DNA damage and oxidative stress induced by imidacloprid exposure in different tissues of the Neotropical fish Prochilodus lineatus

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d Highligh t s

- We evaluated acute effects of Imidacloprid (IMI) on the fish Prochilodus lineatus.
- IMI promoted oxidative damage in different fish tissues.
- IMI promoted hypoglycemia in the exposed fish.
- DNA damage was detected in erythrocytes of fish exposed to IMI.
- Liver, kidney and gill were the most affected organs by IMI exposures.

A b s t r a c t

Imidacloprid (IMI), a systemic neonicotinoid insecticide widely used in worldwide scale, is reported in freshwater bodies. Nevertheless, there is a lack of information about IMI sublethal effects on freshwater fish. Thus, the aim of this study was to identify the potential hazard of this insecticide to the South American fish Prochilodus lineatus exposed for 120 h to four IMI concentrations (1.25, 12.5, 125, and 1250 µg L⁻¹). A set of biochemical, genotoxic and physiological biomarkers were evaluated in different organs of the fish. IMI exposure induced significant changes in the enzymatic profiles of P. lineatus, with alterations in the activity of biotransformation and antioxidant enzymes in different tissues. Redox balance of the tissues was affected, since oxidative damage such as lipoperoxidation (LPO) and protein carbonylation (PCC) were evidenced in the liver, gills, kidney and brain of fish exposed to different IMI concentrations. Fish exposed to all IMI concentrations showed decreased blood glucose indicating an increase of energetic demand. DNA damage was evidenced by the comet test, in the erythrocytes of fish all the concentrations evaluated. We integrated these results in the Integrated Biomarker Response (IBR) index, which evidenced that the organs most affected by IMI exposure were the liver and kidney, followed by the gills. Our results highlight the importance of investigating different target tissues after IMI exposure and show the sublethal effects of IMI in some of them; they also warn to the possible consequences that fish living in freshwater ecosystems can suffer due to IMI exposure.

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1. Introduction

Neonicotinoids are one of the most important chemical classes of insecticides due to their high efficacy against a broad spectrum of insects and the versatility of their use, together with the fact that they are potent at low doses, relatively non-volatile, and highly water-soluble (EXTOKNET, 1996; Tomlin, 2000). Today this class of insecticides comprises at least seven major compounds with a market share of more than 25% of total global insecticide sales (Bass et al., 2015) and is replacing older classes such as organophosphate and carbamate insecticides worldwide (Jeschke et al., 2011). Neonicotinoids act selectively on insect nicotinic acetylcholine receptors (nAChRs), as agonists (Jeschke et al., 2011), competing for these receptors in the postsynaptic membrane (Tomizawa and Casida, 2005; Anderson et al., 2015). Nicotinoids are much more toxic to insects than vertebrates due in part to differences in their interactions with the binding site of receptors (Tomizawa and Casida, 2005). Neonicotinoid insecticides might be contributing to the decline of insectivorous birds in Europe, and fish, amphibians, bats, and birds around the world. Neonicotinoids are much more toxic to insects than vertebrates due in part to differences in their interactions with the binding site of receptors (Tomizawa and Casida, 2005; Anderson et al., 2015). Nicotinoids are much more toxic to insects than vertebrates due in part to differences in their interactions with the binding site of receptors (Tomizawa and Casida, 2005). Neonicotinoid insecticides might be contributing to the decline of insectivorous birds in Europe, and fish, amphibians, bats, and birds around the world.

Imidacloprid (IMI) was the first neonicotinoid launched in 1991 and has become one of the six major products in many pest control programs (Jeschke et al., 2011; Coulson, 2013). In Brazil, IMI was the tenth best-selling active ingredient during 2013, with approximately 8 thousand tons of compound sold (IBAMA, 2013). The use of IMI to control terrestrial pests could potentially result in unintended transport to aquatic habitats and indirect contamination through spray drift, atmospheric deposition, soil erosion, and runoff (CCME, 2007). A number of governmental agencies have developed guidelines on the acceptable IMI concentrations in surface water for the protection of aquatic organisms, ranging between 0.0083 (RIVM, 2014), 0.23 (CCME, 2007), and 1.05 μg L⁻¹ (USEPA, 2014). However, several studies have detected IMI concentrations in surface water greater than the guideline values, in different aquatic environments around the world. Concentrations up to 49 μg L⁻¹ were already measured (Starner and Goh, 2012; Main et al., 2014; Gibbons et al., 2015; Struger et al., 2017), although the highest concentrations mentioned above were detected in water bodies close to farming areas (Sanchez-Bayo and Goka, 2005; Van Kijk et al., 2013; Morrissey, 2015). In Brazil, IMI concentrations of 2.18 (Bortoluzzi et al., 2006) and 3.65 μg L⁻¹ (Becker et al., 2009) have already been detected in aquatic environments influenced by agricultural activities. In a study of the risk classification of different neonicotinoids in Brazilian surface waters, Miranda et al. (2011) reported that IMI presents a greater risk of promoting serious effects on non-target organisms compared to other neonicotinoids. Additionally, according to Albuquerque et al. (2016), neonicotinoids together with other currently used insecticides such as fipronil, represent the greatest potential risk to local aquatic environments when compared to other classes of pesticides.

