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RESEARCH ARTICLE

Glyphosate-based herbicides affect behavioural patterns of the livebearer *Jenynsia multidentata*

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Abstract

Roundup® is one of the most widely marketed glyphosate-based herbicides in the world. There are many different formulations of this brand that differ from each other in glyphosate concentration, salts and adjuvants, including surfactants, which are labelled as "inert" compounds. Several studies have shown that these formulations are highly toxic to fish, even compared with pure glyphosate. However, mechanisms underlying this toxicity are not fully understood. In this context, this study evaluated the effects of exposure to Roundup Original® (RO), Roundup Transorb® (RT), and Roundup WG® (RWG) on the behavioural patterns of the livebearer *Jenynsia multidentata*. This fish naturally inhabits agricultural areas in southern Brazil and Argentina where glyphosate is used extensively. In the experiment, animals were exposed to the herbicides for 96 h, at the environmentally relevant concentration of 0.5 mg/L of glyphosate. Swimming performance, anxiety, aggressiveness, long-term memory and male sexual activity were recorded. The formulation RWG negatively affected swimming performance, thigmotaxia and long-term memory consolidation. Conversely, RT reduced the sexual performance of males. These results confirm that Roundup® formulations are extremely harmful and also that they have different targets of toxicity, affecting behaviours that are essential for fish survival.

Keywords Aquatic environment · Ecotoxicology · Roundup · Neurotoxicity · Behaviour · Fish

Introduction

The increased use of herbicides, together with the disposal of effluent without proper treatment and accidental spills, has detrimental effects on aquatic ecosystems (Caballero-Gallardo et al. 2016; Moustafa et al. 2016). One of the most commonly used herbicides in the world is glyphosate (Caballero-Gallardo et al. 2016; IARC 2015; IBAMA 2014; James 2015; Wang et al. 2016). Glyphosate, N-(phosphonomethyl)glycine, is an aminophosphonate and analogue of the natural amino-acid glycine that acts as a broad-spectrum, post-emergent and non-selective herbicide. The extensive application of glyphosate and its relatively long persistence in water (half-life between 45 and 60 days) lead to its presence in the environment in concentrations that may vary from 0.1 to 1.5 mg/L in water bodies (Annett et al. 2014; Peruzzo et al. 2008; Queiroz et al. 2011; Tzaskos et al. 2012).

Roundup is a widely available commercial glyphosate-based herbicide. Their formulations contain glyphosate, salts and surfactants, which facilitate penetration of the active component into biological membranes (Székács and Darvas 2012; Tsui and Chu 2003). Although the toxicity of pure glyphosate is considered to be low by the World Health Organization (WHO 2005), its formulations are highly toxic to many aquatic species (Melo et al. 2017; Velasques et al. 2016; Pérez et al. 2011; Salbego et al. 2010; Relyea and Jones 2009). This increase in toxicity is

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related to the surfactants and other unspecified compounds labelled as inert ingredients (Sánchez et al. 2017, 2018).

Several studies have shown that glyphosate-based herbicides have neurodegenerative effects on fish (Braz-Mota et al. 2015; Salbego et al. 2010; Glusczak et al. 2006, 2007). Sandrini et al. (2013) showed in an in vitro experiment that pure glyphosate inhibited the acetylcholinesterase (AChE) activity in the brain and muscle of the zebrafish *Danio rerio* and the livebearer *Jenynsia multidentata*. Furthermore, Sánchez et al. (2017) showed that Roundup Original® (RO) and Roundup Transorb® (RT) inhibited this enzyme in the brain and muscle of *J. multidentata* exposed (96 h) to these formulations. It is well known that AChE catalyses the breakdown of the neurotransmitter acetylcholine (ACh), terminating synaptic transmission at neuromuscular junctions and chemical synapses of the cholinergic type.

