(W/L^a) \times 100$, where W: weight of fish body in grams; L: length of fish in centimeters and a: the value obtained from the length-weight equation according to Llamazares et al. (2014). Hepatosomatic index (HSI) was calculated as liver weight x body $weight^{-1}$ x 100 according to Goede and Barton (1990).

Following Rey Vázquez and Guerrero (2007), firstly, a drop of blood was obtained immediately for glucose measurement with a disposable test strip (Accu-Chek® Performa, Roche). Secondly, within the first 2 h after blood extraction, red blood cells (RBC) count was performed with a Neubauer chamber. Hematocrit (Ht%) values were determined by the micromethod using capillary tubes and centrifuged at 1409 g for 5 min. Hemoglobin (Hb) in erythrocytes was determined using the cyanmethaemoglobin method (hemogloWiener®; Wiener Lab). Before measuring the absorbance, Hb test samples were centrifuged to remove dispersed nuclear material. The following indices: mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV) were calculated according to Ranzani-Paiva et al. (2013).

2.6. Biochemical parameters: brain acetylcholinesterase and oxidative stress

2.6.1. Preparation of biological samples

Enzyme extracts from the gills and brain were prepared according to Scarcia et al. (2014). Briefly, −80 °C frozen organs were weighed and homogenized in ice with homogenization buffer pH 7.4 (100 mM NaH2PO4; 150 mM KCl; 1 mM EDTA; 1 mM DTT; 10% v/v glycerol) (Nilsen et al. 1998). Aliquots of the initial homogenate were immediately processed for lipid peroxidation analyses and the remaining

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homogenate was centrifuged (20 min, 10,000 g, 4 °C), and the supernatant fraction was used for biochemical parameters evaluation.

2.6.2. Acetylcholinesterase activity

The acetylcholinesterase activity (AChE) was measured only in brain supernatant fraction, based on the colorimetric method of Ellman et al. (1961) with acetylthiocholine iodide (2.5 mM) as substrate and dithiobisnitrobenzoic acid (DTNB, 0.3 mM) in the assay media. Absorbance was recorded at 412 nm (UV-VIS 1800 Shimadzu) at 10-s intervals for 2 min. AChE activity was calculated as $nmol.min^{-1}$ $mg.protein^{-1}$.

2.6.3. Enzymatic antioxidant response

Oxidative stress condition was assessed by means of enzimatic antioxidant responses and lipid damage. Glutathione S-transferase activity (GST) was measured according to Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, which can be conjugated with the different isoforms of GST in the presence of reduced glutathione (GSH); the presence of the formed conjugate was evaluated by spectrophotometry at 340 nm. The assay conditions were sodium phosphate buffer pH 6.5 (0.1 M), 10 mM GSH, and CDNB 20 mM. Enzyme activity was expressed as GS-CDNB µmoles formed per minute and mg of total protein.

CAT activity was determined according to Beutler (1982), evaluating the breakdown of H_2O_2 over time, at a wavelength of 240 nm. The mixture reaction contained the aliquots of brain or gills homogenates incubated in sodium phosphate buffer pH 7.2 (0.05 M) containing H_2O_2 20 mM. The enzyme activity was expressed as µmoles of H_2O_2 consumed/min/mg of total protein.

The total antioxidant capacity against peroxyl radical (ACAP) was evaluated through ROS determination in brain or gills samples treated or untreated with a peroxyl radical generator, according to Amado et al. (2009) with modifications by Monserrat et al. (2014). Aliquots of the supernatant fraction were incubated in a 96-well microplate with or without the addition of ABAP 4 mM at 37 °C to generate peroxyl radicals. Immediately, the fluorogenic compound H2DCF-DA 40 µM was added to all wells and the ROS formation

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was determined by fluorometry (ex/em 485/525 nm) (BioTek Synergy HT). The results were expressed as the difference in fluorescence units (FU) in 30 min in samples with and without ABAP and standardized to FU in 30 min without ABAP (background area). For interpretation purposes, a large bar in the graph (high values) means a lower competence to neutralize peroxyl radicals thus a lower antioxidant capacity.

2.6.4. Oxidative damage: lipid peroxidation

Lipid peroxidation was determined by the TBARS (thiobarbituric acid reactive substances) assay, described by Oakes and van der Kraak (2003). The initial homogenate was mixed with BHT 1407 mM, 8.1% SDS, 20% acetic acid (pH 3.5), and 0.8% TBA solution, then incubated at 95 °C for 1 h. Fluorometric measurements (excitation, 516 nm; emission, 560 nm) were performed in a microplate reader (BioTek Synergy HT) and malondialdehyde content was expressed as nmol TBARS g of wet tissue^{-1}. TMP was used as an external standard.

2.6.5. Protein estimation

The total protein quantity of the supernatant fraction was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.7. Data analysis

Data are reported as mean ± standard error, and the statistical significance was set at $p < 0.05$. Differences between the control and vehicle groups were assessed by Student t-test. One-way analysis of variance (ANOVA) with appropriate post hoc test (Tukey test) was conducted to assess differences between vehicle control and treatments. The type of ANOVA (parametric or non-parametric) and post hoc test was chosen depending on whether normality and homoscedasticity of data were demonstrated by analysis of the residuals with the Shapiro-Wilks test. Statistics test and analysis of normality were conducted using R (R Core Team, 2020).

