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Biomarker evaluation in jundiara (*Leiarius marmoratus* x *Pseudoplatystoma reticulatum*) after exposure to an agrochemical used on soybean crops

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Abstract: We aimed to investigate the effects of acute exposure to Roundup Original® on the oxidative stress biomarkers, antioxidant defenses and acetylcholinesterase (AChE) of the hybrid fish jundiara (*Leiarius marmoratus* x *Pseudoplatystoma reticulatum*). The fish were exposed to different herbicide concentrations (10%, 20% and 40%) for 96 hours based on the pre-determined LC_{50}. The thiobarbituric acid-reactive substances (TBARS), protein carbonyls and other antioxidant responses were assessed. None of the animals died during experiments. TBARS levels in the liver, brain and muscle increased when fish were exposed to the herbicide, even though protein carbonyl content increased only in the muscle and brain. Among the antioxidant activities, superoxide dismutase decreased in the liver and brain, while catalase decreased in the liver and increased in the brain after exposure to the herbicide. Reduced levels of glutathione (GSH) occurred in the liver and brain, as a response against the toxic agent. Decreased AChE was found in both the brain and muscle. In the plasma analysis, glucose decreased but cholesterol and AST levels increased after exposure. It was observed that Roundup Original® may lead to deleterious changes in the parameters tested, however the responses varied in the tested tissues.

Keywords: aquatic biota, environment, hybrid fish, Roundup Original®, toxic event

Resumo. Avaliação de biomarcadores em jundiara (Leiarius marmoratus x Pseudoplatystoma reticulatum) após exposição a um agroquímico utilizado nas lavouras de soja. Investigamos os efeitos da exposição aguda ao Roundup Original® no peixe híbrido jundiara (Leiarius marmoratus x Pseudoplatystoma reticulatum). Os peixes foram expostos a diferentes concentrações do herbicida (10%, 20% e 40%) por 96 horas com base na LC_{50}, pré-determinada. Foram avaliadas as substâncias reativas ao ácido tiobarbitúrico (TBARS), proteínas carboniladas e outras respostas antioxidantes, bem como a acetilcolinesterase (AChE). Nenhum dos animais morreu durante os experimentos. Os níveis de TBARS no fígado, cérebro e músculo aumentaram quando os peixes foram expostos ao herbicida, embora o teor de proteína carbonil tenha aumentado apenas no músculo e no cérebro. Entre as atividades antioxidantes, a superóxido dismutase diminuiu no fígado e no cérebro, enquanto a catalase diminuiu no fígado e aumentou no cérebro após a exposição ao herbicida. Níveis reduzidos de glutationa (GSH) ocorreram no fígado e no cérebro, como resposta ao agente tóxico.

Diminuição de AChE foi observada tanto no cérebro quanto no músculo. Na análise de plasma, a glicose diminuiu, mas os níveis de colesterol e AST aumentaram após a exposição. Infere-se que o Roundup Original® pode levar a alterações deletérias nos parâmetros testados, porém as respostas variaram dependendo dos tecidos avaliados.

Palavras-Chaves: biota aquática, meio ambiente, peixe híbrido, Roundup Original®, evento tóxico.

Introduction

In the present decade, agribusiness is one of the main economic activities in Brazil, as well as one of the most consolidated, accounting for around 23% of GDP, and generates approximately 350 billion dollars annually (Molin 2017). A significant proportion of this amount can be attributed to the state of Mato Grosso, the largest national producer of grains and other agricultural commodities (MAPA 2017). This comes hand in hand with intensive pesticide use. For soybean crops, the main pesticide used is glyphosate, the first-choice herbicide for weed control in genetically modified crops (Puértolas *et al.* 2010). The exacerbated use of such a product causes resistance in weeds, unbalancing and reducing biodiversity (Moreira *et al.* 2012). The Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) points out that during the period 2000-2004, there was an 87.4% growth in the fifteen major herbicides used in soybean cropping in Mato Grosso, ranging from 7.5 thousand to 14 thousand tons. Glyphosate use increased up to 94% (AgroNotícias 2007).

