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Effects of Glyphosate on Growth Rate, Metabolic Rate and Energy Reserves of Early Juvenile Crayfish, *Cherax quadricarinatus* M.

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Abstract Early juveniles of the crayfish *Cherax quadricarinatus* were exposed for 60 days to 10 and 40 mg/L of pure glyphosate (acid form) in freshwater. Mortality was 33 % at the highest concentration, while no differences in molting were noted among treatments. After the first month of exposure, weight gain was significantly ($p < 0.05$) reduced in the 40 mg/L group. At the end of the assay, lipid levels in muscle, as well as protein level in both hepatopancreas and muscle were significantly ($p < 0.05$) reduced. These results suggest long-term utilization of both lipid and protein as main energetic reserves, likely in response to the chronic stress associated with herbicide exposure. Besides, the lower pyruvate kinase activity in muscle suggests a possible metabolic depression in this tissue. The hemolymphatic ASAT:ALAT ratio showed higher levels than the control at the highest glyphosate concentration, indicating possible damage to several tissues.

Keywords Crayfish · Somatic growth · Glyphosate · Metabolism · Enzymes

Cherax quadricarinatus is a freshwater species of crayfish, native to northern Australia, which is cultured intensively or semi-intensively in other countries having tropical or subtropical climate, such as Argentina. In farms, a first hatchery procedure is commonly used for early, more sensitive juveniles (<1 g body weight) followed by a nursery phase aimed at pre-growing of advanced, more resistant, juveniles (Jones 1997). In Argentina, most Cherax farms are located near or adjacent to agricultural crops and are subject to contamination by pesticides. Glyphosate [*N*-(phosphonomethyl) glycine] is currently one of most widely used systemic herbicides around the world and Roundup® is one of its main commercial formulations; glyphosate half-life ranges from 7 to 70 days (Giesy et al. 2000). In Argentina, glyphosate is currently applied to genetically modified soybean crops which are resistant to this herbicide. Sediment concentrations of glyphosate have also been demonstrated to reach levels as high as 5 mg/kg (dry weight basis) for aquatic ecosystems in Argentina (Peruzzo et al. 2008). Maximum concentrations of glyphosate approaching 3 mg acid equivalents (a.e.)/L have been reported for surface waters in the United States (Solomon and Thompson 2003; Giesy et al. 2000) and up to 7.6 mg a.e./L in Australian waters (Mann and Bidwell 1999).

Both lethal and sublethal toxicity of glyphosate have been studied to some extent in crustacean species. For instance, reduced growth rates have been observed for shrimp exposed to concentrations of commercial glyphosate formulations (Roundup®; Mensah et al. 2012). We have previously reported that advanced juveniles of *C. quadricarinatus* exposed for 50 days to a mixture of glyphosate and the surfactant polyoxyethylenamine, showed a significant reduction in both growth rate and muscle protein level, as well as some other changes in carbohydrate and lipid reserves (Frontera et al. 2011). To research further in this line, the objectives of the current study were: (1) to assess the effects of sublethal concentrations of glyphosate on early, sensitive juvenile instars of the freshwater

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crayfish *C. quadricarinatus*, in terms of growth, metabolic rate and energy reserves levels, (2) to determine how glyphosate affects the activity level of key metabolic enzymes, such as pyruvate kinase and (3) to determine the levels of both alanine and aspartate aminotransferase activities (ALAT and ASAT respectively) as indicative of tissue damage. All the experiments were done with pure glyphosate (acid form), in order to extent the results obtained to any glyphosate-based formulation that could be applied in the environment.

Materials and Methods

Cherax quadricarinatus early juveniles (mean body weight = 1.28 ± 0.06 g, n = 36) were obtained from ovigerous females mated in the laboratory; females were purchased from a commercial hatchery (Pinzas Rojas S.R.L, Tucumán, Argentina). A chronic (60-days) bioassay was conducted in semistatic conditions, according to standard procedures recommended by the American Public Health Association et al. (2005). Temperature was maintained at 26 ± 1°C throughout, while photoperiod was held at 14:10 (L:D). Dilution water used was prepared from tap water (hardness 80 mg/L as $CaCO_3$ equivalents, pH = 8.0 ± 0.5) purified through a series of three filters with replaceable cartridges (Hidroquil®) to retain sediment, organic matter (by activated charcoal), and cations (using a cationic resin). The water was dechlorinated by holding it for at least 48 h in a storage tank. Dissolved oxygen was always >5 mg/L.