Despite IMI is being reported in surface waters at concentrations below those which will cause mortality to freshwater fish (SERA, 2005; Tisler et al., 2009), sublethal effects may occur, such as physiological stress and DNA damage (Gibbons et al., 2015). However, there is a lack of studies concerning IMI sublethal effects on fish. In this context, the aim of the present study was to evaluate the potentially harmful effects of IMI on biochemical, physiological, and genetic parameters of the Neotropical freshwater fish Prochilodus lineatus. This fish is an ecologically and economically important benthic species, widely found in rivers of the South and Southeast of Brazil where it comprises a large part of the ichthyomassa (Taylor et al., 2006). Besides, P. lineatus is commonly used in ecotoxicological studies given its sensitivity to pesticides (Langiano and Martinez, 2008; Modesto and Martinez, 2010; Pereira et al., 2013; Moreno et al., 2014). Additionally, this species meets the criteria of the OECD (1992) for selection of species for acute toxicity tests.

2. Material and methods

2.1. Fish handling, experimental design, and sampling

Juveniles of P. lineatus (n = 40; 23.3 ± 4.7 g; 12.6 ± 0.9 cm; mean ± SD) were supplied by the Fish Hatchery Station of the State University of Londrina. Fish were acclimated for five days in a 300 L tank containing dechlorinated tap water under constant aeration. The room photoperiod was fixed at a 12:12 h light/dark cycle. On the second and fourth days of acclimation, fish were fed with a commercial fish diet containing 36% protein (Guabi, Brazil). Feeding was suspended 24 h prior to the beginning of the experiments and the animals were not fed during the experiments.

After acclimation, the fish were randomly divided into five groups (n = 8 fish per group) and maintained in glass aquaria containing 80 L of dechlorinated tap water. One group was kept under control conditions only in clean water (IM0) and the other four groups were exposed to different nominal concentrations of active ingredient from the commercial formulation of Imidacloprid48% a.i.-Norton S.A. (Brazil): 125 (IMI125), 12.5 (IMI125), 125 (IMI125), and 1250 μg L⁻¹ (IMI1250) for five days, under static conditions and daily water renewal. Imidacloprid is acutely toxic to adult fishes at relatively high concentrations, over 80 mg L⁻¹; however, juvenile fishes are considerably more susceptible (Cox, 2001). Thus, the lower IMI concentration tested was defined as an environmentally relevant concentration (1.25 μg L⁻¹) and the others were defined from this concentration in geometric progression of ratio 10 to a maximum concentration of 1.25 mg L⁻¹, which would be safe for juveniles of P. lineatus.

Water temperature, pH, dissolved oxygen, and conductivity were monitored throughout the experiment using a multiparameter water quality meter (HORIBA U-52, Japan). Every day, water samples (500 mL) were collected from all aquaria for analysis of IMI concentration, before water renewal. Aquaria were partially covered to avoid photo degradation of IMI.

After the exposure period, fish were anesthetized with benzocaine (0.1 g L⁻¹) and blood samples were collected from the caudal vein and processed for comet assay and hematological parameters. After blood sampling fish were quickly sacrificed by medullar sectioning for the removal of liver, gills, posterior kidney, brain, and axial muscle. Organs were immediately stored in liquid nitrogen for biochemical analysis. In addition, subsamples of the liver were processed for comet assay.

Individual tissue samples were homogenized (1:10 w/v) in a phosphate buffer solution (0.1 M; pH 7.0 or 7.5 for AChE analysis). Samples were centrifuged (15,000xg, 20 min, 4 °C) and the supernatants were stored at −80 °C for subsequent biochemical analysis. For all biochemical biomarkers, the protein content ω was determined according to Bradford (1976).

2.2. Chromatographic analysis of imidacloprid in water samples

Water samples were collected daily during the fish exposure period in clean amber glass bottles and were analyzed without the solid phase extraction (SPE) step. The concentrations of IMI in water samples were determined according to the method validated by Montagner et al. (2014). Quantification of the IMI was performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The analysis was performed using an Agilent 1200 Series LC system equipped with binary pump, automatic injector, and thermostatted column compartment. Chromatographic separation was performed with a Zorbax SB-C18 column (2.1 × 30 mm,
particle size of 3.5 μm) at 25 °C. The mobile phase consisted of ultrapure water (A) and methanol (B), previously filtered in membranes with 0.2 μm porosity, containing 0.1% formic acid, which favors the formation of ions. The separation occurred in the isocratic elution mode, with a ratio of 70% water to 30% methanol in 3 min. Identification and quantification of imidacloprid was performed by mass spectrometry in an Agilent triple quadrupole device (model 6410B). The imidacloprid was ionized in a positive mode electrospray source and monitored by the MRM (Multiple Reaction Monitoring) mode. The analytical curves were constructed according to the area obtained for each compound as a function of its mass in the column. The limits of detection, quantification, and coefficient of determination (r²) for IMI were 1.6 μg L⁻¹, 5.4 μg L⁻¹, and 0.999 respectively.

