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Hematological and biochemical alterations in the fish *Prochilodus lineatus* caused by the herbicide clomazone

Lindalva Pereira[b], *Marisa N. Fernandes*[a,b], *Cláudia B.R. Martinez*[c,*]

[a] *Department of Physiological Sciences, Federal University of São Carlos (UFSCar), Rodovia Washington Luis, Km 235, CEP: 13565-905, São Carlos, SP, Brazil*

[b] *Postgraduate Program in Ecology and Natural Resources, Federal University of São Carlos (UFSCar), Rodovia Washington Luis, Km 235, CEP: 13565-905, São Carlos, SP, Brazil*

[c] *Department of Physiological Sciences, State University of Londrina (UEL), Rodovia Celso Garcia Cid, Km 374, CEP: 86051-990, Londrina, PR, Brazil*

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ABSTRACT

The indiscriminate use of herbicides has led to the contamination of water bodies, possibly affecting the health of aquatic biota. Therefore, to evaluate the possible effects of the clomazone-based herbicide (Gamit® 500) on the fish *Prochilodus lineatus*, juveniles were exposed for 96 h to three concentrations (1, 5 and 10 mg L^{-1}) of clomazone, and an analysis was made of their hematological parameters: hemoglobin (Hb); hematocrit (Hct); red blood cell (RBC) count; mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC) and biochemical parameters: glutathione S-transferase (GST); catalase (CAT); glutathione peroxidase (GPx) and acetylcholinesterase (AChE). Hct presented a significant decrease at the concentration of 10 mg L^{-1}, while the parameters Hb, HCM and MCHC presented a significant decrease at the two higher concentrations, indicating an anemic condition. The RBC increased significantly at the lowest concentration, possibly due to the release of new red blood cells into the bloodstream in response to splenic contraction, which may occur as an adaptive response to the stressor agent. *P. lineatus* presented activation of the biotransformation pathway, indicated by augmented hepatic activity of the enzyme GST and hepatic activation of the antioxidant enzyme CAT at the higher concentrations. Liver GPx was significantly inhibited at the higher concentrations, which may indicate the efficient action of CAT in the elimination of H_2O_2 or its competition with GST for the same substrate (GSH). AChE activity in brain and muscle was inhibited at the higher concentrations, indicating the neurotoxic effects of the herbicide in the fish. The hematological and biochemical alterations led to the conclusion that the herbicide clomazone has toxic effects on the species *P. lineatus*, and that its presence in the environment may jeopardize the health of these animals.

1. Introduction

The literature contains numerous reports about the increasing use of toxic agrochemicals in the last few decades. In 2008, Brazil became the worldwide leader in the consumption of agrochemicals (SINDAG, 2009a), among which herbicides are the most widely used. Brazil's consumption of herbicides corresponded to approximately 59% of the total sold worldwide in 2009 (SINDAG, 2009b). One of the most

* Corresponding author.
E-mail addresses: cbueno@uel.br, claudiabrmartinez@gmail.com (C.B.R. Martinez).
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widely used herbicides is clomazone 2-[(2-chlorobenzyl)]-4,4-dimethyl-1,2-oxazolidin-3-one. This herbicide, which belongs to the chemical group isoxazolidinone, acts as an inhibitor of carotenoid synthesis. Its herbicidal activity is associated with its metabolites, such as 5-ketoclomazone (EPA, 2007), activated by the P_{450} monooxygenase system (Ferhatoglu et al., 2005). This herbicide has been widely employed in agriculture for pre- and post-emergent weed control. In 2009, clomazone ranked among the top ten herbicides most widely used in Brazil (IBAMA, 2010).

Chemical products discharged into the environment end up reaching aquatic systems, contaminating and/or affecting the aquatic biota, including fishes, through direct contact of the surface of the body and the gills of these animals with contaminated water, or else through their food. Once these products have penetrated the organism, their effect may be toxic (Erickson et al., 2008). Clomazone is highly water-soluble (1100 mg L^{-1} according to the EPA, 2007), which increases its potential for contaminating surface water and probably also groundwater (Primel, 2005).