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The comparisons between water columns use between treatments were assessed with multivariate analysis of variance (MANOVA) where the independent variables are the groups (treatments) and the dependent variables are the predictors (time spent in each layer of the aquaria). Multivariate normality was evaluated with Mardia's multivariate skewness and kurtosis, with tests based on chi-squared (skewness) and normal (kurtosis) distributions. Equivalence of the covariance matrices was assessed with Box´s M test.

A linear discriminant analysis (LDA) was performed to obtain a holistic interpretation of IVM toxicity. In LDA, the independent variables (biological parameters) are the predictors, and the dependent variables are the groups (treatments). In this study LDA was applied to explain the contribution of original continuous variables (biomarker responses) by group of biomarkers (hematological, biochemical, and behavioral) respect to different treatments. To explain the relative importance of the individual explanatory variables across the functions, the potency index for each variable was calculated (Hair *et al*., 2010). Additionally, a cross-classification analysis based on minimal Mahalanobis distance to the group mean was performed to assess the correct assignment of individuals to each treatment. The Mahalanobis distance is calculated from the pooled within-group covariance matrix, giving a linear discriminant classifier. To establish the bias of the cross-classification analysis a Cohen's kappa test was performed, which estimates the improvement over chance of the percent corrected classification rates (Titus et al., 1984). All multivariate analysis were performed by the Past 3.26 software (Hammer et al., 2001).

3. Results

During the experiments, there was no mortality in any of IVM or control groups. No significant differences were observed between control and vehicle control for swimming behavior endpoints, morphometric, hematological, or biochemical biomarkers. Thus, all statistical comparisons were made against vehicle control data.

3.1. IVM decrease and renewal rate

The measured concentrations of IVM in replicate water samples taken at 0, 24, 48 and 72 h from the aquaria containing 0.05 and 0.5 mg.L^{-1} IVM are indicated in Table 1. The measured concentration at time 0 were 88% and 72% of the nominal concentrations (0.05 and 0.5 mg.L^{-1} respectively). After 24 h the measured concentrations increased 13.9% and 2.7% above the nominal concentrations. At 48 h the measured concentration was around 50% of the original concentration, establishing 48 h as a reasonable time for total medium renewal during the experiment. IVM was not detected in samples from the control aquarium.

3.2. Swimming behavior

Treatment associated effects were observed in swimming behavior (Table 2). Fish treated with the lowest concentration of IVM had a significant decrease ($p=0.046$) in MSS during the escape response compared to control group. Fish treated with the higher concentration of IVM had a slight decay in the TAT, RS and TDT; however, all these differences were not significant ($p\geq0.05$). Water column use from IVM treated fish was not significantly different from the control group (Figure 1).

3.3. Morphometric and hematological biomarkers

Condition factor (CF) showed suboptimal values (≤1) during the entire experiment. However, no significant differences were detected between IVM treatments and vehicle control. Hepatosomatic index (HSI) showed a non-significant tendency to increase with IVM concentrations. Hematological parameters and associated indexes did not show significant differences between IVM concentrations and control groups (Table 3).

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3.4. Biochemical parameters: brain acetylcholinesterase and oxidative stress

A trend of increase ($p>0.05$) in AChE activity was associated to the increase of IVM concentrations (Figure 2). GST levels in gills showed a significant decrease in activity at the lowest concentration of IVM compared to control group ($p=0.022$). Antioxidant and detoxification enzyme activities of CAT and GST, ACAP and TBARS levels in brain and gills did not show significant differences compared to the control group (Figure 3).

3.5. Discriminant analysis (multivariate statistics)

An LDA was conducted separately for each set of biomarkers (hematological, behavioral, and biochemical/stress). Using the discriminant functions, the ordination of behavioral and biochemical data showed differences between control and treatments, despite the occurrence of overlaps between IVM concentrations (Figure 4). The hematological data showed high overlapping between controls and treatments.

High percentage values of cross-classification were observed for the biochemical/stress parameters in brain (91.67%) followed by gills (83.33%) with a high Kappa value (Kappa>0.75) (Table 4). Behavioral data showed an overall percentage of correct cross-classification of 77.78% against the 33.33% of agreement obtained by chance (Kappa=0.66, intermediate value). The hematological set of parameters showed the lowest percentage of correct cross-classification (66.67%) with a low Kappa value (Kappa=0.5) indicating a weak level of non-random agreement.

The potency index showed that ACAP and CAT, in brain and gills, played a dominant role in discriminant analysis with biochemical/stress biomarkers. From behavioral data, the RS played the main role in discriminant analysis showing the highest potency index. In the case of hematological biomarkers, MCH and Hb were the ones that played a greater role in discriminant analysis between treatments (Table 4).

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4. Discussion

Nowadays, a battery of biomarkers is recommended to evaluate potential effects of xenobiotics on cellular functions and processes, behavior, or cell damage (Götte et al., 2020). In the present study, several potential negative effects associated with IVM exposure were explored in *P. lineatus* at different levels of biological organization. Behavioral and biochemical biomarkers showed significant effects even at the lowest concentration.

