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Effects of a glyphosate-based herbicide on the development and biochemical biomarkers of the freshwater copepod *Notodiaptomus carteri* (Lowndes, 1934)

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ABSTRACT

In this work we analyzed the effects of Sulfosato Touchdown®, a glyphosate-based herbicide, on the ontogenic development and biochemical markers of the freshwater copepod *Notodiaptomus carteri*. A 30-days life-cycle experiment was carried out with three different glyphosate concentrations (0, 0.38, and 0.81 mg L^{-1}) to analyze the developmental time from nauplii to adult copepods and their individual growth. An additional 10-days experiment with the same glyphosate concentrations was designed to evaluate the energy reserves (glycogen, proteins and lipids) and the activity of three antioxidant enzymes, superoxide dismutase (SOD), catalase, and glutathione-S-transferase (GST) in adult copepods, separately for females and males. We found that the lowest glyphosate concentration increased the nauplii and total development time. The highest glyphosate concentration prevented copepods from reaching the adult stage, inhibited the growth of the first copepodite stage and increased the GST and SOD activity in adult females. According to our results, the presence of this herbicide in freshwater systems could impose a risk in the ecological role of copepods in nature. This study will contribute to propose the Notodiaptomus genus as model specie for monitoring purposes in the Neotropical aquatic systems.

1. Introduction

Volume of glyphosate-based herbicides applied in field crops has risen approximately 100-fold since the 1970s (Myers et al., 2016). The expansion of genetically modified seeds resistant to glyphosate herbicide (N-(phosphonomethyl) glycine) has produced a constant increase of the production and use of these formulations (Pengue, 2017; Santadino et al., 2014). These herbicides lack selectivity and can, by spray drift or surface runoff, reach surrounding freshwater systems affecting non-target species such as aquatic invertebrates, with consequences for the ecosystem functioning (Schaaf, 2017; Vera et al., 2012). Thus, to describe the effects of pollutants in the aquatic systems, it is necessary to analyze the potential effects on key organisms. Moreover, a multi-assessment approach including biomarkers at different levels of biological organization should be performed to have an integral view of the deleterious effects (Bonifacio et al., 2016).

Within aquatic systems, copepods are dominant members of plankton communities and main component of the secondary production of most marine and freshwater pelagic ecosystems (Hart et al., 1998; Raymont, 1983). They constitute an important link in the food webs (Boxshall and Halsey, 2004) so any alterations in their populations can have several consequences for the whole ecosystem. Among calanoid copepods, the species of the genus Notodiaptomus Kiefer are endemic to the Neotropical region, and have a wide distribution within this area because their populations can inhabit a great variety of environments, from small semi-arid lagoons to large river systems (Paggi, 1995). This particularity makes them one of the most important and representative groups of the neotropical freshwater zooplankton. Due to its small size, sexual dimorphism and a short life cycle including 12 larval stages (six nauplii stages: NI-NVI and five copepodite stages: C1 to C5 and adult stage), this copepod group have been suggested as potential good candidate to assess toxicological effect of pollutants (Gutierrez et al., 2010). In fact, previous studies showed that some species of this group can be highly susceptible to diverse xenobiotic compounds such as heavy metals and pesticides (Gutierrez et al., 2011, 2010). However, knowledge about their sensitivity to

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environmental pollutants, as well as their individual and population responses is still very scarce in comparison with other zooplankters traditionally used in toxicological tests such as those from the Holarctic regions (e.g.: *Daphnia magna*), or estuarine copepods. Furthermore, strikingly, the effects of glyphosate on these copepods are practically unknown, despite being one of the main pollutants of the aquatic systems of the Neotropical region (Peruzzo et al., 2008; Ronco et al., 2008). Thus, information on the effects of glyphosate on neotropical freshwater copepods becomes highly required in order to recognize some potential effects of this herbicide on the neotropical freshwater ecosystems and to provide accurate information to propose these taxa as potential monitoring organisms in this biogeographical region.