2.3. Biomarkers

2.3.1. Physiological assays

To determine the hematocrit (Hct), blood was centrifuged (12000g, 5 min) in a micro-capillary centrifuge (Luguiumac S.R.L, Model LC 5, Argentina) and readings were performed using a standardized chart. The number of red blood cells per mm² blood (RBC) activity was measured using an improved Neubauer hemocytometer under a light microscope. The hemoglobin content (Hb) was estimated using the cyanmethemoglobin colorimetric method (commercial kit, Labtest Diagn. Chrom, United Kingdom). Blood samples were centrifuged (10 min, 1000 x g) and the cellular pellet was suspended in PBS to be used in the comet assay. For blood samples an aliquot of cellular suspension was also mixed with PBS (1:100).

Cellular suspensions were then mixed in low melting point agarose (0.5%), placed on slides precoated with normal (1%) melting point agarose, covered with coverslips and kept under refrigeration (10 °C) for 40 min. The slides were subjected to: a) lysis: 2 h at 4 °C, protected from light, in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauroyl sarcosinate, 10% DMSO, 1 mL Triton X-100, pH 10.0); b) DNA denaturation: 30 min in the dark in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13, at 4 °C); c) electrophoresis: 20 min, 300 mA, 25 V, 0.7–0.8 V cm⁻¹; and d) neutralization: three rinses for 5 min each with buffer (0.4 M Tris, pH 7.5). Slides were fixed with absolute ethanol and kept under refrigeration until analyses. Slides were stained with gelRed and analyzed under a Leica microscope (DM 2500) adapted for fluorescence/epifluorescence at 1000X magnification. All slides were blind-reviewed. The extent of DNA damage was quantified by the length of the tail formed by the migration of DNA fragments and classified into four comet classes, according to Vieira et al. (2016). DNA damage score was obtained by multiplying the number of cells in each class by the damage class, and ranged from 0 (all undamaged) to 300 (all maximally damaged).

The micronuclei (MN) test and the erythrocytic nuclear abnormalities (ENA) test were performed according to the technique described by Ueda et al. (1992). A small drop of blood was smeared over the glass slides, dried overnight and were then fixed with methanol. At the time of analysis, each slide was stained with 0.003% acridine orange in Sorenson's buffer at pH 6.8 and immediately analyzed under epifluorescence at 1000X magnification. For each fish 3000 cells were analyzed, evaluating the presence of micronuclei and ENA (according to Carrasco et al., 1990). ENA were classified into three categories: segmented nucleus, lobulated nucleus, and kidney-shaped nucleus, in addition to the presence of binucleated cells. The mean frequency of MN and ENA, for each group, was calculated and expressed per 1000 cells (%).

2.3.2. Biochemical analysis

Glutathione-S-transferase (GST) activity was determined according to Keen et al. (1976) and adapted for microplates (Gagné, 2014), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, at 340 nm. Enzyme activity was expressed as nmol CDNB conjugate formed min⁻¹ mg protein⁻¹.

Glutathione (GSH) concentration was determined according to Beutler et al. (1983) using 5,5’-dithiobis-2-nitrobenzoic acid (DTNB), at 412 nm against a GSH standard curve. GSH concentration was expressed in μg GSH mg protein⁻¹. Copper–zinc superoxide dismutase (CuZn-SOD) activity was determined according to McCord and Fridovich (1969), based on the measurement of the inhibition of the reduction rate of cytochrome c by the superoxide radical, at 550 nm and 25 °C. SOD activity was expressed in units mg protein⁻¹, with one unit of SOD corresponding to the quantity of enzyme that promoted the inhibition of 50% of the reduction rate of cytochrome c. Catalase (CAT) activity was determined according to Beutler (1975), by monitoring the H₂O₂ decomposition at 240 nm. The activity was expressed in μmol H₂O₂ min⁻¹ mg protein⁻¹. Selenium-dependent glutathione peroxidase (GPx) activity was determined by NADPH oxidation in the presence of GSH and H₂O₂, at 340 nm (Hopkins and Tudhope, 1973) and was expressed in μmol oxidized NADPH min⁻¹ mg protein⁻¹.