During the IVM decay assay, the initial decrease of IVM concentration at time 0 could be attributed to the adsorption to the glass surface of the aquaria. According to Krogh et al. (2008), desorption of IVM can occurr after a certain time. In this experiment, this effect was observed after 24 h approaching the nominal concentrations. After 48 h, the decay of IVM was expected as it has been reported by several authors (Rath et al., 2016; Prasse, et al., 2009; Krogh et al., 2008). The choice of 48 h as the total renewal time was coherent with these findings.

Overall effects of IVM on fish swimming performance were detected by discriminant analysis. Specifically, routine, and maximum swimming speed seemed to be the most altered endpoints even at the lowest concentration of IVM (0.5 µg.L^{-1}). As Weis and Candelmo (2012) mentioned, these endpoints could be directly related with the capacity to escape/avoid predators and, therefore, the ecological impact of changes to this behavior would be even greater. Reduced predator avoidance ability can increase mortality rates and affect the population size and size-structure. Prey exposed to contaminants as IVM may fail to detect predators, take more risks in leaving refuge to find food, and have a poor fast start performance, reduced stamina, inability to school, altered activity patterns and increased conspicuousness (hyperactivity), all leading to increased predator vulnerability (Scott and Sloman, 2004).

Effects of IVM on fish swimming performance were also reported in previous studies. Oliveira et al. (2016) and Domingues et al. (2016) reported behavioral alterations on *D. rerio* including slow movement and erratic swimming with periods of paralysis at concentrations between 0.25 to 200 µg.L^{-1}. Similar effects were reported by Bard and Gadbois (2007) and Mladineo et al. (2006) in short term assessments

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of the behavioral effects of IVM in *Fundulus heteroclitus* (Linnaeus, 1766) and *Sparus aurata* (Linnaeus, 1758), respectively. In both studies, exposed fish showed alterations in behavior including lethargy, postural changes, and loss of activity. Also, administration of IVM via food to juveniles of *Salmo salar* (Linnaeus, 1758) caused similar effects: reduced mobility and longer time to complete feeding (Ucan-Marin et al. 2012). These behavioral changes are similar to those described for anticholinergic agents, which would suggest that AChE may also be affected. However, in our experimental conditions, brain AChE results did not allow to establish such an association. Alternatively, in vertebrates IVM targets GABA receptor chloride channels, leading to hyperpolarization of the membrane potential and reduction of nerve transmission (Stevens and Breckenridge 2001). Since GABA receptor channels are mostly found in the central nervous system and given that the blood-brain barrier in fish did not seem to be effective enough to prevent IVM from accumulating in the brain, as suggested by Horsberg (2012), Katharios et al. (2004) and Høy et al. (1990), a possible explanation for these effects is that IVM can reach the brain, affecting the central nervous system and, consequently, causing behavioral changes.

Hematological parameters can be used as health status indexes to detect the potential response to stress in fish (Oliveira et al., 2019). Few studies have considered hematological parameters for analyzing IVM toxicological effects. Katharios et al. (2002) reported a decrease in hematocrit and an increase in glucose and hemoglobin levels with high doses of intraperitoneally injected IVM (100-800 $\mu g.Kg^{-1}$ of fish) on *S. aurata*. Peng et al. (2012) studied the influence of IVM on blood parameters of *Carassius auratus* (Linnaeus, 1758) after oral administration. However, they did not find significant effects associated with IVM in any blood parameter, similarly to Madrid et al. (2021), that reported that high concentrations of IVM caused weak or no effects in blood parameters in an Amazonian fish *Corydoras schwartzi* Rössel, 1963. In the same way, the results obtained in the present study did not show a significant response of blood parameters of *P. lineatus* exposed to IVM at the assayed concentrations. In addition, the discriminant analysis reports the lowest percentage of correct classification indicating that this group of biomarkers does not discriminate well between treatments.

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In the present study, IVM seems to increase, not significantly, the AChE activity in the brain. These findings seem to contradict the frequently reported inhibitory effect of AChE activity by pollutants (Olivares-Rubio et al., 2021). However, an increase in the activity of AChE in fish from polluted regions (Kopecka et al., 2006) and in experimental conditions (Kist et al., 2012; Oliveira et al., 2015) has also been previously reported. Oliveira et al. (2015) found a significant increase of AChE activity in acute and sub-chronic exposures of *Rhamdia quelen* (Quoy & Gaimard, 1824) to Benzo(a)pyrene, polycyclic aromatic hydrocarbons, and Dichlorodiphenyltrichloroethane. These authors suggest that this increase in AChE activity could affect the level of acetylcholine at nerve terminals and then interfere with neurological responses and, consequently, fish behavior. Ucán-marín *et al.* (2012) reported a similar trend towards increase in AChE activity with IVM exposure through diet, associated with a decrease in mobility behavior and feeding activity of *S. salar* males (Ucan-Marin, 2005). These authors hypothesize that male fish might have fed more intensely than females and thus would have accumulated a higher effective dose of IVM, so that the compensatory benefit of elevated brain AChE might have helped mitigate the lethargy inducing effects of IVM. In other species, such as the springtail *Folsomia candida* (Willem, 1902), a trend of increasing AChE through generations exposed to IVM was reported, also associated with a decrease in avoidance (Guimarães et al., 2019). The results of the present work must be taken with precaution, as more research is needed to clarify the long-term effect of IVM on AChE activity.