Glyphosate-based herbicides can also induce oxidative stress in several organs, including the brain, which may affect the morphology and function of its cells (Moura et al. 2017; Sánchez et al. 2017; Velasques et al. 2016; Braz-Mota et al. 2015; Sinhorin et al. 2014a, b). In this context, Sánchez et al. (2018) observed glial cell proliferation in *J. multidentata* after exposure to different Roundup® formulations. Alterations in the brain influence fish behavioural patterns. Hued et al. (2012) observed a reduction of male sexual activity in *J. multidentata* and Sandun et al. (2015) recorded alterations in nesting and aggressive behaviour of male tilapia, *Oreochromis mossambicus*, both having been exposed to commercial formulations based on glyphosate (Roundup Max Granular® and Roundup®, respectively).

The effects of herbicides on fish behaviour can affect their survival, which ultimately may have ecological consequences, particularly in agricultural areas where agrochemicals are applied regularly. Moreover, it is important to compare toxicity between the different formulations that are marketed to indicate how dangerous they are and to support new rules for environmental protection. Thus, the aim of this study was to evaluate and compare the effects of three commercial Roundup formulations (RO, RT and Roundup WG® (RWG)) on behaviour patterns such as locomotion, aggressiveness, social interaction and reproduction, learning and memory in the neotropical fish *J. multidentata*. This species is often used as a model in biomonitoring studies due to its ability to respond to the presence of environmental contaminants (Ballesteros et al. 2007; Hued et al. 2012; Sandrini et al. 2013; Pinto et al. 2015; Sánchez et al. 2017, 2018) and because it can inhabit environments that are severely degraded by anthropogenic activities (Hued and Bistoni 2005; Chivittz et al. 2016). In addition, this species exhibits sexual dimorphism, facilitating the evaluation of the effects of contaminants on reproductive traits. Adult males develop a gonopodium (copulatory organ), constituted by a modification of the anal fin (Galindo-Villegas and Sosa-Lima 2002), which is introduced into the female genital pore (gonopore), to achieve fertilization. *J. multidentata* is a viviparous species; fecundation and initial development of the brood are internal.

Materials and methods

Herbicides

Three commercial glyphosate herbicides were used in this study: RO, RT and RWG. RO is a liquid formulation composed of glyphosate isopropylamine salt (IPA) (480 g/L), glyphosate acid equivalent (360 g/L GlyAE) and surfactant MON 0818, the Monsanto code for the POEA designation. POEA is a polyoxyethylene amine derived from fatty acids that is added to the herbicides to facilitate the penetration of glyphosate into plant tissues. RT is also a liquid made from glyphosate isopropylamine salt (IPA), but at 648 g/L, glyphosate acid equivalent, at 480 g/L GlyAE, and POEA. However, RWG is a granular formulation that has ammonium salt glyphosate at 792.5 g/kg, glyphosate acid equivalent at 720 mg/kg GlyAE, and unspecified “inert compounds”. In fact, all these formulations have “inert compounds” in which the surfactants and other adjuvants are included. Due to the risk to environment they represent, the surfactants generally represent 15% or less of the formulations (Giesy et al. 2000).

Acclimation and exposure to herbicides

The present study comprises of experiments conducted in Brazil and Argentina. Experiments in Brazil were carried out in the Laboratory of Toxicology of Universidade Federal do Rio Grande using *J. multidentata* (males: weight 0.54 ± 0.03 g, length 2.90 ± 0.06 cm and females: weight 0.69 ± 0.03 g, length 3.05 ± 0.05 cm) collected, using a hand net, during spring and summer of 2017, from pristine streams in Cassino Beach, Rio Grande do Sul, Brazil (licence for collection SISBIO 37129-2) (Chivittz et al. 2016).

In Argentina, the experiments were performed at the IDEA-CONICET Laboratory of Universidad Nacional de Córdoba, Argentina, with specimens of *J. multidentata* (males: weight 0.30 ± 0.02 g, length 2.49 ± 0.05 cm) that were collected with a backpack electrofisher during spring (October) 2018 at Rio Villa de Soto, Córdoba, Argentina, which is surrounded by forest and has no human population or crop fields in the area.

The acclimation period was 2 weeks in Brazil and 4 days in Argentina. During the acclimation, fish were fed with commercial food (Tetra Color Bits®) twice a day. The ratio of 1 g of fish per 1 L of water was maintained in the acclimation period and also during the experiment. In both countries, fish were put in 20-L tanks filled with water with aeration. In Brazil water at 5 ppt was used, obtained by mixing dechlorinated tap water with marine salt. Fish from Argentina were acclimated in dechlorinated tap water only. Water was partially changed every 2 days during the acclimation period.