Among the individuals' biological traits, the development time of copepod from nauplii to copepodite (Supplementary Figure I) has been suggested as an effective variable to detect hormonal alterations in chronic toxicity tests (Andersen et al., 2001). In addition to the development time, the analysis of growth patterns has also been considered for understanding of compensatory changes in the energy metabolism (Gutierrez et al., 2010; Sancho et al., 2009). Although some studies showed that different xenobiotics (e.g. insecticides, heavy metals) are able to alter both individual traits, development time and growth patterns, in copepods including species of the Notodiaptomus genus (Brown et al., 2003; Gutierrez et al., 2010), no information exists about the effects of glyphosate.

A previous study suggested that glyphosate formulations inhibit the physiological recovery of the copepod *Notodiaptomus conifer* after being exposed for 48 h to sublethal concentrations (Reno et al., 2014). It is therefore possible to think that glyphosate can have negative effects on individual attributes such as the development time and growth.

On a smaller scale of biological organization, biochemical parameters have also been considered very sensitive indicators to sublethal concentrations of many stress factors (Connon et al., 2012; Kroon et al., 2017; Yancheva et al., 2016). It has been found that glyphosate-based herbicides are involved in a great variety of deleterious effects on aquatic organisms such as the depletion of energetic reserves in crayfish (Avigliano et al., 2014; Frontera et al., 2011), amphipods (Dutra et al., 2011) and mussels (Milan et al., 2018), and the generation of oxidative stress in crayfish (Banaee et al., 2019; Frontera et al., 2011), larval amphibians (Costa et al., 2008; Lajmanovich et al., 2011), crabs (Hong et al., 2019) and fish (Cattaneo et al., 2011; De Menezes et al., 2011; Glusczak et al., 2012; Guilherme et al., 2012; Lushchak, 2011; Modesto and Martinez, 2010). However, there is a lack of information about the effect of glyphosate-based herbicides in the components of the energetic metabolism and oxidative stress biomarkers of freshwater copepods.

Given the increasing use of glyphosate-based herbicides in agriculture areas, particularly in the Neotropical regions, and the gaps of information regarding their effects on the native fauna, we were interested in evaluating the impact of this herbicide on different biological parameters of an endemic copepod at different biological organization levels. In this study we focused on the freshwater copepod *Notodiaptomus carteri* and evaluated the effects of Sulfosato Touchdown® (ST), a glyphosate-based herbicide, on the development time, individual growth, energetic metabolism (glycogen, proteins and lipids) and antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT and glutathione-S-transferase, GST).

2. Materials and methods

2.1. Sampling and culturing

Individuals of the native copepod *Notodiaptomus carteri* (Lowndes, 1934) were collected with a plankton net (200 μm mesh) from lakes of the Paraná River alluvial plain (31° 37′ S, 60°41' W). For acclimation, a stock culture was carried out in laboratory conditions, using 50% filtered lake water and 50% dechlorinated tap water (physicochemical properties in Supplementary data I). Female copepods carrying egg sacs were separated from the stock culture until the eggs hatch. The culture media for the experiments was dechlorinated tap water and it was changed weekly and oxygenated by bubbling air for at least 24 h before using it. Temperature and lightning conditions were maintained through air conditioning and an automatic photoperiod device, while pH and dissolved oxygen in the water were measured during all the experimental period. Water quality parameters were as follows: pH 7.1; dissolved oxygen 7.32–7.89 mg L^{-1}; temperature 21 ± 2 °C; and photoperiod 16:8 (light:darkness). Copepods were fed three times a week with an algae culture including *Chlorella vulgaris* and *Scenedesmus* sp. cultivated according to Sager and Granick medium (Supplementary data II).