Lipid peroxidation (LPO) was determined by the thiobarbituric acid reactive substances (TBARS) assay, performed according to Camejo et al. (1998). TBARS concentrations were expressed in nmol MDA mg protein⁻¹. Oxidative damage to proteins was measured by quantifying the protein carbonyl content (PCC) according to Levine et al. (1994) at 360 nm and the results were expressed in nmol carbonyl mg⁻¹ protein⁻¹.

Subsamples of brain and muscle tissue were used for the AChE assays, according to Ellman et al. (1961) and adapted for microplates (Alves Costa et al., 2007). AChE activity was determined at 415 nm and expressed in nmol min⁻¹ mg protein⁻¹.

2.3.3. Genotoxic analysis

The alkaline comet assay with erythrocytes and liver cells was performed according to Singh et al. (1988), with some modifications. Liver samples, were mechanical and chemical dissociatitated, according to Cavalcante et al. (2008). Subsequently, the solution was filtered (30 μm mesh size) into a tube containing fetal bovine serum (FBS), centrifuged (10 min, 1000 x g) and the cellular pellet was suspended in FBS to be used in the comet assay. For blood samples an aliquot of cellular suspension was also mixed with PBS (1:100).

C. The mobile phase consisted of ul-

...m mesh size) into a tube containing fetal bovine serum (FBS), centrifuged (10 min, 1000 x g) and the cellular pellet was suspended in FBS to be used in the comet assay. For blood samples an aliquot of cellular suspension was also mixed with PBS (1:100).

...Biomarker Response Index (IBR)

The biomarker results were applied into the “Integrated Biomarker Response Index” (IBR2), described by Beliaeff and Burgeot (2002) and modified by Sanchez et al. (2013). This index is based on the principle of reference deviation between a disturbed and undisturbed state (Sanchez et al., 2013). In the present work, the deviation between biomarkers measured in fish exposed for five days to different IMI concentrations, for each organ, were compared to those measured in fish exposed to clean...
water (IMI0). For each individual biomarker, the ratio between the mean value obtained at each IMI concentration and the respective reference control value (IMI0) was log-transformed (Yi). In the next step, a general mean (μ) and standard deviation (s) was calculated, considering Yi values of a given biomarker measured in each group. Next, Yi values were standardized by the formula: Zi = (Yi – μ)/s and the difference between Zi and Z0 (IMI0) was used to define the biomarker deviation index (A). To obtain an integrated multiple biomarker response, the value of A of each biomarker was calculated for every exposed group and IBRV2 was calculated for each group by the sum of the absolute values of A. For each group (IMI1.25, IMI12.5, IMI125, and IMI1250) and each organ analyzed, A values calculated for each biomarker were reported in a star plot representing the reference deviation of each investigated biomarker. The area above 0 reflects biomarker induction, and the area below 0 indicates biomarker inhibition.

2.5. Statistical analysis

After checking data for normality (Shapiro Wilk test) and homoscedasticity (Levene’s test) mean values for the different treatments, for each organ analyzed, were cross-compared using parametric (ANOVA) or non-parametric (Kruskal-Wallis) analysis of variance, followed by the Student-Newman-Keuls (SNK) or Dunn’s test, respectively, when necessary. A significance level of 0.05 was used in all analyses. The data shown in the graphs represent means ± SEM.

3. Results

3.1. Water parameters

During the tests, there was no mortality in any experimental group. The physical and chemical characteristics of the water for all the experimental periods remained stable. No significant differences between aquaria were detected (Table 1). All quantified imidacloprid concentrations were lower than the nominal concentrations and ranged from 5.45 to 359 μg L⁻¹.

3.2. Biomarkers

3.2.1. Physiological parameters

No significant differences were found for hematological parameters amongst fish exposed to different concentrations of IMI (Table 2). However, plasma glucose was significantly lower in all groups of exposed fish, showing marked hypoglycemia in relation to IMI0 (p = 0.002; Table 2). No significant change was found in plasma ions (Table 2).

3.2.2. Biotransformation and antioxidant defenses

Alterations in GST activity were found in the gills, kidney, and brain. In fact, the gills showed reduced GST activity in IMI12.5 and IMI1250 groups (p < 0.001), as in the kidney of fish exposed to IMI1250 (p < 0.001). In contrast, in the brain a significant GST increase was detected in fish from IMI125 and IMI1250 groups (Fig. 1A) together with a significant increase in GSH content (Fig. 1B). SOD activity showed significant increases in the liver of fish from IMI125 and IMI1250 groups (p = 0.003) and in gills of fish from IMI12.5 and IMI12.5 groups (p = 0.008) (Fig. 1C). For CAT activity the liver of fish from IMI1250 group presented a decrease (p = 0.036) while a significant increase was detected in the kidney of fish from all IMI concentrations (p < 0.001) and in the brain fish exposed to IMI1250 (p = 0.026) (Fig. 1D). GPx activity increased in the gills of fish from IMI12.5 group (p = 0.021) as well as in the brain of fish from IMI125 and IMI1250 groups (p < 0.001) (Fig. 1E).

