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Glyphosate on digestive enzymes activity in piava (*Leporinus obtusidens*)

Glifosato sobre a atividade de enzimas digestivas em piavas (*Leporinus obtusidens*)

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ABSTRACT

*The effects of glyphosate, a nonselective herbicide (1.0 or 5.0mg L^{-1}) on digestive enzymes activity (stomach and intestine) were evaluated in juveniles of piava (**Leporinus obtusidens**) after 90 days of exposure. The activity of acid protease, trypsin, chymotrypsin and amylase increased with the increase of glyphosate concentration. These results indicate that glyphosate affects digestive enzyme activities in this species, and may be an indicator of poor nutrient availability when fish survive in herbicide-contaminated water.*

Key words: *fish, herbicide, acid protease, amylase, trypsin.*

RESUMO

*Os efeitos do glifosato, um herbicida não seletivo (1,0 ou 5,0mg L^{-1}), sobre a atividade de enzimas digestivas (estômago e intestino) foram avaliadas em juvenis de piava (**Leporinus obtusidens**) após 90 dias de exposição. A atividade da protease ácida, tripsina, quimiotripsina e amilase aumentaram com a elevação da concentração de glifosato. Esses resultados indicam que o glifosato afeta a atividade de enzimas digestivas nesta espécie e pode ser indicador da reduzida disponibilidade de nutrientes, quando peixes sobrevivem em água contaminada com este herbicida.*

Palavras-chave: *peixe, herbicida, protease ácida, amilase, tripsina.*

INTRODUCTION

Glyphosate, chemically known as isopropylamine salt of N - phosphonomethyl glycine, is a post-emergent herbicide widely used in several types of cultures (MODESTO & MARTINEZ, 2010a,b) and in South America this herbicide is one of the most widely used following the introduction of glyphosate–resistant transgenic soybean (***Glycine max***) (SOSO et al., 2007). The most known worldwide commercial name for glyphosate is Roundup™ (GIESY et al., 2000).

The piava (***Leporinus obtusidens***) has great potential for fish culture due to its omnivorous feeding habit and ecological and economic importance to the Uruguay River Basin (REYNALTE-TATAJE & ZANIBONI FILHO, 2010). Studies demonstrated that exposure of fish to glyphosate leads to oxidative stress, inhibited brain and muscle acetylcholinesterase activity, cause metabolic changes and impairs growth (MODESTO & MARTINEZ, 2010a,b; SALBEGO et al., 2010; GLUSCZAK et al., 2011). Several studies indicate that growth rate in fish can be partially attributed to digestive capacity (BLIER et al., 2002; FILIPPOV et al., 2013). Therefore, the aim of this study was to verify the effect of chronic exposure to sublethal glyphosate concentrations on the activity of digestive enzymes of piava juveniles.

MATERIAL AND METHODS

Piava juveniles (mean ± SEM; weight = 8.0±0.5g and length = 5.0±1.0cm) were obtained

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from the Fish Culture Laboratory at the Universidade Federal de Santa Maria (UFSM). Fish were acclimated for ten days in 250L continuously aerated tanks with a static system and a natural photoperiod (14h light - 10h dark). After the acclimation period, fish were placed in 250L continuously aerated tanks (30 fish per tank) and exposed for 90 days to 0 (control), 1.0 or 5.0mg L^{-1} Roundup™ (48% acid equivalent of the isopropylamine salt of glyphosate, Monsanto Company, Saint Louis, MO, USA) diluted in water (two replicates). Roundup concentrations were chosen considering that the 0.36-2.16mg L^{-1} range is used in agriculture and based on the estimated half-life of glyphosate (RODRIGUES & ALMEIDA, 1998). The herbicide was reapplied in the tanks at 4-day intervals to maintain the expected concentration (50% of the water was renewed at these moments) Glyphosate and its breakdown product aminomethylphosphonic acid (AMPA) were monitored according to HIDALGO et al. (2004) through eight days (Table 1). Throughout acclimation and experimental period, fish were fed once a day to satiety with commercial fish feed (38% crude protein, Supra, Brazil). Feces and pellet residues were removed daily by suction. The water quality parameters were controlled daily: dissolved oxygen and temperature were measured with a YSI oxygen meter (Model Y5512). The pH was determined with a DMPH-2 pHmeter. Total ammonia nitrogen levels were measured with the salicylate method (VERDOUW et al., 1978). Water hardness was analyzed using the EDTA titrimetric method (EATON et al., 2005). The nitrite concentration and alkalinity were measured using the method of BOYD & TUCKER (1992). The parameters remained within the appropriate levels for the species (Table 2) (REYNALTE-TATAJE & ZANIBONI-FILHO, 2010).

Table 1 - Glyphosate concentration in the water of the experimental tanks through eight days.