Twelve juveniles were randomly assigned to each glyphosate concentration or dilution water control, each juvenile was placed in a 13-cm diameter glass jar, filled with 400 mL of dilution water control and provided with both a small PVC pipe and a tiny plastic net as refuges. Water of all recipients was completely changed three times a week, i.e., mostly every 48 h; 6 h before the change of water, all animals were fed food pellets (35 % protein, 11.12 % lipids, 20.84 % carbohydrates) prepared in the laboratory according to the composition used in a previous study (Chaulet et al. 2012). Food pellets were provided to the crayfish in an amount equivalent to 5 % of body mass, supplemented with *Elodea* sp. fresh leaves, ad libitum.

According to a previous sublethal study (Frontera et al. 2011), and to preliminary range finding tests, the nominal concentrations used were 10 and 40 mg/L of pure glyphosate (acid form, 99.8 % purity, Sigma Co., St. Louis, Missouri). Stock solution was prepared weekly by dissolving the appropriate amount of glyphosate in distilled water. The pH of stock solution was always corrected by adding drops of a 10 N NaOH solution, to achieve the same pH value than the one mentioned for dilution control.

Table 1 Nominal versus measured concentrations of glyphosate in 48 h-aged solutions, as well as mortality and molting at the end of the 60-days exposure period

Nominal concentration (mg/L)	Measured concentration (48 h)	Mortality (%)	Molting (%)	
			1st molt	2nd molt
0 (control)	0	0	91.7	33.3
10	9.03 ± 0.03	16.7	75	16.7
40	40.13 ± 8.83	33.3*	75	50

Each measured concentrations represents the mean of two independent samples

* Significant ($p < 0.05$) differences with respect to control. Initial number of crayfish: 12 per treatment

Nominal concentrations in the experimental recipients were validated after 48 h of aging, in order to estimate the possible loss of glyphosate between two successive water changes. To this purpose, duplicate samples from each glyphosate concentration was measured by means of ionic chromatography: DIONEX (Sunnyvale, CA) DX-100 chromatograph with a conductivity detector and a 25-ll sample loop using a DIONEX AS-4 as analytical chromatographic column and a mixture of $NaOH/Na_2CO_3$ 4 mM/9 mM as eluent with a flow rate of 2 mL/min. Results of this validation are shown in Table 1.

During the assay, both mortality and molting were registered daily. The molted exoskeleton was kept for 48 h in each container to allow the animal to feed on it. Additionally, all animals were weighed every 2 weeks, in order to calculate the weight gain (WG) as WG = (FW−IW)/IW × 100, where IW is the initial weight and FW is the final weight of juveniles. At the end of the assay, oxygen consumption, hemolymphatic glucose level and energy reserves (glycogen, protein, and lipids) were determined in all surviving animals, following the methodology used in previous studies made on the same species (Chaulet et al. 2012; Frontera et al. 2011). Also, specific activity of both pyruvate kinase (PYK) and lactate dehydrogenase (LDH) were assessed in both muscle and hepatopancreas of all surviving crayfish. Briefly, after obtaining a 20,000×*g* supernatant from homogenates of these tissues, LDH activity was measured by the method of Schiedek (1997), while PYK was assessed based on the method of Reitman and Frankel (1957). Finally, both ALAT and ASAT activities were determined in hemolymph extracted from all surviving crayfish following the method of Reitman and Frankel (1957) with 30 min of incubation, measuring the absorbance of the pyruvate-DNPH complex at 490 nm.

To test significant differences between means, a one-way ANOVA followed by LSD multiple comparisons test (Sokal and Rohlf 1981) was used. Data normality and homogeneity of variances were always confirmed. Fisher

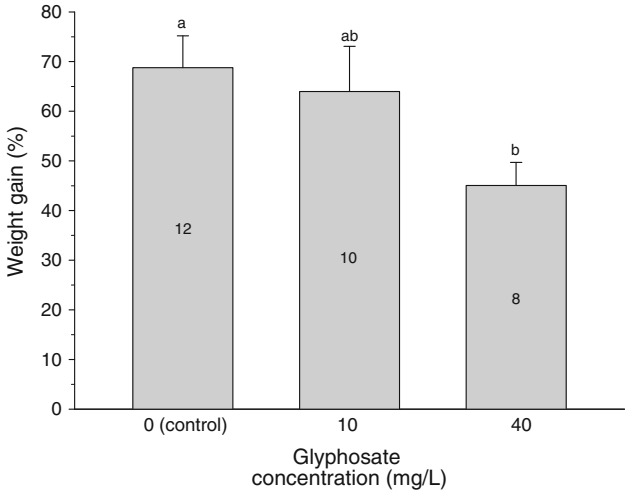

Fig. 1 Weight gain at the end of the experiment. In all cases, mean ± standard error is indicated. *Different letters* indicate significant ($p < 0.05$) differences. *Number of animals* (n) is indicated inside each *bar*

exact test was used to compare the proportion of dead or molted crayfish. A 5 % confidence level was considered in all cases.