2.2. Chemical

Sulfosato Touchdown® (Syngenta Agro), a glyphosate-based formulation (potassium salt, 62%) is one of the main herbicide formulations used in soybean production in the Pampean Region (Argentina). A stock solution containing 620 a.i. mg $^{-}$L was prepared in distilled water previous to the experimental stage. This stock solution was used to prepare each glyphosate actual concentrations, which resulted as follows: 0 mg L^{-1} (C0), 0.38 ± 0.2 mg L^{-1} (C1), and 0.82 ± 0.4 mg L^{-1} (C2), with C2 based on the highest concentration of glyphosate found in water of pampean streams (Argentina) (Peruzzo et al., 2008; Ronco et al., 2008), and half of that value as a lower concentration (C1). The final concentrations were prepared with dechlorinated tap water, which is a technique that applies to most copepod species (Reish and Oshida, 1987) given that drinking water sources comes from the surrounding freshwater bodies were these organisms live, therefore the nutritional content is similar to the natural medium. Glyphosate concentrations at the beginning of the experiment were determined using a chromatograph Dionex DX-100 ion equipped with a conductivity detector Waters 430, a suppressor column Dionex ASRS300, a column Dionex Ion Pack AS4A-SC and a precolumn Ion Pack AG4ASC. The eluent was NaOH 3.2 mM/Na_2CO_3 7.2 mM.

2.3. Experimental design

Life cycle experiment started with the first larval stage (nauplii of 24 h after egg hatch), individually placed in glass vessels with 20 ml of the treatment medium. Experimental design consisted on three treatments (C0, C1 and C2, as mentioned above) with 10 replicas each. Medium was renewed daily during the whole experimental period (30 days). The renewal of the medium was performed as gently as possible with a Pasteur pipette, in order to cause minimum disturbance to the organisms. The fact that no mortality was observed in the control organisms, ensure us that this methodology do not cause major stress. The copepods were daily fed and the larval stage was daily monitored under stereoscopic microscope. The total length (μm) of each larval stage was measured in the molts from the anterior tip of prosome to the end of caudal rami using a compound microscope with a calibrated ocular micrometer (Nikon 41,602).

A parallel chronic test was carried out to evaluate biomarkers response on the adult stages, being males and females separately analyzed. The design consisted of the same procedure as used for the life cycle experiment but limited to 10 days. In this case, ten replicas were also used per treatment, but each replica contained 30 individuals of males or females in glass vessels with 600 ml of the treatment medium (1 ind/20 ml density). Thus, a total of 300 males and 300 females were used for the biochemical analyses. As for the life cycle experiment, during the experimental period, the treatment medium was renewed every day. Even though glyphosate its considered a moderately stable compound (half-life and volatility depending on the formulation), the medium was renewed to avoid the accumulation of waste and to provide more aerated water for the organisms. After 10 days of exposure, copepods were collected with a Pasteur pipette and introduced in

Eppendorf tubes for storage at −80 C in ultrafreezer.

2.4. Biomarkers

Energetic reserves and antioxidant enzyme activities were determined for each replica, consisting on pools of 30 complete individuals, as mentioned above.

2.4.1. Energetic reserves

A homogenization of each pool was carried out with a glass homogenizer in order to disaggregate individuals. Glycogen was estimated according to Seifter et al. (1950). Briefly, homogenates were digested with KOH 30% and KOH 60% in a boiling water bath. After alkaline disruption; glycogen was led to precipitate by ethanol during 24 h at 4 °C. The obtained pellet was resuspended in distilled water and mixed with anthrone reagent in a boiling water bath. Absorbance was measured at 620 nm using a spectrophotometer Metrolab M-330. Lipid content was estimated by the method described by Folch et al. (1957) using a chloroform: methanol (2:1) solvent mixture for extraction, and total protein concentration was estimated according to Lowry et al. (1951) using bovine serum albumin as standard. Values were expressed as µg/individual. Caloric content was indirectly calculated from the biochemical data using the caloric equivalents 9.45, 5.65 and 4.1 cal/mg for lipid, protein and glycogen respectively (Mann and Gallager, 1985), with total caloric content as the sum of the three energy sources per individual.

2.4.2. Antioxidant enzyme activities

For enzyme extracts preparation, individuals were homogenized in an ice-cold 0.1 M sodium phosphate buffer, pH 6.5 containing 20% (v/v) glycerol, 1 mM EDTA and 1.4 mM dithioerythritol. The homogenates were centrifuged at 20,000 x g (4 °C) for 30 min, and the supernatant (enzyme extract) was collected and stored at −80 °C for enzyme measurement.