It has been extensively reported that exposure to pesticides can affect the balance between the generation of reactive oxygen species (ROS) and antioxidant defense in fish (Lushchak, 2011). Thus, GST, CAT, TBARs and ACAP were chosen due to their involvement in a variety of important biochemical pathways in fish (biotransformation, oxidative stress, and antioxidant response). GSTs are phase II enzymes of the biotransformation system. They can catalyze the conjugation of reduced glutathione (GSH) to electrophilic centers of many endogenous or exogenous toxic compounds, as IVM (Özaslan et al., 2018). In the present work, GST activities in gills significantly decreased after exposure to the lowest IVM concentration. The full physiological response of the organism to the compound is always difficult to

predict; the GST "induction window" may have occurred days earlier or be still to occur (Oliveira et al., 2016). Alternatively, as it was reported by Oliveira et al. (2016) and Serafini et al. (2019), GST activity may be reduced due to depletion of GSH, an antioxidant involved in fighting against ROS. During detoxification processes, GSH is converted to oxidized glutathione (GSSG) and thus becomes less available for conjugation with GST. Chronic effects of IVM evaluated in adult *D. rerio* reported an inhibition of GST after the 21-day exposure period (Oliveira et al., 2016; Domingues et al., 2016). The inhibition of GST may compromise the capacity of fish to metabolize pollutants, and responses such as oxidative stress can be triggered (Oliveira et al., 2016). Even though non-significant effects were observed for oxidative stress, the multivariate analysis revealed the highest % of correct classification on both tissues (brain and gills) and a high potency index for CAT and ACAP. In gills, CAT activity decreased in *P. lineatus* after exposure to the highest IVM concentration. In general, the inhibition of CAT activity has been related to the binding of pollutants to -SH groups of enzymes, increased H_2O_2 and/or superoxide radical (Ruas et al., 2008). Thus, the apparent decrease in CAT activity in gills of *P. lineatus* might have resulted from its inactivation by the superoxide radical triggered by IVM exposure. Although a non-significant decrease in ACAP levels was detected, a marked tendency was observed in gills. This tendency was clearly evidenced in the discriminant analysis. As it has been reported by previous authors (Amado et al., 2009; Vinagre et al., 2003), a decrease in ACAP levels can be interpreted as augmented antioxidant competence and is considered a compensatory response. A similar decrease in ACAP levels was observed by Serafini et al. (2019) during acute exposure of *Rhamdia quelen* to eprinomectin, another semisynthetic avermectin. These authors reported a significant decrease in ACAP levels in liver tissue with the highest concentration of eprinomectin (3.976 μg. L^{-1}) that did not return to normal levels after the 48-h recovery period.

The results obtained from the analysis of behavior, hematology and biochemistry biomarkers were integrated through multivariate analysis. A single and isolated biomarker is not enough to reflect the health status of an organism, and therefore using a large battery of biomarkers from different levels of organization is more appropriate to assess the toxicological effects of xenobiotics (Beliaeff and Burgeot,

2002). To identify the contribution of the group of parameters that were important to describe the overall effects in *P. lineatus,* a discriminant function with a classification analysis was used. In these analyses, brain biochemical biomarkers showed the highest percentage of correct classification, followed by gills biomarkers and behavioral endpoints. A high value of cross-classification indicates that most individuals were correctly classified in their treatments considering these sets of data (brain, gills, and behavior) separately. A confirmation that the correct/successful assignment was significantly higher than likely by chance were the high values of Cohen's Kappa statistic. An additional interest lay in finding the biomarkers that played a greater role in discrimination, which was achieved through the potency index. This index is an aggregative measure because it summarizes information across the different canonical functions. A high potency index means that these variables contribute the most to the discrimination analysis among the treatments. Brain and gills ACAP showed the highest potency index among the biochemical biomarkers and routine swimming among the behavioral biomarkers. Individually, these parameters did not show significant differences in the ANOVA analysis despite showing a non-significant trend (Ghisi et al. 2017).

Despite the large-scale use, the environmental concentrations of IVM in the water are uncertain and highly variable. Both assayed concentrations (0.5 and 1.5 $\mu g.L^{-1}$) seemed to be high compared to predicted environmental concentrations (PEC) of 0.2–2.5 $ng.L^{-1}$ calculated for surface water nearby animal pasture sites by Liebig et al. (2010). In addition, a PEC of 13 $ng.L^{-1}$ was determined for surface water by Metcalfe et al. (2008). However, since IVM is highly hydrophobic, it is rapidly removed from the aqueous phase, and could be accumulated in the suspended organic matter and/or in the water column (Liebig et al., 2010; Kövecses & Marcogliese, 2005). Consequently, water bodies with high levels of suspended organic matter, like Paraná and Uruguay rivers from Rio de la Plata basin (Bergamino et al., 2017; Drago, 2007), could report higher IVM concentrations in water. In this sense, Mesa et al. (2020) found values of 1.24 $ng.g^{-1}$ IVM in water from floodplain lakes of Paraná river adjacent to cattle breeding areas. As previously mentioned, these floodplains play a key role on *P. lineatus* population dynamics and

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recruitment processes (Lozano et al., 2019). Thus, the assayed concentrations and selected life-stage of *P. lineatus* for this experiment represent a probable environmental scenario.