The mean values of the physicochemical parameters measured in the acclimation and experimental waters were pH 7.2 ± 2, temperature 21.4 °C ± 0.69, dissolved oxygen 6.7 mg/L ± 1.37 (measured with a multiparameter probe, HANNA HI9146, Brazil), nitrite 0.20 ± 0.04 (LaboconTest, Brazil) and ammonia 0.4 ± 0.08 (LaboconTest, Brazil). The photoperiod was fixed at 12L:12D.

Fish were exposed to a fixed concentration of 0.5 mg/L of glyphosate in all the tests, calculated on the basis of the glyphosate contained in the three formulations used (RO, RT and RWG, see Acclimation and exposure to herbicides). This is considered a concentration of environmental relevance (see Introductory). Moreover, it was chosen due to the non-mortality of *J. multidentata* after 96 h of exposure to this concentration, regardless of whether the formulation was RO, RT or RWG (Sánchez et al. 2017). A control group (without glyphosate in the medium) was maintained throughout the test. The animals were fasted during the tests. RO, RT and RWG were put in the water only at the beginning of the test and the media were not renewed. An aliquot of water was collected daily from each treatment for glyphosate analysis. Temperature and photoperiod were reported above in this section. At 96 h, the fish were submitted to behavioural tests and later euthanized with an overdose of 500 ppm Benzocaine® (Sigma-Aldrich). The behavioural patterns analysed in Brazil were social interaction, space exploration (open field test), swimming, aggressiveness and long-term memory (LTM). In Argentina, males were submitted to analysis of sexual activity.

Performances of social behaviour, open field aggressiveness and avoidance

Twenty fish were used for the analysis of behavioural patterns. They were exposed to each treatment (96 h), being randomized males (n = 10) and females (n = 10). RO, RT and RWG were put in the water only at the beginning of the experiment and media were not renewed. An aliquot of water was collected daily from each treatment for glyphosate analysis. At 96 h, the fish were submitted to behavioural tests and later euthanized with an overdose of 500 ppm Benzocaine® (Sigma-Aldrich). The behavioural patterns of social interactions, space exploration (open field test), swimming and aggressiveness were conducted with fish sampled in Brazil, as was the avoidance inhibitory test that measured LTM.

Social behaviour

The protocol used for social interaction was proposed by Gerlai et al. (2000) and adapted to our fish model and experimental conditions. After 96 h of exposure to the herbicides, each fish was put in an aquarium (25 cm wide × 44 cm long) filled with 5 cm of clean water (without herbicide) and divided into three compartments using nets (Fig. 1a). The compartment on the left side was empty of fish, the compartment on the right side contained a group of five fish (stimulus) and in the middle was the fish submitted to the experiment. Fish were tested individually. Before recording started, each fish was habituated to the experimental aquarium for 1 min. The preference of the animal for each side (left or right) was recorded for 10 min (SONY SSC-G118) and then analysed using the Software SMART 3.0, Panlab/Harvard Apparatus, Spain.

Open field test

The open field test can be used to analyse fear and swimming performance (Egan et al. 2009; Maximino et al. 2010; Prut and Belzung 2003; Rosemberg et al. 2011). The apparatus consisted of a white aquarium (25 cm wide × 44 cm long) with uniform colour, filled with only 3 cm of clean water so fish swam only at the bottom (Fig. 1b). Each fish was placed in the centre of the aquarium and its activity was recorded for 10 min (Panasonic DMC-FZ40). The fish's spatial exploration behaviour and swimming performance were examined. The following variables were considered: total distance covered (mm/s), swimming speed (cm/s), percentage of exploration rate, percentage of mobility rate and freezing. This last was considered to occur when the fish was immobile for more than 5 s. Videos were analysed using the ToxTrac® software. In addition, the preference of the fish for the central or peripheral area of the aquarium was recorded. A fish remaining permanently in the peripheral area or exhibiting thigmotaxis were behaviours associated with fear. On the other hand, a permanent presence in the central area was associated with predatory susceptibility. Thigmotaxis was analysed using SMART 3.0 software (Panlab/Harvard Apparatus, Spain). The method was adapted for *J. multidentata*.