Studies have already proven the presence of clomazone in waters located in areas where sugarcane (Armas et al., 2005) and rice (Primel, 2005) are cultivated and residual clomazone concentrations, ranging from 0.2 to 0.4 mg L^{-1} were found in 90% of water samples collected from rivers near rice cultivation (Zanella et al., 2002). Studies have also showed that this herbicide is toxic to Neotropical fish species such as *Rhamdia quelen* (Miron et al., 2005, 2008; Crestani et al., 2006, 2007; Menezes et al., 2011) and *Leporinus obtusidens* (Moraes et al., 2007).

Exposure to contaminants can cause biological changes in organisms. These changes can be measured and used as indicators of exposure to or effects of environmental pollutants, which are called biomarkers. These biomarkers enable the rapid assessment of the health of organisms and warn about possible environmental risks (Hugget et al., 1992; Mayer et al., 1992; Van der Oost et al., 2003).

Among biological changes, hematological parameters are considered potential biomarkers of exposure to chemical agents, since the latter can induce an increase or decrease in the various hematological components (Heath, 1995; Van der Oost et al., 2003). The effects of pesticides have been observed in blood parameters of fishes. In a study of the herbicide atrazine, which belongs to the same chemical group as clomazone (the triazine group), hematological alterations were found in *Oreochromis niloticus* (tilapias) (Hussein et al., 1996). Curimba (*Prochilodus lineatus*) exposed to the glyphosate-based herbicide Roundup Transorb® also presented hematological alterations (Modesto and Martinez, 2010b).

The most extensively investigated biochemical biomarkers are the enzymes involved in the detoxification of toxic agents and their metabolites, i.e., biotransformation and antioxidant defense enzymes. Like other animals, fish have biotransformation enzymes, whose principal function is to catalyze the conversion of liposoluble organic compounds into more easily excretable hydrosoluble metabolites (Livingstone, 1998). This biotransformation involves phase I of the detoxification process, hydrolyzing the toxic compound, which may be eliminated or continue on the biotransformation pathway (Van der Oost et al., 2003; Di Giullio and Hinton, 2008). Phase II involves the conjugation of the metabolites produced in phase I with endogenous compounds from the cells, such as peptides, sugars or sulfates, resulting in water-soluble products that are easily excreted (Di Giulio et al., 1995). Enzymes of the glutathione-S-transferase (GSTs) family act in phase II of the detoxification process, reducing the probability of these compounds binding to other cell macromolecules (Stegeman et al., 1992).

During the biotransformation of xenobiotics, reactive oxygen species (ROS) are formed that can damage cell structures via oxidation. To minimize the effects of these ROS, cells have enzymes such as catalase and glutathione peroxidase, which are involved in the antioxidant process (Storey, 1996; Van der Oost et al., 2003). Certain agrochemicals induce augmented activity of the biotransformation enzyme GST and oxidative stress (Dorval et al., 2003; Monteiro et al., 2006). Therefore, enzyme activity may be a useful indicator of the presence of contaminants in the organism.

Acetylcholinesterase (AChE) is an important enzyme that participates in the transmission of the nerve impulse of cholinergic neurons. This enzyme is a well known biomarker for organophosphate-based insecticides, which are known as powerful inhibitors of its activity (Payne et al., 1996; Fulton and Key, 2001; Van der Oost et al., 2003; Oruç and Usta, 2007). In addition to these pesticides, AChE also responds to a series of other contaminants, presenting a very promising potential for environmental assessment and monitoring (Payne et al., 1996).

Studies of the effects of toxic agents on aquatic organisms enable the establishment of maximum acceptable concentrations of toxic substances in the environment without causing significant damage to the resident biota. Such studies are therefore indispensable for assessing risks and outlining guidelines for the use of pesticides (Rand and Petrocelli, 1985; Rand, 1995). Among the aquatic organisms, fishes occupy an important position in the field of aquatic toxicology (Di Giullio and Hinton, 2008). The fish *P. lineatus* (Order Characiformes) is a Neotropical species that has gained increasing commercial relevance in the south of Brazil because it is easily bred on fish farms (Winkaler et al., 2007). Toxicity tests with *P. lineatus* have shown that this species is sensitive to a variety of pesticides, and it is currently considered a potential bioindicator species (Langiano and Martinez, 2008; Pereira Maduenho and Martinez, 2008; Modesto and Martinez, 2010a).