The multiple-endpoint approach by using different sets of parameters allowed an integrated interpretation and further understanding of the effects of IVM in fish. Low concentrations of IVM caused significant effects on fish escape response (MSS) and antioxidant response through inhibition of GST activity of *P. lineatus*. Furthermore, the multivariate analysis via discriminant function allowed to identify overall effects of IVM treatments on biochemical/stress and behavioral biomarkers.

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FIGURE CAPTIONS

Fig. 1. Time spent by *P. lineatus* in each layer of the aquarium by IVM concentration. Data are expressed as mean ± standard error.

Fig. 2. Brain acetylcholinesterase activity of *P. lineatus* by IVM concentration. Data are expressed as mean ± standard error.

Fig. 3. Biochemical parameters: (A) glutathione-S-transferase, (B) catalase, (C) thiobarbituric reactive substances (TBARs) and (D) Total antioxidant competence against peroxyl radicals (ACAP levels). For interpretative purposes, a small area in the bar graph of ACAP levels means a higher antioxidant capacity and vice versa. * Indicates significantly different from control treatment (Tukey test $p<0.05$). Data are expressed as mean ± standard error.

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Fig. 4. Ordination of data from linear discriminant analysis (LDA) summarizing the overall effect for each set of biochemical/oxidative stress (brain and gills), hematology and behavioral biomarkers. Black area: control group; blue area: low IVM concentration (0.5 µg L^{-1}) and red area: high IVM concentration (1.5 µg L^{-1}).

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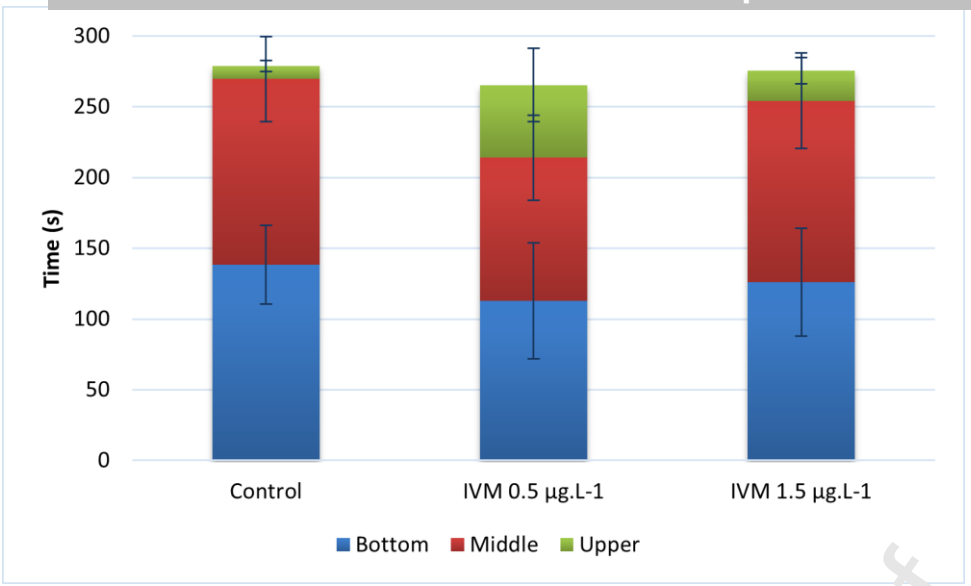

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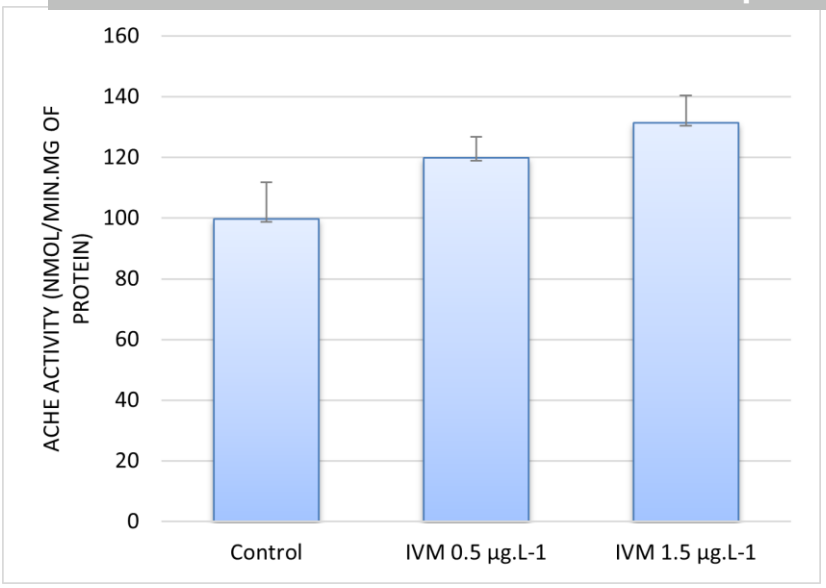