Contamination by xenobiotic compounds has been rising alarmingly in aquatic ecosystems as a result of anthropogenic activities in recent years. In this context, given the constant exposure of this medium to a large number of toxic substances from several sources, aquatic biota becomes increasingly important in detecting the degree of impact (Sauco *et al.* 2010). Depending on the level of impact and exposure time, toxic substances interact with living organisms in aquatic environments, including fish, causing numerous changes and severe ecological imbalances (Dang *et al.* 2016).

In addition, indiscriminate fishing has been reducing the occurrence of native species in Brazil, leading to a decrease in their trade. As a response to this problem, fish farmers have invested in the hybridization of fish (Gomes & Sato 2010). This technique aims to produce animals with better physical and marketing performance than their parentals (Helfman *et al.* 2009). The hybrid fish, commonly called jundiara or *pintado-da-Amazônia*, is a result of the artificial crossing between two large species of neotropical catfish – the painted jundiá (*Leiarius marmoratus*) and cachara (*Pseudoplatystoma reticulatum*) (Ventura *et al.* 2013). This hybrid is considered an important fish of great commercial interest due to its carcass yield and pleasant taste.

Considering that fish farms are commonly found in agricultural regions, and that during the planting of various crops there is intensive pesticide use within areas close to the rivers and streams that supply the tanks in fish farms, fish exposure to these chemicals may lead to the contamination and accumulation of these substances, resulting in provoked pathologies (Moura *et al.* 2017a,b). Based on the high index of herbicide use in the state of Mato Grosso, this study aimed to describe the effects of Roundup® on some biochemical and metabolic parameters of the hybrid fish jundiara, using from different rates of exposure.

Materials and Methods

Fish: Juvenile animals (60.0 ±7.0 g and 16.0 ±2.0 cm) were purchased from a fish farm in Sinop, Mato Grosso, Brazil. Fish were distributed in 300-L fiberglass tanks containing aerated and dechlorinated water, under natural photoperiod conditions (12 h light / 12 h dark) for 10 days to acclimate to laboratory conditions before the experiment. During this stage the fish were fed once per day with fish pellets containing approximately 42% crude protein. The water quality parameters were measured as following: temperature 28 ±1.0 ºC, pH 6.7 ±0.3, dissolved oxygen 6.35 ±0.5 mg L^{-1}, hardness 17 ±1.0 $CaCO_3$, nonionized ammonia 0.06 ±0.02 and nitrite 0.07 ±0.01 mg L^{-1}. Pellet residue and feces were removed by suction every other day.

Chemicals: For our experiments, the agrochemical used was the glyphosate-based herbicide Roundup Original® (480 g L^{-1} containing isopropylamine salt of glyphosate, 360 g L^{-1} acid equivalent, N-(phosphonomethyl) glycine (glyphosate) and 684 g L^{-1} of inert ingredients), Monsanto, St. Louis, MO, USA). Bradford reagent, bovine serum albumine, hydrogen peroxide (H_2O_2), sodium dodecyl sulfate

(SDS), reduced glutathione, trichloroacetic acid (TCA), Trizma® hydrochloride solution, 1-chloro-2,4-dinitrobenzene (CDNB), Triton X-100, 2-thiobarbituric acid (TBA), monobasic potassium phosphate, dibasic potassium phosphate, monobasic sodium phosphate, dibasic sodium phosphate, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), 5,5'-dithio-bis(2-nitro-benzoic acid) (DTNB), malondialdehyde (MDA), 2,4-dinitrophenylhydrazine (DNPH) and the other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) at a high purity percentage (95-99%).