Aggressiveness

Aggressiveness was evaluated by observation of the animal and its reaction to its own reflected image in a mirror, as described by Gerlai et al. (2000). A mirror was glued on one wall of the aquarium at an angle of 22.5° with the base (25 cm wide × 44 cm long and filled with 3 cm of clean water) (Fig. 1c). The image of the animal itself was reflected in the mirror, looking larger when the animal was positioned near to it. Fish were analysed individually after 1 min of habituation.

The proximity of fish to the mirror segment indicated a preference for the "opponent" and the contrary meant "avoidance of the opponent". Aggressive behaviour was evaluated from the number of episodes of approaching the mirror with a

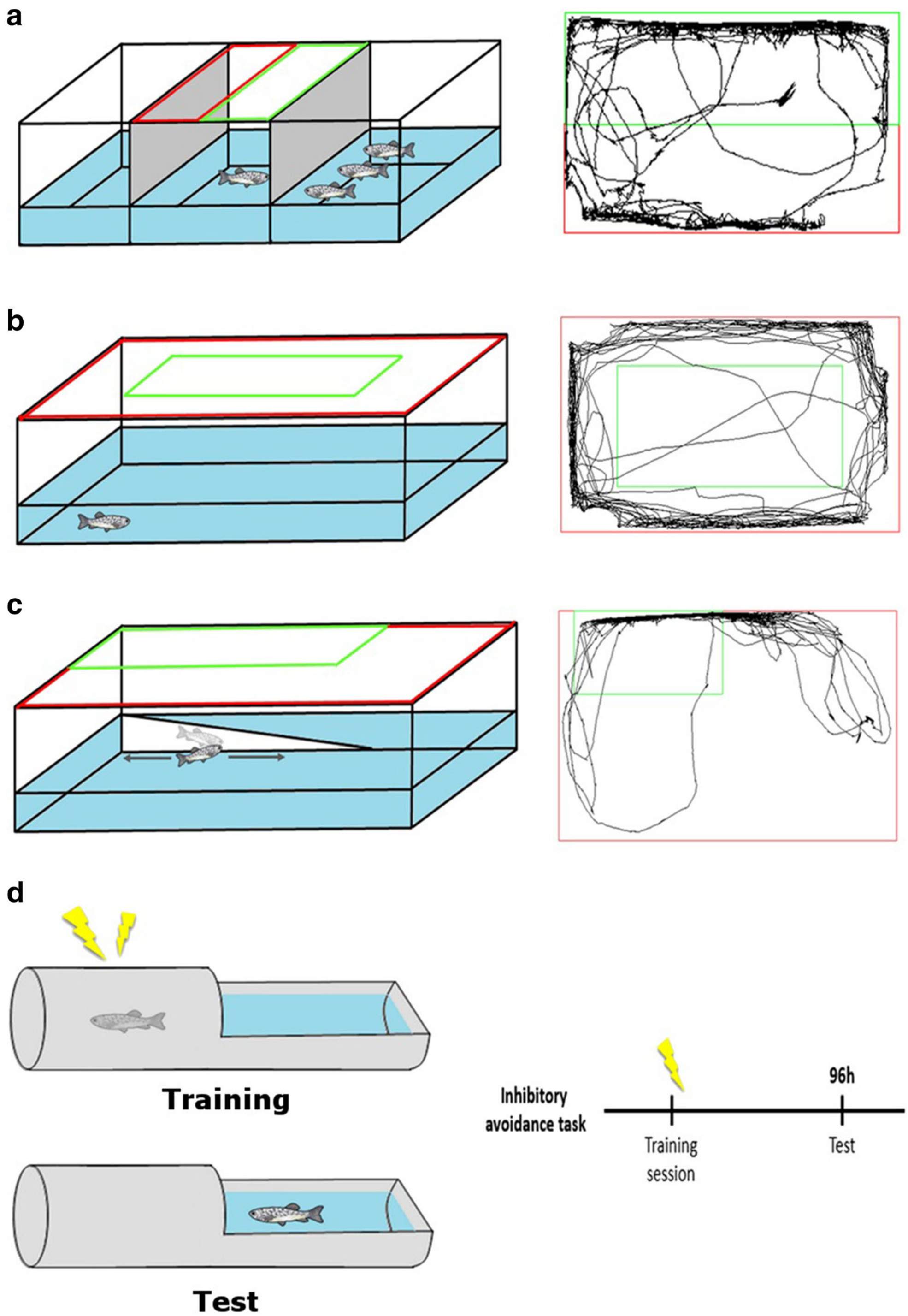

Fig. 1 Illustrative images of the methodological approaches carried out for behavioural analyses of *J. multidentata*: **a** social interaction; **b** open field; **c** aggressiveness; and **d** inhibitory avoidance. In **a**, **b** and **c**, the green areas correspond to the stimuli areas and the red areas correspond to non-stimuli areas. On the side of **a**, **b** and **c** are pictures created by Smart Software® at the end of the analysis by the control fish. **d** represents the apparatus of avoidance inhibitory test in the training phase when the fish received shocks (after 72 h of exposure), and in the test phase without shock (after 96 h of exposure)

combative posture (swimming fast towards the “opponent”) and “bites” in the mirror over 10 min (after 1 min of habituation), recorded with a video camera (SONY SSC-G118).

Avoidance inhibitory test

Avoidance inhibitory apparatus was used to examine the consolidation of long-term memory (LTM) (Castro et al. 2009). The apparatus was divided into a dark and light area separated by a gate. The dark area was a tool that generates electric shocks (5 mA, 6 V, unconditioned stimulus) (Fig. 1d). For this test, fish were trained 1 day before the end of the herbicide exposure. During the training stage, animals were placed on the light side of the apparatus with the gate closed. After 3 min in the apparatus (habituation), the gate