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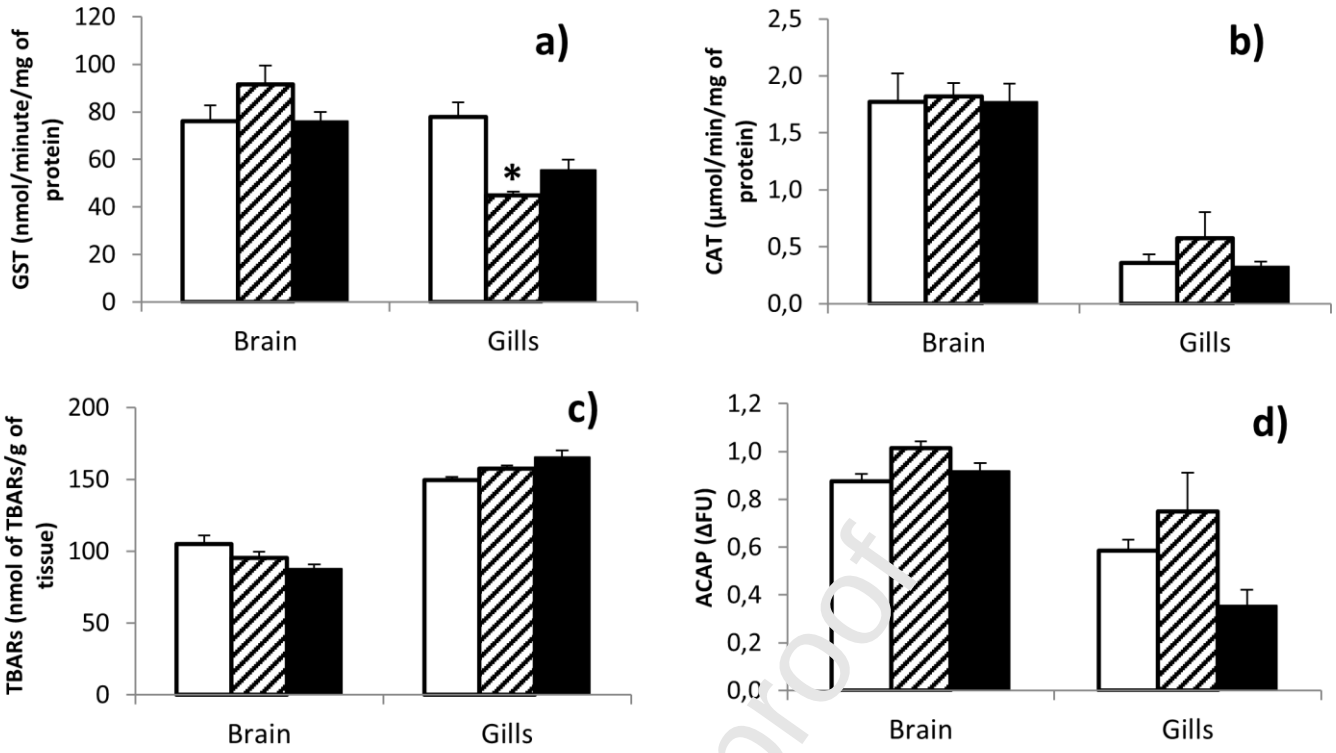

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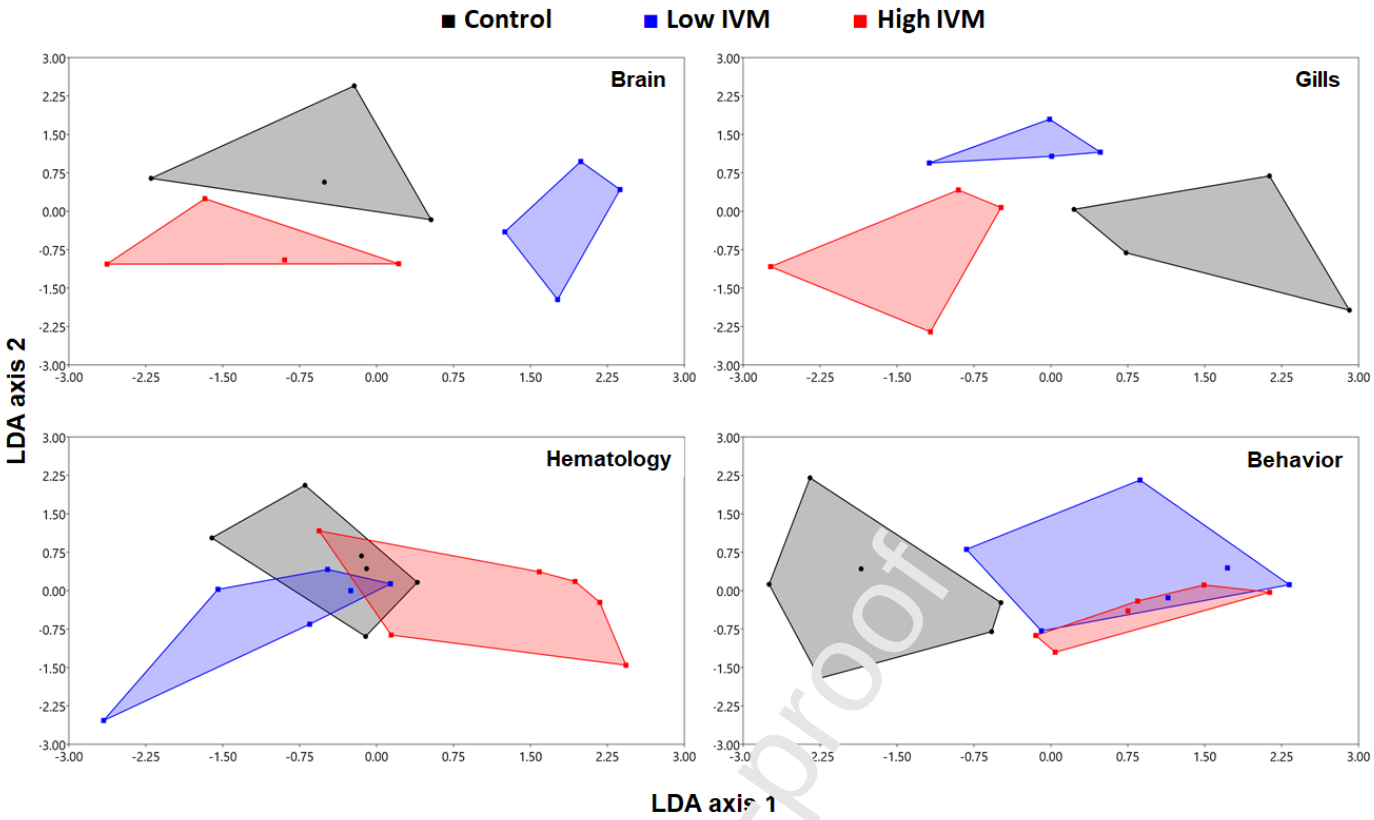