Enzyme activities were assayed spectrophotometrically using a microplate reader. Superoxide dismutase (SOD) activity was determined according to Misra and Fridovich (1972), soluble glutathione S-transferase (GST) activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974), and catalase activity (CAT) was determined according to Beutler (1982). Enzymatic activity was calculated in terms of the enzyme extract protein content (Bradford, 1976) and the results were expressed as mU mg prot^{-1} (GST) or U mg prot^{-1} (CAT and SOD).

2.5. Statistical analysis

Data obtained of each experiment were first tested for normality and homoscedasticity using Kosmogorov-Smirnov and Levene tests respectively. Differences among treatments in each variable analyzed were assessed using analysis of variance (ANOVA). When variables could not be normalized through logaritmization, the non-parametric Kruskal–Wallis test was used. A Tukey post-test was used for comparison among groups ($p < 0.05$). Differences between females and males were assessed through Student T Test or the non-parametric Mann-Whitney *U* Test.

3. Results

3.1. Development time and body length from nauplii to adult

The mean total development time of *N. conifer* from the control was 23 days (5 ± 1 days for nauplii and 18 ± 3 days for copepodites). Both glyphosate concentrations caused an increase in the mean nauplii development time (8 ± 3.5 days for C1, and 13 ± 4.5 days for C2), but only in C2 the difference from the control was statistically significant ($F = 19.03$; $p = 0.0001$; Fig. 1). As regard the copepodite stages, the highest glyphosate concentration (C2) prevented the organisms to reach the copepodite 2 stage, indicating that any organism could pass to the copepodite 3 stage. The lowest glyphosate concentration caused a significant increase in the copepodite (26 days) and total developmental time (34 days) respect to the control group ($W = 286$; $p = 0.0003$) (Fig. 1).

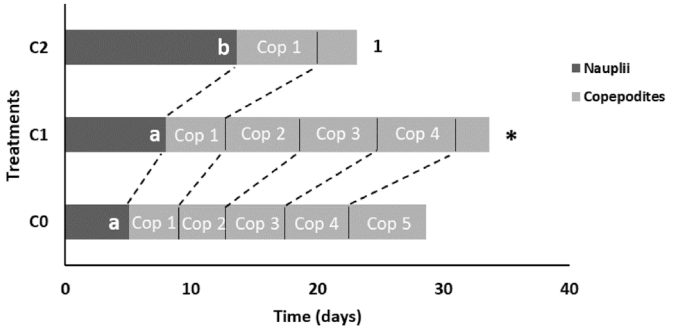

Fig. 1. Mean development time of *N. carteri* exposed to glyphosate concentrations. Black areas are related to nauplii development time and grey areas are related to copepodite development time, from the copepodite 1 to copepodite 5, except in C2 where no copepod reached the third copepodite stage (1). Letters symbolize significant difference respect to the control in the nauplii development time (Kruskal-Wallis test). * Indicate significant differences respect to the control ($p < 0.05$ for total development time). CO: Control, C1: Concentration 1, C2: Concentration 2.

The mean body length of copepodite 1 in C2 was significantly smaller respect to C1 and C0 ($F = 12.73$; $p = 0.0006$). No differences in the body length were recorded between C0 and C1 in any copepodite stages (Fig. 2).

3.2. Energetic reserves in adult copepods

All analyzed macromolecules in control organisms showed higher levels in females than in males (Table 1), being this difference statistically significant for glycogen ($T = 4.58$; $p = 0.0446$).

The caloric content per individual was also higher in females than in males but no statistical differences were found. Energetic reserves and caloric content did not vary significantly between treatments (ANOVA, $p > 0.05$) (Fig. 3).