Experimental design: Animals were exposed to different concentrations of the herbicide to obtain the lethal dose as LC_{50} at 13.57 mg L^{-1} of Roundup Original®(glyphosate nominal concentration), which was estimated by the trimmed Sperman-Karber method as described by Hamilton *et al.* (1977). For this experiment we chose the concentrations equivalent to 10%, 20% and 40% of LC_{50}. After the acclimation period, fish were distributed among four different groups (0, 10%, 20% and 40%) for 96 hours. The fish were placed in 50 L tanks with a total of 4 fish per tank. Water characteristics were the same as described for the acclimation phase and did not change during the experimental period. Fish were kept in a static system. All tests were carried out in duplicate and the fish did not receive food during the experimental period, in accordance with Aguiar et al., 2004. Stock solution was prepared by dissolving Roundup Original®in water and then added to the experimental tanks. Following exposure, animals were removed from the tanks, anesthetized with eugenol (50 mg L^{-1}) (Kreutz *et al.*, 2011) and killed by medular section. Samples of liver, brain and muscle tissue were removed by dissection. The samples were stored at -80 ºC for posterior biochemical assays. This study was approved by the Committee Guidelines (Ethics in Animal Research of the *Universidade Federal de Mato Grosso*, reference number: 23108.780290/11-5).

Biochemical analysis: Oxidative stress parameters - Thiobarbituric acid reactive substances (TBARS) and protein carbonyl levels (PC): Liver and muscle samples were homogenized in 20 mM PPB (potassium phosphate buffer), pH 7.5 for lipid peroxidation (LPO) analysis, centrifuged at 1000 g at 4 ºC and and the supernatant removed after centrifugation for examination. LPO in liver and muscle was estimated by spectrophotometrical determination of the thiobarbituric acid reactive substances (TBARS) levels. This method was described by Buege & Aust (1978) and TBARS concentration was expressed in nmol MDA mg $protein^{-1}$ following the calibration curve for MDA. For protein carbonyl analysis, liver and muscle samples were homogenized in 10 mM Tris-HCl buffer (pH 7.4). The protein carbonyl (PC) content in liver and muscle was determined by spectrophotometry after DNPH derivation according to Yan et al. (1995), with some modifications. The total carbonyl content was assessed using a molar extinction coefficient of 22.000 M^{-1} cm^{-1} and expressed as nmol carbonyl mg $protein^{-1}$.

Enzymatic and nonenzymatic antioxidants: Superoxide dismutase (SOD) activity in liver and brain tissue was measured according to Misra & Fridovich (1972). Catalase (CAT) activity in liver and brain tissue was measured according to Nelson & Kiesow (1972). Reduced glutathione (GSH) levels were quantified following the method of Sedlak & Lindsay (1968).

Acetylcholinesterase assay: Acetylcholinesterase (AChE) activity in muscle and brain tissue was measured using the method as described by Ellman *et al.* (1961). Brain and white muscle tissue were homogenized in 50 mM sodium phosphate buffer pH 7.2, containing 1% Triton X-100, and after centrifugation the supernatant was removed for analysis. The supernatant was incubated at 25 ºC for 2 min with 100 mM phosphate buffer pH 7.5, and 10 mM DTNB was used as chromogen. The substrate, 1 mM acetylthiocholine (ASCh), was added after 2 min to promote the reaction. Absorbance was measured at 412 nm for a duration of 2 min and the activity was expressed as µmol acetylthiocholine hydrolyzed min^{-1} mg of $protein^{-1}$.

Protein determination: Protein content was estimated by spectrophotometry according to Bradford (1976) by using bovine serum albumin as a standard. Absorbance of the samples was measured at 595 nm.

Plasma metabolic parameters: Following collection, blood samples were centrifuged and the plasma removed and stored at -80 ºC for posterior analysis. Plasma glucose was analyzed by colorimetric commercial kit based on the glucose oxidase method, while cholesterol was determined using enzymatic commercial kits based on esterase-oxidase reactions. Moreover, the aspartate transaminase (AST) activity was assigned through kits based on Ultraviolet-International Federation of Clinical Chemistry and Laboratory Medicine (UV-IFCC) kinetic reactions.

Data analysis: Data was presented as mean ±standard deviation (n = 7-8) by one-way analysis of variance (ANOVA), followed by a *Tukey's* test. Statistical significance was accepted at $P < 0.05$. A Bartlett's test was performed to compare the homogeneity of variances among the groups.