was opened, allowing the animal to pass to the dark area. This area should be the area preferred by the fish, possibly seeking “protection” (Serra et al. 1999). When fish swam into the dark area, two sequential electrical shocks were triggered. Immediately after the shock, the fish was removed from the apparatus and returned to its respective experimental aquarium for the continuation of the experiment to complete 96 h of exposure to the herbicides. After 96 h of pesticide exposure (1 day after the training), fish were submitted to the same protocol, but no shock was triggered. LTM formation was determined by the time required for the fish to enter into the dark area, with a maximum of 300 s. The inhibitory avoidance apparatus was filled with water without herbicides.

Male sexual activity

After the 96-h herbicide exposure period (eight fish per treatment-control, RO, RT and RWG), males were individually placed together with a female that had not been exposed to any glyphosate formulation. They were placed in a 5-L aquarium filled with 4 L of clean dechlorinated tap water. After 10 min of habituation, the male's sexual behaviour was recorded for 20 min through direct observation and recording (Panasonic DMC-FZ40). When the males showed no interest in the female, the female was removed and replaced by another female, with a maximum of four substitutions. If the male did not manifest interest in any of the females, it was considered as presenting no sexual activity. Parameters considered as the sexual performance of males of *J. multidentata* were based on Bizarra et al. (2000). They are listed below:

Total pursuit time (TP): Time, expressed in seconds, during which the male fish pursues the female in order to access its gonopore.
Total number of pursuits (NP): Number of times that a male fish pursues a female to access the female's gonopore, making contact with the female through the repetitive chase.
Copulation attempts (CA): Number of times that a male fish enlarges its gonopodium to make contact with the female gonopore.
Number of copulations (C): Number of times that a male fish's gonopodium made direct contact with the female gonopore.