3.2.3. Oxidative damage

Except for muscle, all the analyzed tissues demonstrated oxidative damage in lipids or proteins at least in one of the concentrations tested. LPO was significantly higher in fish liver (p = 0.001) and kidney (p = 0.024) from IMI1250 group; in the gills of fish from IMI12.5 and IMI125 groups (p < 0.001); and in the brain of fish from IMI125 and IMI1250 groups (p = 0.005) (Fig. 2A). Similarly, a significant increase was detected in PCC in the liver of fish from IMI1250 group (p = 0.036); in the gills of fish from IMI125 group (p < 0.001); and kidney of fish from IMI125 and IMI1250 groups (p = 0.009) (Fig. 2B).

### Table 1

| Physical and chemical parameters and imidacloprid concentrations in the water used in the experiments. The values are represented as mean ± SD (n = 5). |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Water parameters | IMI0            | IMI 1.25        | IMI 12.5        | IMI 125         | IMI 1250        |
| Temperature (°C) | 22.61 ± 2.25    | 22.62 ± 1.80    | 22.86 ± 1.76    | 23.33 ± 2.00    | 23.49 ± 2.19    |
| pH               | 7.93 ± 0.19     | 7.92 ± 0.14     | 7.88 ± 0.15     | 7.87 ± 0.19     | 7.80 ± 0.21     |
| Dissolved oxygen (mgO₂ L⁻¹) | 7.10 ± 0.08 | 7.14 ± 0.13     | 7.14 ± 0.16     | 7.15 ± 0.09     | 7.09 ± 0.07     |
| Conductivity (µS cm⁻¹) | 100.6 ± 9.93 | 101.4 ± 10.38   | 100.8 ± 10.52   | 100.8 ± 10.37   | 101.6 ± 10.73   |
| Imidacloprid (µg L⁻¹) | <DL            | <DL             | 5.45 ± 0.21     | 37.15 ± 0.49    | 359.15 ± 0.77   |

Values are mean ± SD (n = 5). DL (Detection limit) = 1.6 μg L⁻¹

### Table 2

| Physiological parameters of *P. lineatus* kept under the control condition (IMI0) or exposed to IMI (nominally: 1.25, 12.5, 125, and 1250 μg L⁻¹). |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Physiological parameters | IMI0            | IMI 1.25        | IMI 12.5        | IMI 125         | IMI 1250        |
| Hemoglobin (mg dl⁻¹) | 10.42 ± 0.33    | 10.12 ± 0.38    | 10.47 ± 0.14    | 11.09 ± 0.17    | 10.67 ± 0.46    |
| Hematocrit (%)    | 45.14 ± 0.88    | 43.14 ± 1.30    | 41.29 ± 1.32    | 48.00 ± 0.87    | 43.43 ± 1.78    |
| Number of RBCs (x 10⁶ cells mm⁻³) | 3.50 ± 0.10 | 3.78 ± 0.18     | 3.61 ± 0.17     | 4.18 ± 0.11     | 3.78 ± 0.16     |
| Glucose (mg dl⁻¹) | 75.24 ± 4.24²   | 58.15 ± 3.28²   | 48.87 ± 2.12²   | 55.04 ± 5.41²   | 56.73 ± 4.01²   |
| Na⁺             | 150.94 ± 3.50   | 153.72 ± 4.17   | 158.24 ± 3.51   | 149.20 ± 3.42   | 154.43 ± 2.13   |
| K⁺              | 4.78 ± 0.20     | 4.48 ± 0.41     | 4.89 ± 0.40     | 3.69 ± 0.26     | 4.13 ± 0.29     |
| Cl⁻             | 105.67 ± 4.86   | 104.71 ± 3.77   | 114.36 ± 5.51   | 98.79 ± 4.34    | 104.02 ± 5.00   |
| Ca²⁺            | 2.91 ± 0.56     | 1.61 ± 0.11     | 2.30 ± 0.28     | 1.79 ± 0.11     | 2.14 ± 0.25     |
| Mg²⁺            | 1.00 ± 0.10     | 0.93 ± 0.03     | 0.94 ± 0.072    | 0.96 ± 0.05     | 0.83 ± 0.07     |

Results are expressed as mean ± SEM (n = 5). Different letters indicate significant differences between groups (p < 0.05).
3.2.4. AChE activity

AChE activity was not affected by IMI exposure in brain or muscle. Fig. 3 shows AChE activities in these tissues at different IMI exposure concentrations.

3.2.5. Genotoxic biomarkers

The comet assay showed that all IMI concentrations promoted significantly higher DNA damage scores in *P. lineatus* erythrocytes in comparison to fish exposed to IMI0, indicating damage to the genetic material (*p* = 0.018; Fig. 4A). On the contrary, hepatocytes did not present any significant changes in DNA damage scores (Fig. 4A).