3.3. Antioxidant enzyme activities

Regarding enzymatic activity, CAT and SOD registered higher activity levels in males than in females of the control group ($T = -2.72$;

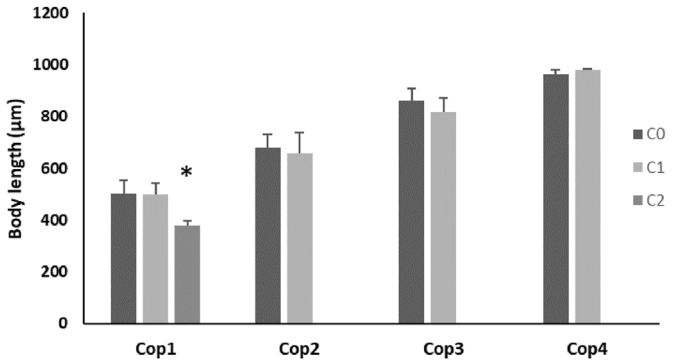

Fig. 2. Mean body length at each copepodite stage. C0: Control, C1: Concentration 1, C2: Concentration 2, Cop 1: Copepodite 1, Cop 2: Copepodite 2, Cop 3: Copepodite 3, Cop 4: Copepodite 4, Values expressed as Mean ± SD. * indicate significant differences respect to the control (p-value < 0.05). Please, note that for C2, only the copepodite 1 stage could be evaluated because any individuals reached the copepodite 2 stage.

Table 1
Energetic reserves and caloric content for males and females of *N. carteri* in control treatments. Caloric equivalents from Mann and Gallager (1985). * indicates significant difference between females and males ($p < 0.05$).

		Mean value	Caloric equivalent	Caloric content
Parameter	Gender	(μg/ind)	(cal/mg)	(mcal/ind)
Glycogen	Female	0.35	4.1	1.43
	Male	0.12	4.1	0.49 *
Lipids	Female	0.89	9.45	8.41
	Male	0.76	9.45	7.18
Proteins	Female	5.36	5.45	29.21
	Male	4.72	5.45	25.72
Total caloric	Female			39.05
Content (mcal/ind)	Male			33.39

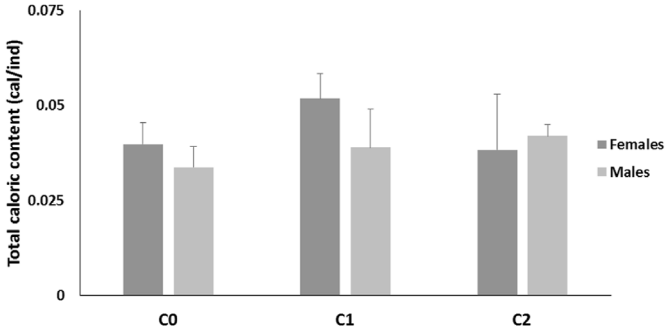

Fig. 3. Total caloric content values in organisms exposed to Sulfosato Touchdown®. Values are expressed as mean ± S.E.M. C0: control. C1: concentration 1. C2: concentration 2.

$p = 0.053$ and $T = -4.5$; $p = 0.045$), while the highest GST activity levels was found in control females (Fig. 4). The presence of glyphosate caused an increase in GST in females at both concentrations, as well as in SOD activity at the highest concentration ($F = 11.94$; $p = 0.0081$ and $H = 7.2$; $p = 0.0036$ respectively), while catalase did not show any difference between treatments ($p > 0.05$) (Fig. 4).

4. Discussion

4.1. Life cycle

The adequate development of immature phases of arthropods determines the recruitment to the following life stages (Quinn, 2019). Thus, the characterization of the morphologic alterations, developmental time and growth models during life cycle tests may help to predict the population dynamic (Boissonnot et al., 2016; Brown et al., 2003). Although glyphosate mode of action in animals is not clear enough, this herbicide has been found to affect the normal development of crustaceans and larval insects. A higher metabolic rate and a thinner exoskeleton (Vesela and Vijverberg, 2007) combined with less effective detoxification mechanisms (Koivisto, 1995) lead to a higher sensitivity to toxicant exposures in the immature phases than in the adults. Accordingly, in this study we found that both glyphosate concentrations caused an increase in the development time of larval stages with an excessive energy cost that was manifested in a smaller size of the first copepodite stage. This concurs with some previous studies where glyphosate imposed a trade-off between detoxification and growth in other invertebrates, such as the snail *Pomacea canaliculata* (Xu et al. (2017). An increment of the energy cost due to the investment in detoxicant and antioxidant systems, can also lead to a negative effect on the fitness (Monaghan et al., 2009). Although we did not evaluate other individual traits of this copepods, our results showed that at the highest glyphosate concentration the organisms that reached the first copepodite stage could not survive to the second one. This highlight that other physiological mechanisms could be affected during the intermolt period with negative consequences for their fitness.