Results

We observed changes in most of the parameters evaluated in the fish and in different tested concentrations of the herbicide after 96 hours of exposure. There was a significant increase in liver and muscle TBARS levels (only at the 40% concentration) and in the brain (for all tested concentrations) (Table 1). Protein carbonyl increased in muscle tissue at a concentration of 20%, whereas in the fishes' brain the increase occurred at concentrations of 10 and 40% (Table 1). In liver tissue there was no change in this biomarker (Table 1). GSH levels increased in the liver and brain of fish at concentrations of 10 and 40%, similar to protein carbonyl (Table 1). SOD activity decreased in the liver at all exposure concentrations when compared to the control group (Fig. 1). For brain tissue this reduction occurred only at the lowest concentration (Fig. 2). CAT showed decreased hepatic activity at all exposure concentrations (Fig. 3); whereas in brain tissue its activity increased only at 20% herbicide exposure (Fig. 4). Muscle AChE activity decreased at concentrations of 10 and 40% (Fig. 5a), however activity within the brain decreased only at 10% concentration (Fig. 5b). In addition, glucose decreased at plasma concentrations of 10 and 20%, while plasma cholesterol increased at these same concentrations of the herbicide (Table 2). Aspartate transaminase (AST) increased only at concentrations of 10 and 40% (Table II).

Discussion

Jundiara is a hybrid fish with important commercial appeal in the northern region of Mato Grosso, and previous results have already shown its sensitivity to the toxic action of the Roundup Original® herbicide (Moura *et al.* 2017a). Based on these facts, the aim of this study was to compare the effects of three sublethal concentrations of the herbicide on the biomarkers of oxidative stress and some metabolic parameters. The concentrations used may be considered environmentally relevant as repeated applications of this herbicide may induce higher concentrations within the aquatic environment, as mentioned by Nwani *et al.* (2013), and similar to those used in the study of Dutra *et al.* (2011), whose values varied between 0.36 and 2.16 mg L^{-1} for 7 days of exposure on the crustacean *Hyalella castroi*, and also Topal *et al.* (2015), who exposed *Oncorhynchus mykiss* to 2.5, 5 and 10 mg L^{-1} of glyphosate for periods of 6 to 96 hours. Lipid peroxidation (LPO) and protein carbonylation are phenomena frequently observed during the manifestation of oxidative stress (Sinhorin *et al.* 2014a, Loro *et al.* 2015). Increased levels of TBARS (liver and brain) and PC (brain) in fish in this study are in accordance with the results of Sinhorin *et al.* (2014a) and Moura *et al.* (2017a).

Table I. Liver and brain content of GSH (µmol GSH mg protein^{-1}), liver, muscle and brain content of TBARS (nmol MDA mg protein^{-1}) and liver, muscle and brain levels of protein carbonyl (PC, nmol carbonyl mg protein^{-1}), in jundiara exposed to different concentrations of Roundup Original® for 96 h.

	Liver			
	Control	10%	20%	40%
GSH	5.97 ± 0.60	7.29 ± 0.60*	6.86 ± 1.10	8.85 ± 0.93*
TBARS	1.38 ± 0.21	1.63 ± 0.41	1.58 ± 0.36	1.83 ± 0.23*
PC	6.07 ± 1.05	6.16 ± 1.09	5.31 ± 0.71	5.56 ± 0.78
	Muscle			
	Control	10%	20%	40%
TBARS	0.56 ± 0.07	0.53 ± 0.08	0.53 ± 0.12	0.71 ± 0.15*
PC	3.76 ± 0.71	2.94 ± 0.64	6.39 ± 0.67*	3.29 ± 0.38
	Brain			
	Control	10%	20%	40%
GSH	6.74 ± 1.10	8.99 ± 1.69*	8.07 ± 1.63	9.24 ± 1.80*
TBARS	3.44 ± 0.68	4.74 ± 0.47*	4.68 ± 0.68*	5.07 ± 1.05*
PC	1.55 ± 0.30	2.27 ± 0.59*	1.73 ± 0.32	2.26 ± 0.44*

Data represent the mean ±S.D (n = 7-8). Asterisks indicate a difference between groups and control values (ANOVA followed by Dunnett's test) $P < 0.05$.