Thus, the purpose of making an integrated analysis of the hematological and biochemical parameters of *P. lineatus* was to evaluate the possible effects of this clomazone-based herbicide (Gamit® 500) on *P. lineatus* (curimba), using biochemical and physiological parameters as indicators of toxicity.

2. Material and methods

2.1. Test organisms

Six months old juveniles of *P. lineatus* (Valenciennes, 1847), weighing 4.15 ± 1.78 g and with a total length of 7.82 ± 0.59 cm (mean ± standard deviation) were obtained from the Experimental Fish Farm of Londrina State University (EPUEL), Parana,

Brazil. The fish were acclimated for five days in 300-liter tanks containing dechlorinated water, under constant aeration and a 12-h light-dark cycle. During this acclimation period, the animals were fed with commercial fish food (36% protein content – Guabi®, BR) twice, after the first and the fourth day of acclimation. Fish were not fed during the toxicity tests. The physical and chemical parameters of the water were monitored daily using a multiparameter water quality monitor (Horiba U-50) and remained constant along the acclimation period (mean ± standard deviation): temperature: 22.25 ± 0.64 °C; pH: 7.12 ± 0.12; dissolved oxygen: 6.54 ± 0.03 mg O_2 L^{-1}; conductivity: 62 ± 4.32 μS cm^{-1}.

2.2. Acute toxicity tests

After the acclimation period fish were transferred to 100-liter glass aquaria filled with 80 L of water, divided into four groups of 10–12 animals per aquarium (in order to maintain a maximum density of 1 g of fish per liter of water): a control group (0 mg L^{-1}) which was exposed only to water, and three experimental groups which were exposed for a period of 96 h to water containing the formulated Gamit® 500 product with different nominal concentrations of the herbicide clomazone (1, 5, and 10 mg L^{-1}). The water in the aquariums was not changed during the period of the tests. Clomazone concentrations were defined based on previous studies on clomazone toxicity to other Neotropical species. Miron et al. (2005) exposed jundiá (*R. quelen*) to 1, 5, 10, 20 and 50 mg L^{-1} of clomazone for 96 h and Crestani et al. (2006, 2007) exposed the same fish species (*R. quelen*) to 0.5 and 1.0 mg L^{-1} for up to 192 h. These authors defined clomazone concentrations based on the herbicide concentrations usually recommended in rice fields, that is of 0.5–1.0 mg L (Rodrigues and Almeida, 1998). The physical and chemical parameters of the water were examined daily during the experimental period (mean ± standard deviation): temperature 23.10 ± 0.29 °C; pH 7.07 ± 0.25; OD: 6.86 ± 0.47 mg O_2 L^{-1}; conductivity: 64 ± 2.94 μS cm^{-1}).

2.3. Sampling

After the 96-h experimental period, the fish were anesthetized with benzocaine (0.1 g L^{-1}) and blood was drawn from the caudal vein, using heparinized syringes. Blood samples were stored refrigerated (4 °C) for the subsequent analysis of the hematological parameters. After collecting the blood, the animals were killed by medullary section and the liver and brain removed and immediately stored at −80 °C for subsequent enzyme analysis. The procedures were performed following to the protocol approved by the Ethics Committee on the Use of Animals of Londrina State University, according to the Brazil's National Council for the Control of Animal Experimentation.