The following relative indices were calculated using these variables, following to Hued et al. (2012) and Roggio et al. (2014):

Attempts at copulation as a function of the total pursuit time: CA/TP
Ratio of pursuits involving copulation attempts: CA/NP
Copulations as a function of the total time of pursuit: C/TP
Ratio of pursuits that ended in copulations: C/NP
Proportion of copulations as a function of total copulation attempts: C/CA

Gonadosomatic index and Fulton's condition factor

After the evaluation of male sexual behaviour, fish were euthanized and body weight (BW) (g) and standard length (SL) (cm) were recorded in order to assess the general condition of the fish using Fulton's condition factor (Eq. (1)). Then, the gonads were dissected and weighed to estimate the gonadosomatic index (Eq. (2)).

$$K = \left(\frac{\mathrm{BW}}{\mathrm{SL}^3}\right) * 100 \quad (1)$$

where K is Fulton's condition factor; BW is the body weight and SL is the standard length of the fish. $K > 1$ means that fish are growing.

$$GI = \left(\frac{\mathrm{GW}}{\mathrm{BW}}\right) * 100 \quad (2)$$

where GI is the gonadosomatic index; GW is gonad weight and BW is the body weight of the fish.

Data analysis

Data were expressed as mean ± standard error (SEM). Comparisons between treatments or between training and test, in the case of the inhibitory avoidance test, were evaluated by one-way analysis of variance (ANOVA) followed by the Tukey test. Normality (Shapiro-Wilk) and homoscedasticity (Levene's test) were checked previously. A significance level of 95% ($p < 0.05$) was adopted for all analyses. Statistical analyses were performed using Sigma-Plot 11.0 software.

Results

During the experiments, there was no mortality, regardless of the formulation tested. Mean values of glyphosate measured in water were 0.59 ± 0.07, 0.58 ± 0.14 and 0.56 ± 0.16 mg/L for RO, RT and RWG, respectively (Sánchez et al. 2018). Glyphosate was not detected in the control groups. The herbicide was measured only in the experiments performed in Brazil. It was not possible to measure it in Argentina, but the same stock solution and work solution were used for all the tests (in both Brazil and Argentina).

Figure 2 shows the behaviour patterns of social interaction, open field exploration, aggressiveness and LTM. Figure 2a shows that *J. multidentata* is a social fish as it preferred to interact with other fish than to be reclusive. But, RWG caused