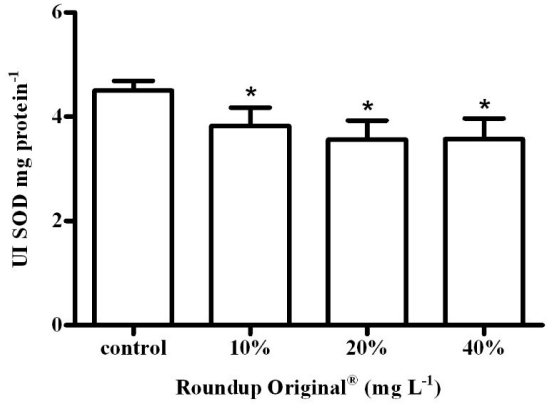

Figure 1. SOD activity in liver of jundiara exposed to different concentrations of Roundup Original® or only water (control) for 96 h. Data are means ±standard deviation (n = 7). Asterisks indicate significant difference from respective control group ($P < 0.05$).

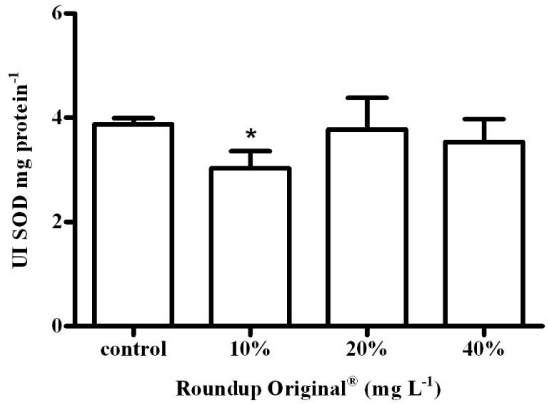

Figure 2. SOD activity in brain of jundiara exposed to different concentrations of Roundup Original® or only water (control) for 96 h. Data are means ±standard deviation (n = 7). Asterisks indicate significant difference from respective control group ($P < 0.05$).

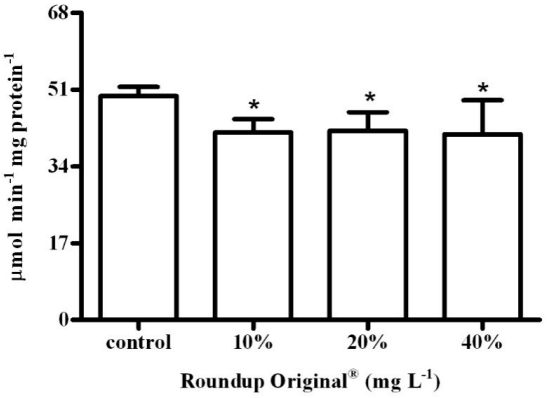

Figure 3. CAT activity in liver of jundiara exposed to different concentrations of Roundup Original® or only water (control) for 96 h. Data are means ±standard deviation (n = 8). Asterisks indicate significant difference from respective control group ($P < 0.05$).

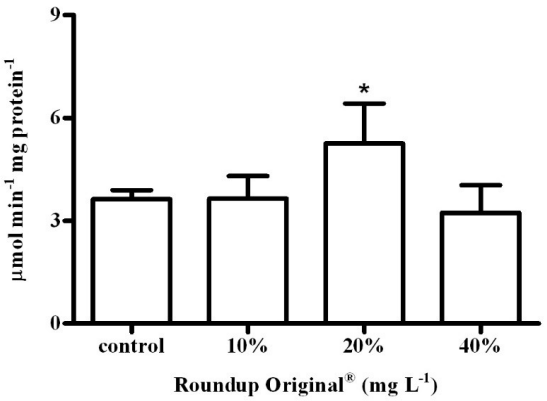

Figure 4. CAT activity in brain of jundiara exposed to different concentrations of Roundup Original® or only water (control) for 96 h. Data are means ±standard deviation (n = 7). Asterisks indicate significant difference from respective control group ($P < 0.05$).