2.4. Hematological analyses

Hematocrit (Hct) was determined by blood centrifugation (5 min, 1400 g) in heparinized glass capillaries, using a microhematocrit centrifuge (Luguimac S.R.L., Model LC 5, Argentina). The hemoglobin (Hb) concentration was estimated by the cyanmethemoglobin method, using a commercial kit (Labtest Diagnóstica, Brazil) and a spectrophotometer (Libra S32, Biochrom, U.K.) at 540 nm. The number of red blood cells per cubic millimeter of blood (RBC) was determined from samples of blood fixed in formalin citrate buffer (sodium citrate diluted in 0.4% formalin solution), using a Neubauer chamber and an optical microscope (400× magnification). These blood parameters were then used to derive the following hematimetric indices: mean corpuscular volume (MCV, fl), calculated as (Hct × 10)/RBC; mean corpuscular hemoglobin (MCH, pg), calculated as (Hb × 10)/RBC; mean corpuscular hemoglobin concentration (MCHC, %) calculated as (Hb × 100)/Hct.

2.5. Biochemical analyses

The samples of hepatic tissue were weighed and homogenized in potassium phosphate buffer (0.1 M, pH 7.0) in a proportion of 1:10 (w/v). They were then centrifuged (20 min, 10,000 g, 4 °C) and the supernatant was used for the analyses.

Catalase (CAT) activity was determined from the speed of decomposition of hydrogen peroxide (H_2O_2), based on the decrease in absorbance at 240 nm (Beutler, 1975). Enzyme activity was expressed in μmol of metabolized H_2O_2 min^{-1} mg $protein^{-1}$.

Glutathione peroxidase (GPx) activity was determined by the method of Hopkins and Tudhope (1973), based on the oxidation of NADPH + H^+ in the presence of hydroperoxide at 340 nm. The values of the activity of this enzyme were expressed in μmol of oxidized NADPH min^{-1} mg $protein^{-1}$.

GST activity was determined by monitoring the complexation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Keen et al., 1976). The values of GST activity were expressed in nmol of conjugated CDNB min^{-1} mg $protein^{-1}$.

Samples of brain tissue were homogenized in potassium phosphate buffer (0.1 M, pH 7.5) in a proportion of 1:10 (w/v). The homogenate was centrifuged (10.000 g, 20 min, 4 °C) and the supernatant used for the AChE assays by Ellman et al. method (1961) adapted for microplates, as described by Alves Costa et al. (2007). AChE activity was determined using the substrate acetylthiocholine iodide (9 mM) and 5,5′-dithiobis(2-nitrobenzoic acid) DTNB color reagent (0.5 mM) at 415 nm, using a multilabel plate reader (Victor^3, PerkinElmer, USA). AChE activity was expressed in nmol min^{-1} mg $protein^{-1}$.

The protein concentration of all the homogenates was determined in a spectrophotometer at 700 nm, according to the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard, and was expressed in mg L^{-1}.

2.6. Statistical analysis

The mean values obtained for each variable analyzed at the different experimental concentrations were compared to each other by parametric analysis of variance (ANOVA), after performing a normality test (Shapiro–Wilk test). Whenever necessary, the differences were identified by the Student–Newman–Keuls multiple comparison test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Hematological parameters

A significant decrease in Hct ($P = 0.006$) was observed at the concentration of 10 mg L^{-1} in comparison with the other groups (Fig. 1A), and in Hb ($P \leq 0.001$) at concentrations of 5 and 10 mg L^{-1} in comparison with groups 0 and 1 mg L^{-1} (Fig. 1B). The RBC showed a significant increase ($P = 0.024$) only at the concentration of 1 mg L^{-1} in group 0 mg L^{-1} (Fig. 1C), while the MCV remained unchanged ($P = 0.455$) (Fig. 1D). However, HCM and MCHC decreased significantly ($P = 0.001$ and $P \leq 0.001$, respectively) at concentrations of 5 and 10 mg L^{-1} in comparison to groups 0 and 1 mg L^{-1} (Fig. 1E and F).