In relation to frequency of micronucleus (MN), only 1 erythrocyte with MN was detected in fish exposed to the highest IMI concentration. In addition, there was a significant increase in ENA frequency in fish exposed to IMI1250 (*p* = 0.043), relative to the other groups (Fig. 4B).

3.3. IBR

IBR values indicated that the liver and kidney showed greater variations in biomarker responses, with average IBR values of 11.29 and 11.15, respectively, followed by the gills (10.71) and brain (8.22) (Fig. 5). Muscle did not demonstrate any significant variation in biochemical responses and, therefore, was not applied in the index. Mean IBR values increased as IMI concentrations increased: IMI1250 = 8.90 < IMI125 = 9.66 < IMI125 = 10.58 < IMI1250 = 12.23, indicating that the highest concentration promoted more pronounced effects in the different organs evaluated, with the exception of the gills.

4. Discussion

This study is the first to report oxidative and DNA damage in different organs of a fish after IMI exposure. Previous studies conducted with a small number of fish species have concluded that fish are typically less sensitive to neonicotinoids than insects or other vertebrate groups (Anderson et al., 2015). However, the results of the present work show that *P. lineatus* was severely affected by the exposure to different IMI concentrations.

In the present work, the concentrations of IMI determined by LC-MS/MS were lower than the nominal concentrations (about 30% of nominal concentration). This discrepancy between nominal and measures concentrations is possibly related to different factors, such as photolytic degradation of the active ingredient in the commercial formulation and/or in the water of the aquaria, liquid formulation used, neutral pH and the interference of inert ingredients present in the formulation in IMI determination. Imidacloprid decomposes reasonably fast in aqueous solution under the influence of UV-light producing a number of photoproducts. According to Moza et al. (1998), irradiation at 290 nm resulted in 90% substrate transformation in 4 h, and 6-Chloronicotinaldehyde, N-methyl nicotinacidamide, 1-(6-chloronicotyl) imidazolidone and 6-chloro-3-pyridyl-methylthylethylendiamine were the main photoproducts identified by CG-MS analysis. The type of the formulation can also interfere on the persistence of IMI. Sarkar et al. (1999) observed higher half-lives of IMI in the powder formulation when compared to the liquid formulation. These same authors investigated the effect of pH on IMI persistence in water and found that the mean half-life value was higher at pH 9.0, indicating a longer persistence of the insecticide residues under alkaline conditions.

Hematological parameters and plasma ions of *P. lineatus* remained unaffected after IMI exposures. However, fish showed decreased glycemia after exposure to all IMI concentrations. Under stress conditions, an extra supply of energy substrates is required to compensate the increased energy demand and carbohydrates are usually the first energy supply in these situations (Saravanan et al., 2011; Cazenave et al., 2014). Another hypothesis to explain the observed hypoglycemia would be the decrease of glycogen in consequence of the high amount of energy required for the hepatic synthesis of detoxification enzymes, as pointed out by Begum and Vijayaraghavan (1995). In the fish *Piaractus mesopotamicus* acutely exposed to the organophosphate trichlorfon, Venturini et al. (2015) observed an increase in glucose in the liver and muscle, which suggests an increased glucose uptake during exposure. A
hypoglycemic response in freshwater fish exposed to other insecticides is frequently reported (Singh et al., 1993; Borah and Yadav, 1995; Agrahari et al., 2007) and is related to the rapid use of blood glucose during hyperexcitability, tremors and convulsions, due to the effects of the insecticides on fish (Singh et al., 1993). Our results suggest that IMI exposure, regardless the concentration, promotes an increased metabolic demand in organs such as liver and muscle leading to hypoglycemia. Additional metabolic and behavior analyzes in exposed fish would be important to better elucidate these results.