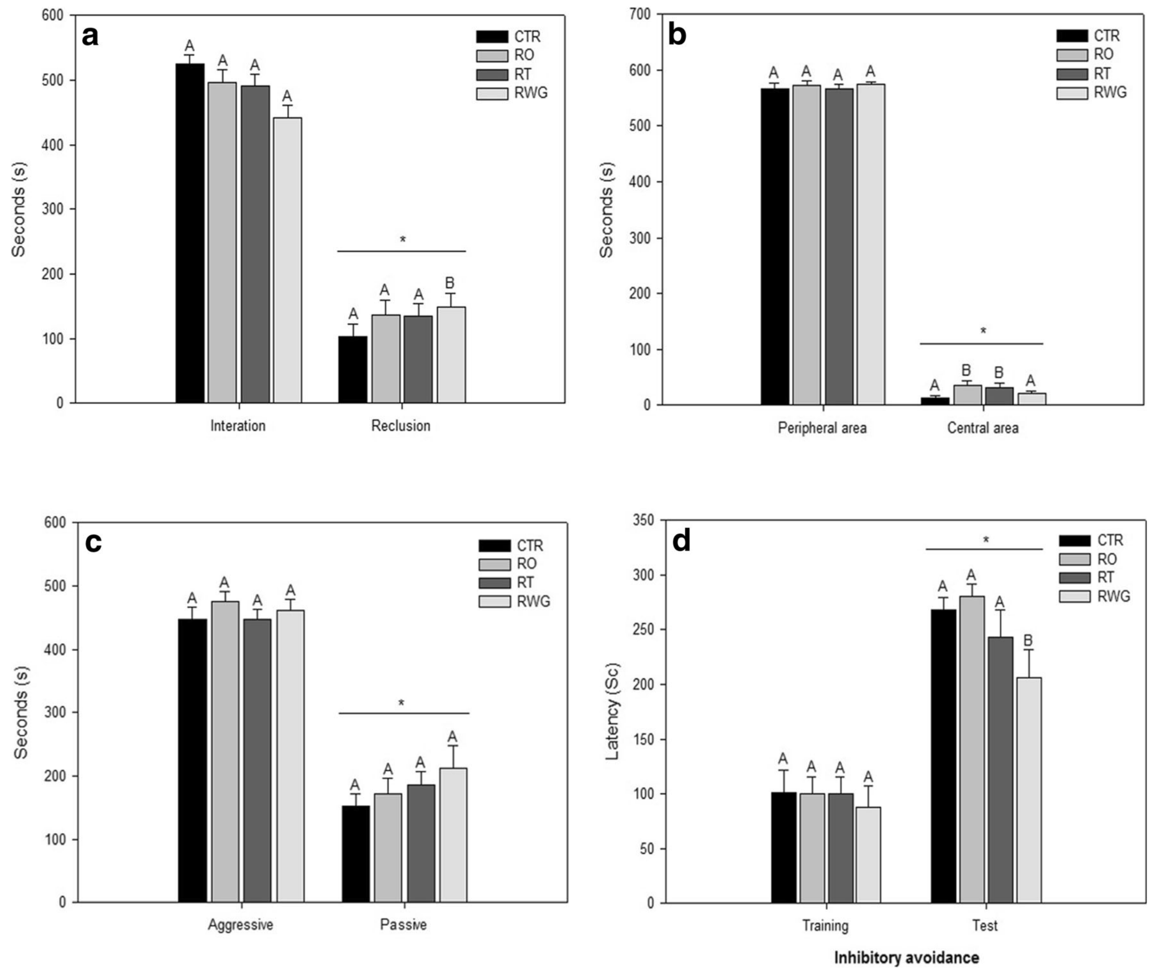

Fig. 2 Effects of Roundup Original® (RO), Roundup Transorb® (RT) and Roundup WG® (RWG), at a concentration of 0.5 mg/L of glyphosate, on the behavioural patterns of *J. multidentata*. **a** Degree of social interaction based on the length of time fish spent next to the group of five fish (interaction area) or that it was reclusive. **b** Results of anxiety and depression, based on the preference of fish for the peripheral or central area in the open field test, respectively. **c** Results of aggressive behaviour, given by the time the fish spent close to the mirror (aggressive performance) or away from the mirror (passive behaviour). **d** Period of time (expressed in seconds (s)) spent by the fish in the light area of the inhibitory avoidance apparatus during the training phase (after 72 h of exposure) and the test phase (after 96 h of exposure). Data are expressed as mean ± standard errors. Capital letters represent significant differences between treatments. Asterisk (*) indicates differences between fish preference areas in **a**, **b** and **c**, and differences between training and test periods in **d**. Significant differences were assigned by ANOVA and Tukey as *a* posteriori test, with $p < 0.05$

a significant increase in the time of reclusion when compared to controls and other formulations. With respect to open field exploration, Fig. 2b shows that the time spent by the fish in the periphery area was higher compared to time spent in the central area. RO and RT increased the time that fish stayed in the central area, which was considered as behaviour indicative of depression.

Regarding swimming performance (Table 1), total distance covered, mobility and exploration were significantly affected by the herbicides. RWG reduced the distance travelled with respect to the control group. As for mobility and space exploration, RWG was the most harmful, followed by RT and then by the RO. Other variables, such as speed and freezing, were not significantly affected by the herbicides.

Figure 2c shows that the period of time spent by the fish at the side with the mirror, close to the "opponent", was significantly higher compared with the side without the mirror ("avoidance of the opponent"), meaning that *J. multidentata* is a naturally aggressive fish species. There were no effects of herbicides on this behaviour. For the LTM test (Fig. 2d), it was observed that during the training (after 72 h of exposure), the length of time spent in the light area, before exploring the dark area, was lower when compared to the test (after 96 h of exposure). This means that *J. multidentata* had developed LTM. They learned that if they go to the dark side they would receive a shock. However, RWG significantly reduced the amount of time spent by the fish on the light side.