3.2. Biochemical parameters

The results obtained for hepatic GST indicated a significant increase ($P \leq 0.001$) in the activity of this enzyme in the animals exposed to clomazone concentrations of 5 and

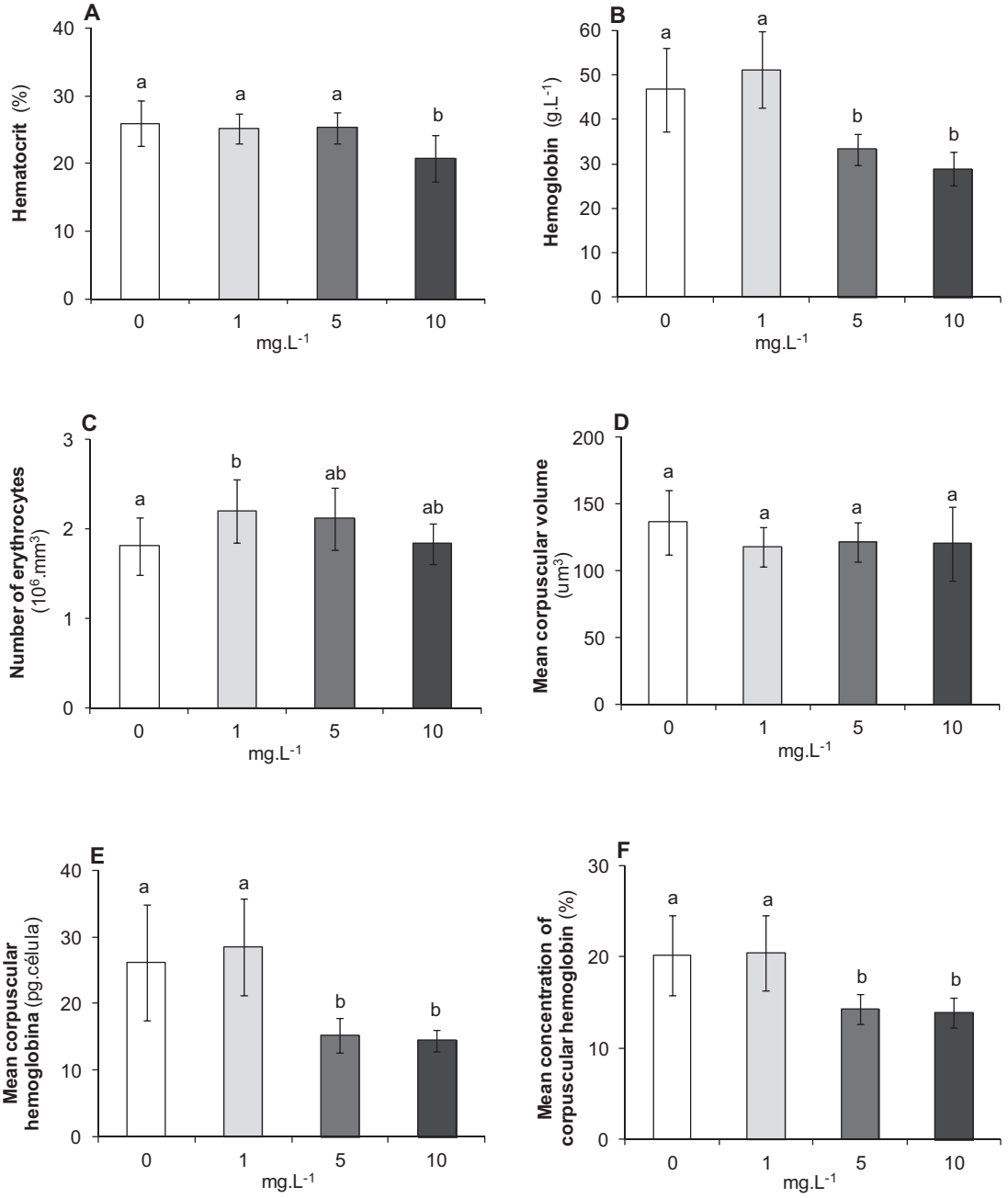

Fig. 1 – Mean values and standard deviation of hematocrit (A), hemoglobin content (B), number of erythrocytes (C), mean corpuscular volume (D), mean corpuscular hemoglobin (E) and mean concentration of corpuscular hemoglobin (F) of *P. lineatus* exposed to different concentrations (1, 5 and 10 mg L^{-1}) of clomazone or only to water (0 mg L^{-1}) for 96 h. Different letters indicate significant difference ($P < 0.05$).