Results and Discussion

The highest mortality value (33 %) was seen in animals exposed to 40 mg/L of glyphosate, this value was significantly ($p < 0.05$) different from that of control; no significant ($p > 0.05$) differences were noted in the molting percentages between either glyphosate concentration and the control (Table 1).

Figure 1 shows the WG values at the end of the assay, for both concentrations of glyphosate and the control. A clear and significant decrease in weight gain (35 % lower than control) was seen after the first month of exposure to 40 mg/L of glyphosate. Although Frontera et al. (2011) observed a decrease in weight gain in advanced (approx. 5 g body weight) juveniles of the same species exposed to 22.5 mg/L of pure glyphosate for 50-days, this effect was not statistically significant. Apart from the different concentrations and time or exposure, the significant drop in the weight gain of the early juveniles used in the current study could be also related to the higher sensitivity of these smaller and younger juveniles (approx. 1 g body weight), compared to the advanced juveniles used in the previous study. Several studies made on both sizes of juveniles have clearly shown a difference in sensitivity between them (Jones 1997; Barki and Karplus 2004).

Concomitant with the observed decrease in weight gain, a significant ($p < 0.05$) decrease in total protein content (Fig. 2) was observed in both muscle, at 40 mg/L, and hepatopancreas, at both assayed concentrations. Besides, a significant ($p < 0.05$) decrease in total lipid content was clearly observed in muscle, at the highest glyphosate concentration, with respect to control (Fig. 2). Both lipids and proteins are closely involved with the energy available for crustacean growth; moreover, several crustaceans mobilize protein under stressful situations such as starvation. The crustacean hepatopancreas is able to carry out gluconeogenesis from muscle protein as substrate, to elevate glycemia for supporting a higher metabolic demand (Sánchez Paz et al. 2006). In *C. quadricarinatus*, both protein and lipids have been identified as the main energy reserves for either growth or reproduction (Ghanawi and Saoud 2012). No significant ($p > 0.05$) differences were noted among treatments, concerning, glycemia or glycogen content (data not shown) suggesting that under chronic stress, glycogen is maintained as an energy reserve ready for immediate utilization, as seen in other crustaceans (Zhou et al. 2011).

At the 10 mg/L exposure, muscle pyruvate kinase activities were significantly lower ($p < 0.05$) relative to the control group (Fig. 3), while no differences ($p > 0.05$) were seen in the hepatopancreas. Concerning lactate dehydrogenase, no significant ($p > 0.05$) differences were seen in any case, suggesting that the anaerobic pathway in this tissue remains inactive. Taken together, these results were indicative of a reduced total metabolic intensity of cells. Given that no differences were seen in the oxygen consumption of the whole organism (data not shown), such reduction can not be a priori extended to tissues other than muscle. A drop in the aerobic metabolism had been reported for advanced *C. quadricarinatus* juveniles exposed to 22.5 mg/L of glyphosate (Frontera et al. 2011).

ASAT:ALAT ratios measured in hemolymph at 40 mg/L exposure concentrations were significantly ($p < 0.05$) higher relative to controls (Fig. 3). Since ASAT is a non-specific cytosolic and mitochondrial enzyme found in most tissues, and ALAT is a cytosolic enzyme mostly present in the liver, the increase in ASAT:ALAT ratio in the hemolymph has been considered as indicative of a generalized tissue damaged (Mayer et al. 1992). Therefore, the significantly higher ASAT:ALAT ratio found at the highest glyphosate concentration suggests that many organs are being affected by glyphosate in their cellular integrity. Both increased blood levels of ALAT and ASAT have been reported in rats treated with either Roundup® or pure glyphosate, although a stronger effect was seen with the former (El-Shenawy 2009). Pro-oxidant effects of glyphosate, likely enhanced by the surfactant presented in the Roundup® formulation, has been proposed to explain tissue damage (El-Shenawy 2009).