It has been extensively reported that exposure pesticides can affect the balance between the generation of reactive oxygen species and antioxidant defense in fish (Lushchak, 2011); therefore, alterations in the activity of antioxidants enzymes in different organs suggest from adaptive response to oxidative stress condition to irreversible damage in the organ (Livingstone, 2001). Enzymes such as SOD and CAT play an important role in the elimination of ROS produced during the biotransformation of xenobiotics and the induction of the SOD/CAT system may be the first defense mechanism against ROS (Lushchak, 2016). In P. lineatus exposed to IMI, an increase in hepatic SOD activity was detected at both IMI 125 and IMI1,250 indicating the generation of superoxide anions. Increased SOD activity was also observed in zebra fish exposed to neonicotinoid thiamethoxam for 7 and 14 days (Yan et al., 2015) and after 7 days of exposure to a range of 0.3–3 mg L\(^{-1}\) of IMI (Ge et al., 2015). In contrast, hepatic CAT activity decreased in P. lineatus after exposure to IMI1,250. Shukla et al. (2017) also detected reduced CAT activity in the liver of zebra fish exposed to IMI for 24 h. In general, the inhibition of CAT activity has been related to the binding of toxicants to -SH groups of enzyme, increased H\(_2\)O\(_2\) and/or superoxide radical (Ruas et al., 2008). Thus, the significant decrease in CAT activity in liver of P. lineatus might have resulted from its inactivation by the superoxide radical triggered by IMI exposure. Navarro and Martinez (2014) also observed these opposite responses of the two primary antioxidant defenses in P. lineatus exposed to surfactant polyoxyethylene amine, supporting the hypothesis that SOD activity increases until the superoxide anion radical increases to a threshold and after that the excess of this radical promotes the inhibition of CAT. Taking together, these alterations in the antioxidant enzymes SOD and CAT might indicate a high production of ROS, which triggers a state of oxidative stress, as evidenced by the highest levels of LPO and PCC detected in P. lineatus exposed to IMI1,250. LPO reflects loss of membrane integrity (Valavanidis et al., 2006); while PCC reflects loss of protein function (Wong et al., 2010). Therefore, results obtained in the liver demonstrate irreversible damage, which can lead to a reduction in its functionality, intensifying the toxic effects of IMI.

Fig. 2. Lipid peroxidation (A) and protein carbonyl content (B) in the liver, gill, kidney, brain, and muscle of P. lineatus kept under the control condition (IMI0) or exposed to Imidacloprid at 1.25, 12.5, 125, and 1250 µg L\(^{-1}\). Data are mean ± SEM, n = 8. Different letters indicate significant differences between groups (p < 0.05).

Fig. 3. Acetylcholinesterase activity in muscle and brain of P. lineatus kept under the control condition (IMI0) or exposed to Imidacloprid at 1.25, 12.5, 125, and 1250 µg L\(^{-1}\). Data are mean ± SEM, n = 8.
Gills are organs involved in the excretion of xenobiotics but they also represent an important tissue for the uptake of xenobiotics as they are in direct contact with pollutants (Monteiro et al., 2005). In the present study, gills were one of the main organs that exhibited changes in the majority of the antioxidant defenses evaluated. Significant increases in SOD and GPx activities were evident in the gills of fish exposed to the lower concentration of IMI tested. Presumably, these increments in both SOD and GPx were effective for preventing oxidative damage in gills of fish exposed to IMI 1.25, but not to IMI 12.5. Moreover, gills of fish exposed to IMI 125 showed an increase in LPO and PCC levels. Detrimental effects on lipid and protein may be related to a decrease in branchial GST activity in fish exposed to both IMI 12.5 and IMI 125. The role of GST is to catalyze the detoxification of harmful compounds via conjugation of GSH (Hellou et al., 2012). Despite the levels of GSH in the gills remained unchanged in relation to the control fish (IMI0), the decrease in GST activity suggests a decline in detoxification at the gills, making possible the emergence of peroxidative damage.

As in the liver and gills, oxidative damage was evident in the kidney of *P. lineatus* exposed to the highest IMI concentration, along with decreased GST and increased CAT activities. IMI has a high water solubility (0.51 g.L at 20 °C) and low log Kow value (0.57), indicating a low potential for bioaccumulation (Moza et al., 1998). Due to these characteristics, in mammals neonicotinoids are excreted unchanged in urine (Tomizawa and Casida, 2005). Rats excreted 96% of radio-labeled IMI within 48 h following an unspecified oral dosing, with 90% excreted in the first 24 h (Klein, 1987). Even if urinary elimination of xenobiotics is less important in fish than it is for mammals, due to the contribution of the gills to chemical elimination, urine may represent an important route for excretion of some compounds (Kleinow et al., 2008). Given its hydrophilic characteristic and importance of the kidney for its elimination, IMI could quickly reach this organ and promote biochemical disorders. An overproduction of ROS in the kidney was suggested by the increased CAT activity and high levels of protein carbonylation (PCC), which may also inactivate the activity of enzymes, such as GST.