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Author statement

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Table 1

Ivermectin decay results. Actual concentrations of IVM measured in the aquarium water at 0 h, 24 h, 48 h and 72 h after addition of the chemical.

	Nominal concentration							
Time (h)	0.05 mg/L				0.5 mg/L			
	Actual concentration (mg/L)			%	Actual concentration (mg/L)			%
0	0.044	±	0.004	88.0	0.360	±	0.033	72.1
24	0.057	±	0.012	113.9	0.513	±	0.087	102.7
48	0.025	±	0.009	51.0	0.281	±	0.028	56.2
72	0.014	±	0.006	27.9	0.214	±	0.031	42.9

Values are means ± standard deviations of the concentrations recorded in two samples taken from an aquarium containing two concentrations of IVM. % are values for the measured concentrations expressed as percentage of the nominal concentration.

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Table 2

Effects of IVM exposure on swimming activity of *P. lineatus*. RS (routine swimming), TDT (total distance travelled by each fish), TAT (total activity time).

	Vehicle control			0.5 µg.L^{-1}			1.5 µg.L^{-1}		
RS (SL.s^{-1})	1.46	±	0.48	1.29	±	0.41	0.52	±	0.12
TDT (m)	21.04	±	6.02	17.14	±	5.27	7.27	±	1.98
TAT (%)	48.20	±	15.25	71.17	±	7.60	23.85	±	6.43
Maximum swimming speed (SL.s^{-1})	43.25	±	4.98	27.46*	±	2.08	29.21	±	4.53
Water column use (s)									
Bottom	128.30	±	27.82	112.76	±	41.05	125.96	±	38.02
Middle	131.30	±	30.05	101.25	±	30.07	128.18	±	33.78
Upper	9.00	±	3.95	51.30	±	25.90	21.35	±	9.44

Values are means ± standard errors. Speed are expressed as body standard lengths per second. *indicates significant differences ($p<0.05$) with vehicle control.

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Table 3

Effects of IVM exposure on hematological parameters of *P. lineatus*. CF (condition factor), HSI (Hepatosomatic index), Ht (hematocrit), Hb (hemoglobin concentration), RBC (red blood cells count), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration)

	Vehicle control			**0.5 µg.L^{-1}**			**1.5 µg.L^{-1}**		
CF	0.94	±	0.05	0.92	±	0.03	0.91	±	0.03
HSI	1.08	±	0.22	1.26	±	0.28	1.25	±	0.22
Blood glucose (mg.dL^{-1})	38.5	±	7.0	35.0	±	3.3	37.7	±	4.6
Ht (%)	18.3	±	2.7	18.2	±	1.1	15.2	±	2.1
Hb (g.dL^{-1})	5.39	±	1.58	7.24	±	1.88	4.30	±	1.17
RBC (x10^{6} µL^{-1})	1.36	±	0.65	1.53	±	1.42	0.89	±	3.84
MCV (fL)	189.83	±	50.28	119.52	±	14.43	422.39	±	182.68
MCH (pg)	4.36	±	0.61	4.73	±	1.19	9.42	±	3.66
MCHC (%)	26.05	±	5.38	42.09	±	13.09	23.16	±	3.47

Values are means ± standard errors.

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Table 4

Results of Discriminant analysis on the data set of biochemical/stress (brain and gills), hematological and behavior biomarkers of *P. lineatus*.