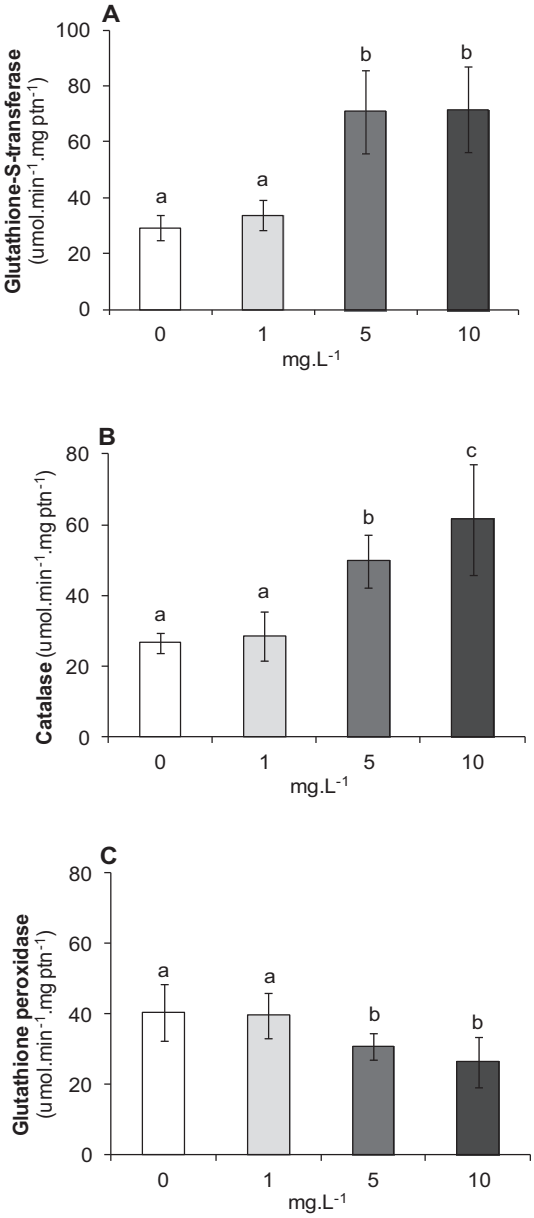

Fig. 2 – Mean values and standard deviation of hepatic activity of glutathione-S-transferase (A), catalase (B) and glutathione peroxidase (C) of *P. lineatus* exposed to different concentrations (1, 5 and 10 mg L^{-1}) of clomazone or only to water (0 mg L^{-1}) for 96 h. Different letters indicate significant difference ($P < 0.05$).

10 mg L^{-1} in relation to groups 0 and 1 mg L^{-1} (Fig. 2A). CAT also increased significantly ($P \leq 0.001$) at concentrations of 5 and 10 mg L^{-1}; this increase differed at these two concentrations as well as in comparison to groups 0 and 1 mg L^{-1} (Fig. 2B). GPx activity was significantly reduced ($P = 0.004$) at the concentrations of 5 and 10 mg L^{-1} when compared with groups 0 and 1 mg L^{-1} (Fig. 2C). The results revealed significant inhibition ($P \leq 0.001$) of brain AChE activity in fish exposed to clomazone concentrations of 5 and 10 mg L^{-1} for 96 h in relation to groups 0 and 1 mg L^{-1} (Fig. 3).

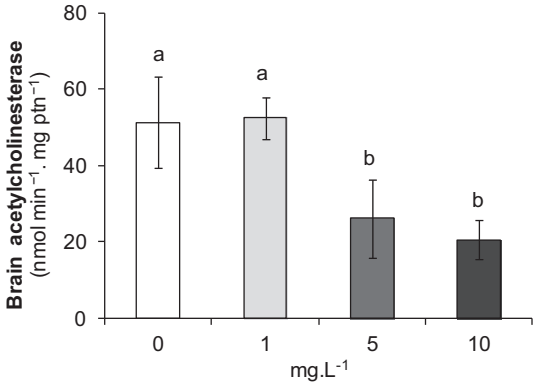

Fig. 3 – Mean values and standard deviation of brain acetylcholinesterase (A) of *P. lineatus* exposed to different concentrations (1, 5 and 10 mg L^{-1}) of clomazone or only to water (0 mg L^{-1}) for 96 h. Different letters indicate significant difference ($P < 0.05$).