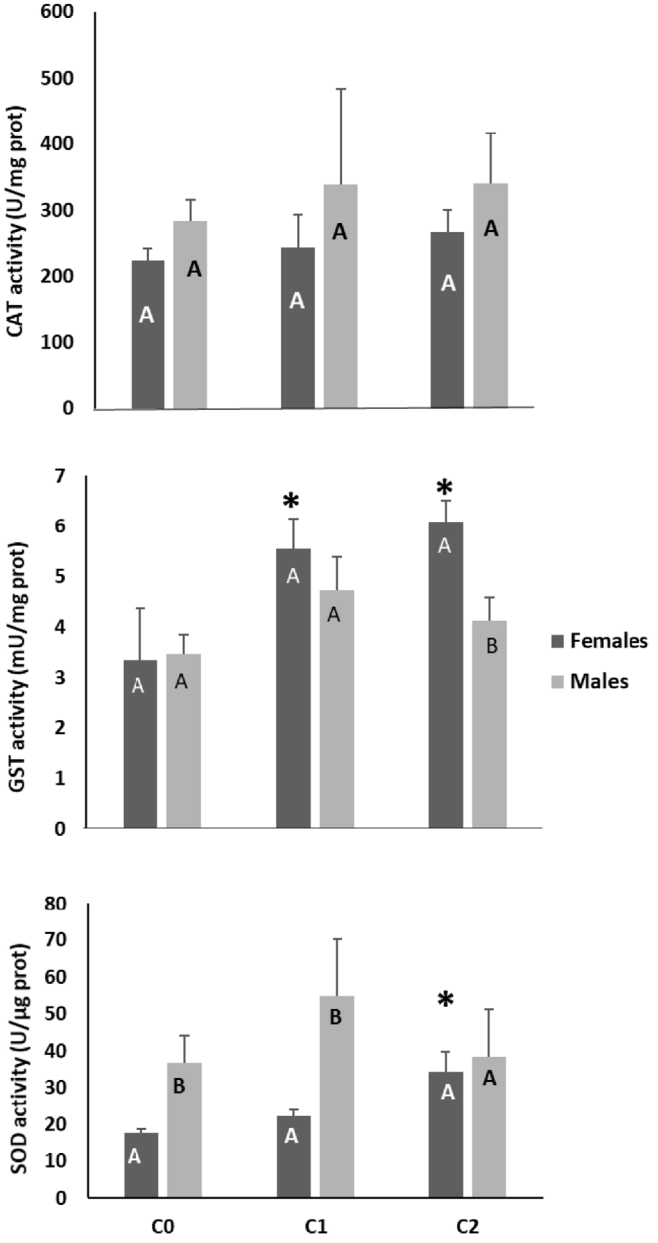

Fig. 4. Catalase, GST and SOD activity in organisms exposed to glyphosate concentrations. Values are expressed as mean ± SD. Bars with different letters means significant difference between females and males ($p < 0.05$). * indicates significant difference respect to the controls ($p < 0.05$).

4.2. Energetic reserves

Biological metabolism is the main responsible for the complex and dynamic structures of ecological systems, where organisms transform energy to carry out their own activities (Brown et al., 2004). In this context, analyzing the metabolic components of organisms is a helpful tool as an indicator of its health status and its ability to develop properly the ecological functions within the ecosystem. Our results showed that *N. carteri* exhibit higher values of glycogen in females than in males. These results fits with the biological function of females because they are responsible of reproduction, and energy storage is of high importance for this function given that the eggs production require

high energy amounts, primarily glycogen (Lease and Wolf, 2011; Reim et al., 2006). Theoretically, larger energy storage would allow a higher fertility as well as a greater chance of leaving successful offspring (Epp and Lewis, 1979; García-Barros, 2000; Sato and Suzuki, 2010). In this study we did not found differences in the glycogen content among glyphosate treatments, suggesting that the energetic investment in the offspring production is not affected by this herbicide at the tested concentrations.