Costa *et al.* (2008) reported that the occurrence of lipid peroxidation may be a direct consequence of the impairment of SOD and CAT enzyme activity, a scenario in which the body's ability to detoxify reactive species is compromised (Nwani *et al.* 2013). In addition, Glucszak *et al.* (2011) reported similar results for *Leporinus obtusidens* at concentrations of 3 to 20 mg L^{-1} of this same herbicide for the same period of time (96 h), and also reported increased TBARS and PC in the liver of that species.

Regarding GSH, this substance is considered an important non-enzymatic antioxidant defense in the body, by combining several xenobiotics and facilitating their excretion, as well as being a catalytic substrate for the enzymes glutathione peroxidase (GPx) and glutathione-S-transferase (GST) (Haluzová *et al.* 2011)

The increase in GSH levels in the present study has previously been observed by other authors after experiments with herbicides containing glyphosate (Ferreira *et al.* 2010, Sinhorin *et al.* 2014a, Moura *et al.* 2017a). In view of the findings of this experiment, this elevation suggests a possible protective reaction, since when increased, GSH represents a timely response to an intoxication event, due to its purpose of preventing cellular damage (Sinhorin *et al.* 2014a).

Reduced activity of SOD (liver and brain) and CAT (liver) enzymes has previously been documented in a study by Moura *et al.* (2017a) with

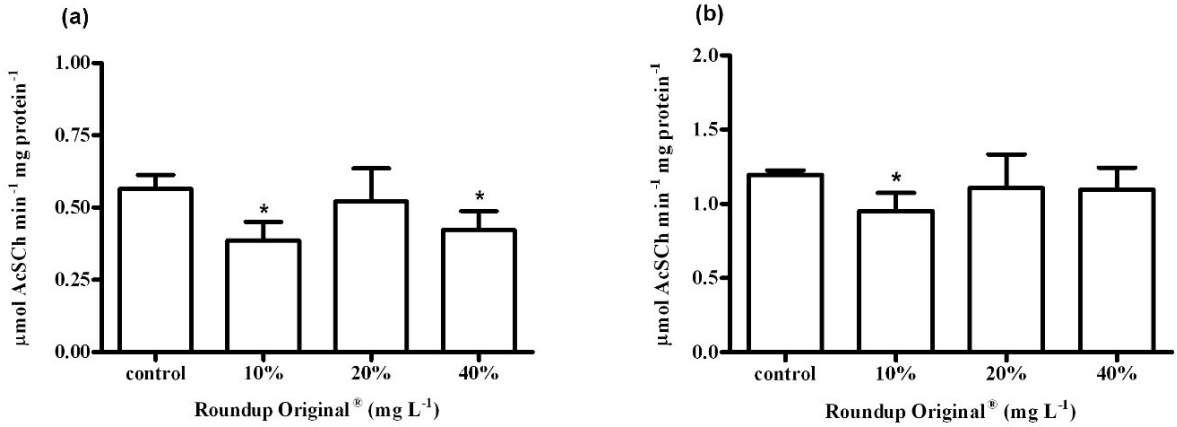

Figure 5. AChE activity in muscle (a) and brain (b) of jundiara exposed to different concentrations of Roundup Original® or only water (control) for 96 h. Data are means ±standard deviation (n = 7). Asterisks indicate significant difference from respective control group ($P < 0.05$).

Table II. Plasma levels of glucose (GLU, mg dL^{-1}), cholesterol (CHOL, mg dL^{-1}) and aspartate transaminase (AST, U L^{-1}) in jundiara exposed to different concentrations of Roundup Original® for 96 h.