4. Discussion

Hematological parameters are potential biomarkers of exposure to agrochemicals due to their sensitivity to certain toxic agents (Heath, 1995). In the present analyses, the fish exposed to clomazone presented a significant decrease in Hct at the concentration of 10 mg L^{-1} in comparison to the groups tested at the concentrations of 0, 1 and 5 mg L^{-1}. The content of hemoglobin (Hb), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) also showed significantly lower values at concentrations of 5 and 10 mg L^{-1} than group 0 mg L^{-1} and the experimental group subjected to the concentration of 1 mg L^{-1}. Clomazone has also been reported to cause Hct to decrease in the Neotropical fish species *R. quelen* exposed to concentrations of 0.5 and 1 mg L^{-1} for 96 h (Crestani et al., 2006). Since no significant changes were found in the red blood cell (RBC) count at concentrations of 5 and 10 mg L^{-1}, it is possible that the quantity of hemoglobin in the erythrocytes decreased (Heath, 1995). These results express a probable condition of anemia, since it is known that many chemical pollutants, including agrochemicals, can induce anemia in fish (Heath, 1995; Min and Kang, 2008). This anemia may be due to ROS-induced oxidative injury via oxidation of hemoglobin or other cellular components (Bloom and Brandt, 2008). However, the RBC count increased significantly at the lowest concentration (1 mg L^{-1}) when compared with the other groups. An increase in this parameter may occur in situations of acute stress, when the adrenergic stimulus triggers splenic contraction, releasing large quantities of red blood cells into the bloodstream (Heath, 1995). The results of the present study indicate that clomazone causes hematological changes that can interfere in oxygen uptake, which may jeopardize the animal's overall health.

The liver is the main detoxification site in fishes (Hinton et al., 2008). The process of detoxification of toxic agents requires a series of reactions that involve biotransformation enzymes such as GST and antioxidants such as CAT and GPx. The GST group of enzymes catalyzes the conjugation of GSH with a variety of endogenous compounds, which are less

permeable to the membranes and more easily eliminated, by increasing their hydrophilicity (Heath, 1995; Di Giullio and Hinton, 2008). Therefore, the work of these enzymes prevents oxidative damage in cellular components (Van der Oost et al., 2003).

The results of this study demonstrate that GST activity was higher in fish exposed to 5 and 10 mg L^{-1} of clomazone than in groups 0 and 1 mg L^{-1}. Augmented GST activity has been associated with an adaptation of the organism to a variety of organic compounds in the environment (Van der Oost et al., 2003). A significant increase in hepatic GST activity has also been reported in *P. lineatus* exposed to 10 mg L^{-1} of the herbicide Roundup® for periods of 24–96 h (Modesto and Martinez, 2010a). The data obtained in the present study indicate the induction of phase II of the detoxification process and the action of the enzyme GST in the metabolization of the herbicide clomazone. These results are corroborated by Menezes et al. (2011) who showed that longer exposure (8 days) to two clomazone concentrations (0.45 and 0.91 mg L^{-1}) increased GST activity in liver of *R. quelen*, reinforcing the GST role in biotransformation process helping fish tissues to detoxify clomazone.