----------Glyphosate 1 mg L^{-1} ----------		
Day	Glyphosate (mg L^{-1})	AMPA (mg L^{-1})
1	1.01± 0.001	1.05 ± 0.005
2	0.95 ± 0.05	1.02 ± 0.06
4	0.95 ± 0.004	0.95 ± 0.005
8	0.96 ± 0.01	0.97 ± 0.05
---------- Glyphosate 5 mg L^{-1} ----------		
Day	Glyphosate (mg L^{-1})	AMPA (mg L^{-1})
1	5.1 ± 0.002	5.05 ± 0.005
2	4.5 ± 0.05	4.4 ± 0.06
4	4.5 ± 0.005	4.65 ± 0.05
8	4.4 ± 0.04	4.55 ± 0.06

n = 3 for each tank; values are expressed as mean ± SEM.

Table 2 - Overall water quality parameters through 90 days of exposure of piava (***Leporinus obtusidens***) to different glyphosate levels (control, 1.0 or 5.0mg L^{-1}). Data are reported as the mean ± SEM. There was no significant difference between treatments.

Parameters	
Dissolved oxygen (mg L^{-1})	7.2 ± 0.2
Temperature (°C)	22 ± 0.5
pH	7.40 ± 0.05
Total ammonia (mg L^{-1})	0.05 ± 0.001
Hardness (mg $CaCO_3$ L^{-1})	32.0 ± 1.1
Nitrite (mg L^{-1})	0.06 ± 0.01
Alkalinity (mg $CaCO_3$ L^{-1})	39.0 ± 3.2

At the end of the experimental period (90 days) eight fish from each replicate were sampled and killed by section of the spinal cord. Stomach and intestine were sampled, the content removed and the tissues placed in liquid nitrogen for posterior analysis of digestive enzymes activity. In the stomach amylase and acid protease activities were determined and in the intestine amylase, trypsin and chymotrypsin activities. Amylase activity was assayed in 0.2M phosphate-citrate buffer, pH 7.0, 0.5% NaCl with a starch concentration of 2.5%. The reaction was stopped by adding Ba $(OH)_2$ 0.3N and $ZnSO_4$ 5%. The experimental protocol was modified according to BERNFELD (1955). The determination of starch hydrolysis was done following PARK & JOHNSON (1949). The absorbance was recorded at 660nm. One unit of enzyme was defined as 1mmol of glucose released from starch per min per mg of protein. Total acid protease activity was measured using non-specific substrate (casein) according to HIDALGO et al. (1999). The assay was carried out using 0.2M KCl buffer, pH 2.0. The absorbance of the enzyme extract was recorded at 280nm. All samples were assayed in duplicate and readings corrected for blank solutions. Tyrosine was used as standard, and one unit of enzyme was defined as the amount of enzyme needed to catalyze the formation of 1.0mg of tyrosine per min per mg protein, according to HIDALGO et al. (1999). Trypsin activity (E.C.34.21.4) was assayed with toluenesulphonyl-L-arginine methyl ester hydrochloride (TAME). Crude extracts were incubated for 2min (25ºC) in 2mL of Tris/$CaCl_2$ buffer, pH 8.1. Chymotrypsin activity (E.C.34.21.1) was assayed with benzoyl tyrosine ethyl ester (BTEE). Crude extracts were incubated for 2min in 2mL of Tris/$CaCl_2$ buffer, pH 7.8. Both trypsin and chymotrypsin were assayed in duplicate and enzyme activities were recorded at 247 and 256nm, respectively, according to HUMMEL (1959). One unit of enzyme was defined as the amount of enzyme needed to hydrolyze 1µg of substrate (TAME

or BTEE) per min per mg protein. Total protein content of crude extracts was measured following LOWRY et al. (1951), using bovine serum albumin as a standard. Data were assessed for normality using a Shapiro–Wilk test and submitted to one-way ANOVA followed by Duncan test ($P<0.05$), and values expressed as mean ± standard error (n=8). All statistics were carried out using SAS® (1997) software.

RESULTS AND DISCUSSION

Fish survival was not affected by glyphosate levels, being 100% in all treatments. Glyphosate in water of experimental tanks was monitored for eight days (Table 1) and glyphosate concentration in the water was adjusted according to treatments.

Digestive enzymes activities in all portions of the gastrointestinal tract increased significantly in piavas exposed to both glyphosate concentrations except amylase in the stomach of those exposed to 1.0mg L^{-1} (Figure 1). The higher amylase activity in the intestine than in the stomach of piavas (Figure 1A and 1B) is expected for typically omnivorous species (NAMULAWA et al., 2013). Long-term exposure (90 days) to glyphosate 1.0 or 5.0mg L^{-1} did not affect feed intake but reduced growth and caused hematological and metabolic disruption in piavas (SALBEGO et al., 2010).