High glyphosate concentrations have the potential to depress lipids, glycogen and protein levels in many crustaceans species (Avigliano et al., 2014; Frontera et al., 2011). Yet, we found no negative effects on these energetic components for females or males of *N. carteri*. Since this study was developed with a nutritious culture medium and continuous feeding, it is likely that a possible toxic effect of glyphosate on the metabolism has been compensated with an optimal quality of the medium. However, this hypothesis should be tested under different nutritional conditions before proposing the energy metabolism of these organisms as a potential indicator in monitoring.

4.3. Antioxidant enzyme activities

Living organisms have evolved an antioxidant defense system to protect themselves against oxidative stress (Yoon et al., 2019). It has been demonstrated that glyphosate may alter the antioxidant defense system in fish (Langiano and Martinez, 2008; Nwani et al., 2013; Sinhorin et al., 2014), mussels (Iummato et al., 2013) and shrimps (Hong et al., 2018). The enzymes SOD and CAT constitutes an indispensable part of the first line antioxidants, controlling superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) generation, respectively (Chainy et al., 2016). Our results showed an increase in SOD activity in *N. carteri* females exposed to glyphosate, which indicates an activation of the antioxidant system to cope with an increase of O_2^-, while CAT activity showed no variation between treatments. Literature reports a considerable variability on CAT response to glyphosate exposure in invertebrates, possibly related to potential antioxidant changes according to the species, the tissue, and the environment (Glusczak et al., 2007). Pala (2019) and Hong et al. (2018) found that a glyphosate-based herbicide decreased the catalase activity at sub lethal concentrations in an amphipod and a shrimp respectively, while in a study with mussels, Matozzo et al. (2018) found no effect in catalase activity under similar glyphosate concentrations. Therefore, authors suggest that it is important to analyze the activity of different enzymes in order to gain a more accurate perspective of the organism antioxidant responses (Hong et al., 2018; Van der Oost et al., 2003).

As well as for SOD, GST activity showed a significant increase in females exposed to the glyphosate concentrations over the controls. This result is in accordance with previous studies in which exposures to glyphosate affected GST activity in crayfish (Frontera et al., 2011). The higher levels of SOD and GST in females of *N. conifer* exposed to glyphosate suggest that females may have better defense capability against oxidative stress than males. Similar results have been found in a previous study with spiders (Wilczek et al., 2008), and amphipods (Sroda and Cossu-Leguille, 2011), where females showed higher antioxidant activity than males. Authors proposed that the antioxidant defense system in females is more complex with a greater range of detoxifying mechanisms acting to maintain their reproductive fitness. Even when there is a lack of information on gender-related differences in SOD activity in copepods, Matozzo and Marin (2010) found that female clams previous to spawning period, which is the most stressful phase of their reproductive cycle, displayed higher SOD activity than males, suggesting that female clams has a more efficient defense line against oxidative stress. However, further information and studies are required to better understand the defensive strategies of freshwater zooplankton, and particularly copepods, against environmental pollution.

In summary, we conclude that glyphosate (ST) imposed a stress in *N. carteri*, increasing the development time, inhibiting individual growth in the first copepodite stage and activating antioxidant enzymes. These effects suggest that glyphosate can have consequences for the populations and ecological role of this copepod group in the nature. However, further studies are necessary with other pollutants to propose them as bioindicators for monitoring in Neotropical aquatic systems.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Credit author statements

Noelia Fanton: Designed the experiment and developed the experimental set up, performed the statistical analyses and wrote the manuscript. Carla Bacchetta: Contributed with the oxidative stress (catalase, GST and SOD) and with the revisions of the manuscript. Andrea Rossi: Contributed with the evaluation of the energy reserves (glycogen, proteins and lipids) and with the revisions of the manuscript. María Florencia Gutierrez: provided the idea of the study, identified the copepod specie, designed the experiment, contributed with the statistical analyses and writing of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110501.

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