	Plasma			
	Control	10%	20%	40%
GLU	79.22 ± 17.39	48.46 ± 11.92*	58.02 ± 13.28*	77.28 ± 14.85
CHOL	119.10 ± 10.71	141.00 ± 14.71*	140.00 ± 18.48*	134.40 ± 10.15
AST	110.40 ± 19.40	162.90 ± 36.75*	144.00 ± 26.70	156.00 ± 37.80*

Data represent the mean ± S.D (n = 8). Asterisks indicate a difference between groups and control values (ANOVA followed by Dunnett's test) $P < 0.05$.

this same species, as well as by other authors when working with the same herbicide in other fish species (Lushchak *et al.* 2009, Modesto & Martinez 2010, Sinhorin *et al.* 2014a). The increase of free radicals flowing from the intoxication event has already been considered responsible for deficits in the antioxidant system, compromising the performance of these enzymes (Modesto & Martinez 2010). Meanwhile, the induction of brain catalase activity is a probable detoxification response to the overproduction of peroxide radicals promoting a compensation against the neurotoxicity generated, as reported by Dey *et al.* (2016) by exposing teleost fish to 1.2 mg L^{-1} of a herbicide containing glyphosate.

Animals exposed to Roundup Original® also showed reduced activity of the acetylcholinesterase enzyme, a phenomenon similarly verified by Modesto & Martinez (2010) when exposing *Prochilodus lineatus* to 1 or 5 mg L^{-1} of Roundup Transorb®, and Cattaneo *et al.* (2011) who studied the effects of four different concentrations (0.5, 2.5, 5 and 10 mg L^{-1}) of Roundup on *Cyprinus carpio*. This decrease causes accumulation of acetylcholine, with subsequent stimulation of its receptors, causing changes in the neurotransmission process as well as leading to behavioral changes and even death (Menéndez-Helman *et al.* 2012). These results are also in line with those of Moura *et al.* (2017a), in which this same species presented reduced AChE activity when compared to the control group after being exposed to 1.357 mg L^{-1} of Roundup Original® for different periods of time. Rodrigues *et al.* (2013) mentioned that disturbances to the activity of this enzyme can be used as biomarkers of the indication of intoxication in fish by organophosphates or insecticides based on carbamate.

Among the parameters evaluated in plasma, glucose was the only variable that decreased. A similar situation has already been observed by several authors (Glusczak *et al.* 2006, Sinhorin *et al.* 2014b, Loro *et al.* 2015, Moura *et al.* 2017a) and is a probable indication of fish hyperexcitability during the intoxication process, promoting the use of

glucose resources in blood for rapid energy and life support. According to Martínez-Porchas *et al.* (2009), some chemicals can affect metabolic pathways leading to alterations in the tissues functions, even though such responses may vary. Furthermore, raised cholesterol levels, an essential structural component of membranes and the precursor of all steroid hormones, reinforce the assumption that intoxication processes may cause the release of cholesterol into the blood due to liver and kidney failure (Firat *et al.* 2011). Finally, the increase in AST enzyme corroborates the hypothesis that Roundup Original® is potentially toxic to fish liver. This enzyme plays an important role in the indication of cellular damage, and this variation allows the proposition that it is an interesting indicator of glyphosate intoxication, since this same scenario was verified by Loro *et al.* (2015) with two species of fish (*Rhamdia quelen* and *Leporinus obtusidens*) when submitted to 0.2 or 0.4 mg L^{-1} of Roundup.

Our results indicate that the herbicide Roundup Original® is able to induce significant changes in the markers of oxidative stress and also in some metabolic parameters of the species evaluated in the laboratory, in concentrations pertinent to environmental levels. Thus, and in addition to other works of this same theme, the deleterious effects of this substance are exposed here to non-target organisms, indicating the need for their conscious and relevant management and use in order to obtain the proposed outcome, with no harm in the short or long term.

Conclusively, Roundup Original® caused significant deleterious effects on these aquatic vertebrates. Our findings are supported by alterations observed in the oxidative stress biomarkers, as well as AChE and AST, showing an accute toxic response. Through the above-mentioned data we point that short-term exposure to environmentally realistic concentrations of this herbicide can be harmful to fish health. Future studies must be performed to clarify the physiological mechanisms involved in this process.

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