The elimination of hydroperoxides is part of the cellular defense mechanism against oxidative stress and consequent lipid peroxidation (Van der Oost et al., 2003). CAT and GPx are enzymes that play an important role in the inhibition and/or prevention of oxidative damage (Hermes-Lima, 2004). CAT acts specifically in the elimination of H_2O_2, producing O_2 and H_2O, while GPx catalyzes the reduction of a variety of organic hydroperoxides, among them H_2O_2. In the present study, significantly augmented CAT activity was found at clomazone concentrations of 5 and 10 mg L^{-1} when compared with groups 0 and 1 mg L^{-1}. Similar results have been reported for piava (*L. obtusidens*) after 30 days of exposure to 0.5 mg L^{-1} of the same herbicide (Moraes et al., 2007). However, an experiment with *R. quelen* exposed to 0.5 and 1.0 mg L^{-1} of clomazone for 12, 24, 48 and 96 h produced a different result (Crestani et al., 2007), indicating that different species respond differently to the same contaminant. The increase in CAT activity found in this study indicates the intense production of H_2O_2, probably originating from the metabolization of clomazone, as a defense of the organism against oxidative damage, since this enzyme acts specifically on H_2O_2. Because catalase is a peroxisomal enzyme, its augmented activity may also reflect the proliferation of peroxisomes due to the activation of antioxidant pathways (Syakalima et al., 2006). The decreased GPx activity in the liver of *P. lineatus* exposed for 96 h to clomazone concentrations of 5 and 10 mg L^{-1} may be ascribed to the depletion of GSH substrate used in the conjugation reactions catalyzed by GST (whose activity was augmented at these concentrations). It may also be attributed to the competition of GPx and CAT for the same substrate (H_2O_2), since both act in the conversion of hydrogen peroxide (Dröge, 2002) and CAT activity increased at the higher concentrations, i.e., it was active on the H_2O_2 substrate.

Some pesticides such as organophosphates and carbamates are known as powerful inhibitors of acetylcholinesterase (Fulton and Key, 2001; Van der Oost et al., 2003; Oruç and Usta, 2007). The inhibition of AChE activity prevents the hydrolysis of acetylcholine, causing excessive stimulation of the cholinergic pathways, and hence, decreased neural and muscle control (Dutta and Arends, 2003). In the present study, AChE activity in the brain tissue of fish exposed to clomazone was lower at concentrations of 5 and 10 mg L^{-1} than in the fish of groups 0 and 1 mg L^{-1}. A study of *R. quelen* exposed for 96 h to different concentrations of the herbicide clomazone (5, 10 and 20 mg L^{-1}) also found inhibition of AChE (Miron et al., 2005). In a subsequent study, the same species was sensitive to lower concentrations of the product, presenting inhibition of brain and muscle AChE when exposed to 0.5 and 1.0 mg L^{-1} in different experimental periods (Crestani et al., 2007). *L. obtusidens* has also been reported to show significantly reduced AChE activity in brain tissue when exposed to a clomazone concentration of 0.5 mg L^{-1} for periods of 96 and 192 h (Moraes et al., 2007; Miron et al., 2008).

Studies have shown that the inhibition of AChE may lead to altered behavior such as hyperactivity, tremors, convulsions, and lethargy, and may even lead to death (Miron et al., 2005; Rahimi and Abdollahi, 2007). These alterations are related with higher energy expenditure by the animal (Rahimi and Abdollahi, 2007). The fish species *P. lineatus* is a long-distance migrator that travels hundreds of kilometers to its spawning sites (Sivasundar et al., 2001), which requires energy and good neuromuscular conditions. AChE inhibitors such as clomazone can interfere in the reproductive process of this species, because, by affecting the cholinergic pathways, it can also impair muscle control. The present study demonstrates that clomazone interferes in AChE activity by promoting its inhibition, thus potentially affecting the health of the animal. Therefore, this enzyme can be indicated as a biomarker to evaluate the toxicity of this herbicide in aquatic environments.

5. Conclusions

The findings of this study demonstrate that exposure for 96 h to the herbicide clomazone at concentrations of 5 and 10 mg L^{-1} can lead to important alterations in the hematological and biochemical parameters of *P. lineatus*, as well as being neurotoxic to this species. The data obtained here indicate that the presence of clomazone in aquatic environments may be harmful to the health of *P. lineatus* and probably also to other aquatic organisms.

Conflicts of interest

Nothing declared.

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