Group of biomarkers		Variables	Weights for first discriminant function	Weights for second discriminant function	Potency index	% of correct classification
Hematological		Blood glucose	0.029112	-0.015714	0.0007	66.67
		Ht	-0.23193	-0.044226	0.0449	
		Hb	0.12981	1.4101	0.3553	
		RBC	1.91E-12	-3.07E-12	0.0000	
		MCV	-0.014407	0.013862	0.0002	
		MCH	1.0499	-0.9532	1.0691	
		MCHC	-0.16649	0.1479	0.0267	
Biochemical	*Brain*	GST	0.079743	0.016858	0.0038	91.67
		TBARs	0.071555	0.082535	0.0058	
		ACAP	14.115	-3.6565	121.4523	
		CAT	-1.423	-0.61023	1.3438	
	Gills	GST	-0.057743	-0.057857	0.0022	83.33
		TBARs	-0.064529	-0.049355	0.0035	
		ACAP	4.3839	3.4436	16.5173	
		CAT	-3.0126	-1.7141	6.8232	
Behavior		RS	7.2275	1.5586	48.3110	77.78
		TDT	-0.0065027	4.30E-05	0.0000	
		MSS	-0.10156	-0.043887	0.0097	
		TAT	0.012019	-0.0070739	0.0001	

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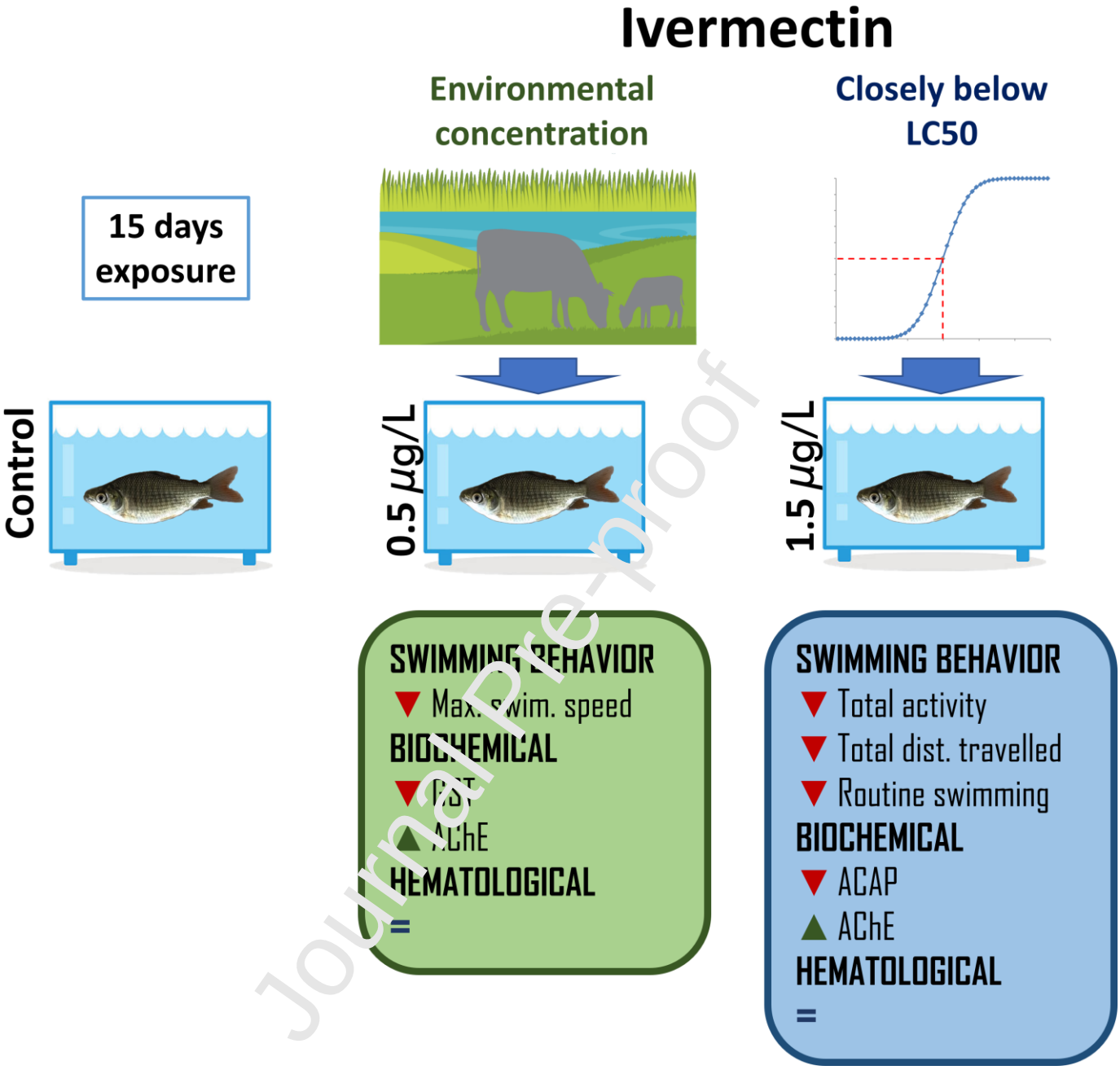
Graphical abstract



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Highlights

* Environmental concentrations of IVM altered fish escape response.

* IVM concentrations tend to induce brain acetylcholinesterase activity.

* GST activity was altered by the IVM environmental concentration.

* Multivariate analysis showed overall effects of IVM treatments in biochemical/stress and behavioral endpoints.

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