The growth and food efficiency in fish depends on their physiological and biochemical

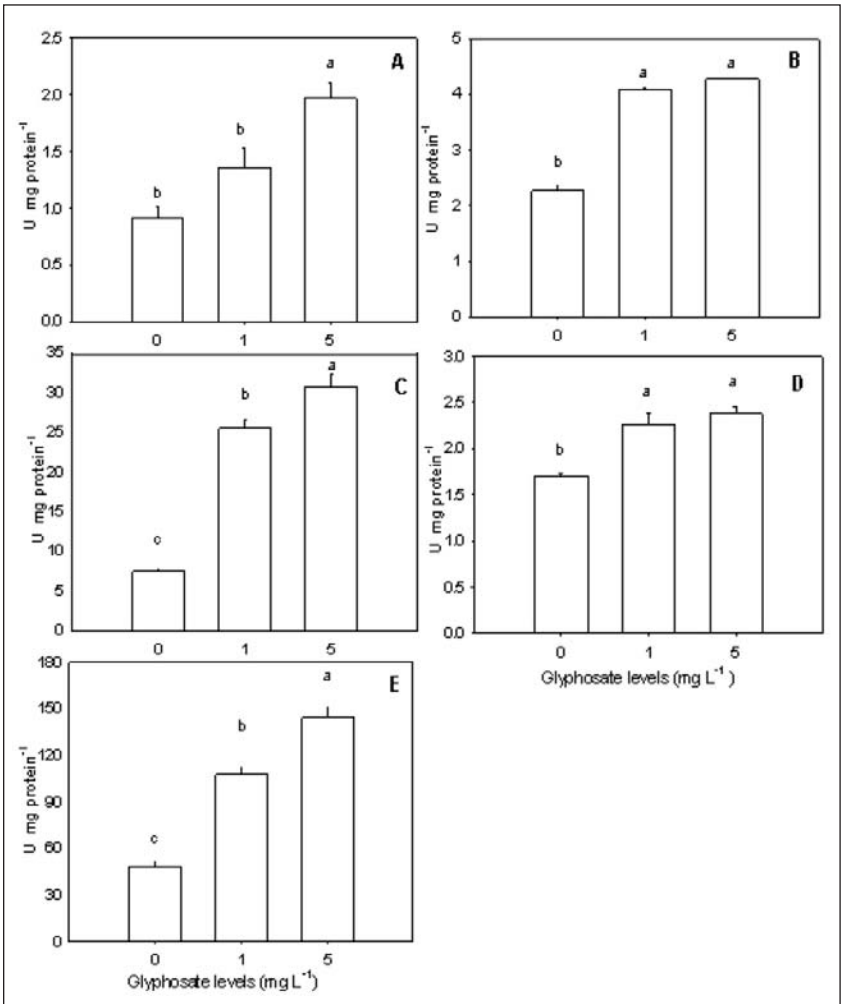

Figure 1 - Digestive enzymes in the stomach and intestine of piava (***Leporinus obtusidens***) after 90 days of exposure to different glyphosate levels. Amylase (A) and acid protease (C) activity in the stomach, and amylase (B), trypsin (D) and chymotrypsin (E) activity in the intestine. Where: U= 1mmol of glucose per min per mg protein^{-1}, to amylase; U= 1.0mg of tyrosine per min per mg protein, to acid protease and U= 1µg of TAME or BTEE per min per mg protein to trypsin or chymotrypsin respectively. Data are reported as the mean ± SEM (n = 8). Different letters indicate significant differences between the groups by the Duncan test ($P\leq0.05$).

capacities to digest and transform ingested nutrients (SWEILUM, 2006; FURNÉ et al., 2008; LAZZARI et al., 2010). BLIER et al. (2002) suggests that growth can be determined by some factors: the activity of digestive enzymes, the availability of oxygen for metabolism and the protein synthesis. The herbicides and pesticides in general can affect oxygen availability for the tissues (ORUÇ & UNER, 1999; GIMENO et al., 1995; BEGUM, 2004).

Metabolic alterations can also intervene in the growth process. SALBEGO et al. (2010) attributed the growth reduction in piavas exposed to glyphosate to several factors: lower exploitation of the available nutrients, reduction of the oxygen in tissues confirmed by fermentative response in the liver and muscle. These alterations suggest that for the maintenance of the fish in glyphosate-contaminated water, a great demand of energy is required. The increase in the activity of digestive enzymes may be an attempt of the organism to increase the exploitation of the food, compensating other possible losses caused by the poisoning.

The digestive enzymes activity reflects in the capacity of exploitation of the food by the fish and that the induction of determined enzymes is directly related to the type of diet (TORRISSEN & SHEARER, 1992). The obtained results allow concluding that low growth observed in glyphosate-exposed piavas by SALBEGO et al. (2010) may be related (at least partially) to the change in digestive enzymes activity.

CONCLUSION

In conclusion, present study demonstrated that long-term exposure to Roundup® increased digestive enzymes activity in piavas probably as a compensatory mechanism to obtain nutrients, indicating Roundup® toxicity. The piava and digestive enzymes activity in this fish may be useful as biondicator and biomarkers of long-term exposure to commercial formulations containing glyphosate.

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ETHICAL STATEMENT

We declare to whom correspond that we assume any responsibility about any process performed during the development of the research entitled "Effects of glyphosate on digestive enzyme activity in piava (***Leporinus obtusidens***)". Likewise we are available to answer any questions that may be needed.

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