In the brain, a rise in LPO levels was observed in fish exposed to IMI 1.25 and IMI 12.5, while at IMI125 and IMI1250 μg L⁻¹, an increase in GSH activity concomitant with increased GST levels was seen, suggesting that activation of antioxidant defenses prevented oxidative stress in this organ at IMI high concentrations. In addition, an increase in brain GPx activity was observed in fish exposed to IMI 125 and IMI 1250. This enzyme catalyzes the reduction of H₂O₂ and lipid hydroperoxides at the expense of GSH (Lushchak, 2016) and its increase could have contributed to prevent oxidative damage in the brain of fish exposed to higher IMI concentrations. Shukla et al. (2017) observed an increase of GSH in the brain of *Danio rerio* exposed to IMI and consequently, an increase of MDA in this organ was not observed. Glutathione peroxidases (GPxs) are known to provide protection against LPO by terminating lipid peroxidation cascade through the reduction of fatty acid hydroperoxides (FA-OOH) and phospholipid hydroperoxides (PL-OOH) (Sharma et al., 2004). In addition to GPxs, some of the GST isozymes can efficiently reduce FA-OOH as well as PL-OOH and can interrupt the autocatalytic chain of lipid peroxidation by reducing these hydroperoxides that propagate lipid peroxidation chain reactions (Zhao et al., 1999; Yang et al., 2001). In addition, a subgroup of GST isoforms with substrate preference for α, β-unsaturated carboxyls (e.g., 4-HNE and acrolein) can effectively detoxify these toxic end products of lipid peroxidation (Singhal et al., 1994; Hubatsch et al., 1998). Thus, GSTs not only complement GPxs in attenuating lipid peroxidation by reducing hydroperoxides, but also protect cells from toxic end products of lipid peroxidation. In the present study, the increase or maintenance of normal levels of GST activity in the liver, brain and muscle may have played an important role in the prevention of oxidative stress. Thus, GST in fish brain can be considered as a beneficial tool for handling stress conditions. Iturburu et al. (2017) highlight the presence of IMI in the brain tissue of exposed fish, indicating that the compound may overpass the blood-brain barrier, which is an essential structure to maintain brain homeostasis and protect the organ from toxic substances.

No changes were observed in AChE activity in muscle and brain of fish exposed to any IMI concentration. Neonicotinoid insecticides act by interfering with the insect nervous system. Specifically, they exhibit insect nicotinic acetylcholine receptors (nAChRs) acting as ‘false neurotransmitters’ (Tomizawa and Casida, 2005). However, IMI binds more strongly in insect than mammalian or other vertebrate nAChRs, for this reason the toxicity in vertebrates is...
lower than in insects (Matsuda et al., 2001); and presumably because of this no effects were observed in AChE activity in *P. lineatus*. Contrary results were found by Topal et al. (2017) who observed AChE inhibition in the brain of rainbow trout after IMI exposure (10 and 20 mg L\(^{-1}\)) for 21 days, indicating that longer exposures periods to higher IMI concentrations can promote disturbances in the neurological activity of exposed fish.

The comet assay showed that exposure to IMI caused an increase in DNA damage in erythrocytes, but not in hepatocytes; the highest concentration of IMI tested also promoted the highest frequency of ENAs. Ge et al. (2015) working with *D. rerio* detected DNA damage in cell suspensions of livers exposed to IMI. Similarly, IMI exposure caused DNA damage in erythrocytes of frogs *Rana N. Hallowell* (Feng et al., 2004). Moreover, in the highest concentrations of IMI tested, lipid peroxidation was also observed in liver, gill, and kidney; this process can generate lipid superoxide, free and alkyl radicals that intensify DNA damage (Wong et al., 2010; Hook et al., 2014). Similarly, Xia et al. (2016) observed a concentration and time-dependent increase in the frequency of MN and ENAs in the fish *Misgurnus anguillicaudatus* after exposure to IMI, in addition to DNA breaks at all concentrations tested (43–115 mg L\(^{-1}\)) after six days of exposure.

Iturburu et al. (2017) observed an increased MN frequency in a Neotropical fish exposed to IMI at 100 \(\mu\)g L\(^{-1}\) and 1000 \(\mu\)g L\(^{-1}\). Together, these results clearly indicate that IMI promotes genotoxic effect on different species of fish.

Any of the analyzed parameters has changed in muscle tissue, this fact can be related to the characteristics of IMI, such as the low lipid solubility of the compound. Due to its hydrophilic nature, we hypothesize that the main route that drives IMI to muscle would be from the blood supply, rather than the skin, and as this compound is rapidly excreted via urine, it does not accumulate in non-target tissues, not causing effects on the muscle.

The IBR values showed that the organs most affected by IMI exposure were the liver and kidney, followed by the gills, which are the main organs responsible for the metabolism, excretion and uptake of xenobiotics in fish, respectively. The biochemical biomarkers responded in a concentration-dependent manner in almost all organs, except for the muscle that did not present any alteration in the analyzed parameters. Our results highlight the importance of investigating different target tissues after exposure to toxicants, once specific tissue responses were observed in the activity of antioxidant enzymes after IMI exposure, indicating different rates of ROS generation and antioxidant capacity of these tissues.

**5. Conclusion**

The results obtained in this study indicated that high concentrations of IMI induced a marked increase in LPO and PCC in different tissues of fish. Although adverse effects were observed at higher exposure concentrations than habitually detected in surface water, IMI exposure caused oxidation of various biological molecules, including lipid, proteins and possible oxidative DNA damage, which leads to inactivation of their functions. Given the constant increment in the use and sale of IMI it is probable that its concentration in surface water will also increase, becoming particularly harmful to fish vulnerable to the effects of chronic exposure to IMI.