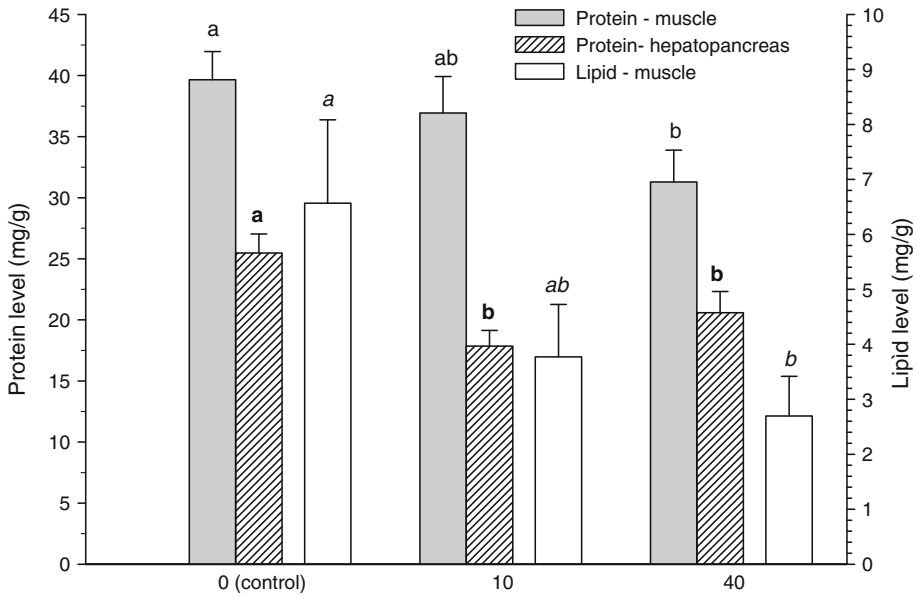

Fig. 2 Total protein levels (mg/g) in abdominal muscle and hepatopancreas, and total lipids in muscle at the end of the experiment. In all cases, mean ± standard error is indicated. *Different letters* (same style) indicate significant ($p < 0.05$) differences. n = same as Fig. 1

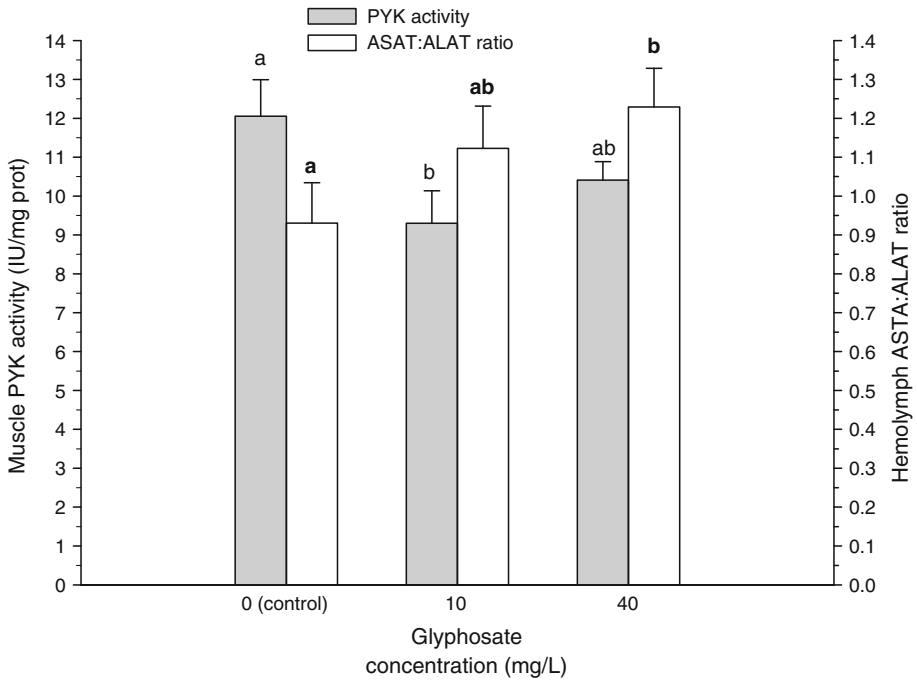

Fig. 3 PYK activity (IU/mg protein) in abdominal muscle, as well as hemolymphatic ASAT:ALAT ratio, at the end of the experiment, In all cases, mean ± standard error is indicated. *Different letters* (same style) indicate significant ($p < 0.05$) differences. n = same as Fig. 1

We conclude that the acid form of glyphosate, the principal active ingredient in most commercial formulations, is able to reduce growth rates and protein and lipid reserves in chronically exposed early juvenile crayfish. We have not seen these effects in advanced juveniles of the same species, chronically exposed to 22.5 mg/L of pure glyphosate (Frontera et al. 2011). Although most of the effects (decrease in weight gain, protein reserves in both hepatopancreas and muscle and lipid reserves from muscle, as well as the increase in ASAT:ALAT hemolymphatic level) were seen at a glyphosate concentration of 40 mg/L, some effects (decrease in protein reserves in hepatopancreas and an apparent metabolic depression in muscle) were observed at 10 mg/L, a concentration near reported environmental concentrations.

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