Table 2 shows the effects of herbicides on male sexual activity in *J. multidentata*. It was observed that 100% of male

Table 1 Swimming performance of *J. multidentata* acutely exposed (96 h) to three commercial glyphosate-based herbicides: Roundup Original® (RO), Roundup Transorb® (RT) and Roundup WG® (RWG) at a 0.5-mg/L concentration of glyphosate contained in each formulation. Results are present as mean values ± standard errors. Letters indicate a significant difference between treatments (ANOVA, $p < 0.05$)

	Control	RO	RT	RWG
Total distance (cm/s)	2529.4 ± 132.4a	2424.1 ± 157.9a	2112.6 ± 157.3a	1913.3 ± 105.5b
Av. speed (cm/s)	4.23 ± 0.42a	4.29 ± 0.40a	4.01 ± 0.36a	3.98 ± 0.46a
Tot. time freezing (s)	0.58 ± 0.56a	2.03 ± 2.02a	1.19 ± 0.80a	Non-observed
Mobility rate (%)	96.58 ± 0.01a	96.91 ± 0.01b	96.61 ± 0.01c	96.42 ± 0.01d
Exploration rate (%)	86.37 ± 0.03a	83.05 ± 0.05b	81.66 ± 0.02c	78.68 ± 0.03d

fish in the control group responded positively to the presence of a female, but this percentage was reduced by exposure (96 h) to the herbicides, this being a reduction to 85.7%, 75% and 88.5% for RO, RT and RWG, respectively. However, in Table 2, we include only the fish that responded positively to the presence of a female for the variables shown. In this context, RT reduced NP, CA and C and the C/NP ratio (see item 2.8).

Figure 3 represents Fulton's condition factor (*K*) and the gonadosomatic index (IGS) There was no difference in Fulton's condition factor calculated for the groups (Fig. 3a). However, Roundup formulations reduced GSI (Fig. 3b), and the largest effect was seen in fish exposed to the RWG formulation, which affected gonadal maturation of males of *J multidentata*.

Discussion

In their natural environment, fish are under stressful conditions and they are able to respond to them at different levels, from molecular to behavioural. These natural responses can be affected by the presence of pollutants. They are considered biomarkers, used to indicate mechanisms of toxicity and predict effects of pollutants in the environment. Depending on the compound, the doses and exposure time, pollutants can be more or less harmful. In this context, the present study has shown that glyphosate-based herbicides negatively affect the behavioural patterns and GSI of *J. multidentata*, at different levels, depending on the formulation. This indicates RO, RT and RWG herbicides exert a neurotoxic action on *J. multidentata* since behaviours are processed by the nervous system. Importantly, the 0.5 mg/L of glyphosate exposure concentration is considered environmentally relevant, if we consider data reported for water bodies in Brazil (> 100 μg/L and 1.48 mg/L) and Argentina (0.1–0.7 mg/L) that are located close to agricultural areas (Silva et al. 2003; Peruzzo et al. 2008; Tzaskos et al. 2012).

The negative effects of herbicides on swimming performance, presented in Table 1, are in accordance with the data reported by Bridi et al. (2017) who showed that Roundup, at exposure concentrations of 0.5 mg/L and below, prejudiced the swimming performance of zebrafish. Decreases in swimming performance were previously associated with the inhibition of AChE in tissues of *J. multidentata* exposed to the insecticides endosulfan (Ballesteros et al. 2009), chlorpyrifos and cypermethrin (Bonansea et al. 2016). Rao et al. (2005) also observed that chlorpyrifos inhibited AChE activity in the

Table 2 Sexual activity of males of *J. multidentata* acutely exposed (96 h) to three commercial glyphosate-based herbicides: Roundup Original® (RO), Roundup Transorb® (RT) and Roundup WG® (RWG) at concentrations of 0.5 mg/L of glyphosate. Results are presented as mean values ± standard errors. Letters indicate a significant difference between treatments

	Control	RO	RT	RWG
TP	215.91 ± 72.04ab	274.00 ± 80.10a	98.08 ± 24.50b	158.26 ± 42.12ab
NP	43.75 ± 6.12a	40.56 ± 8.67ab	21.38 ± 6.09b	36.78 ± 7.55ab
CA	74.71 ± 18.02a	62.00 ± 12.36a	28.25 ± 10.70b	40.22 ± 9.60ab
C	20.13 ± 6.49a	20.00 ± 4.08a	6.50 ± 1.98b	11.00 ± 2.16ab
CA/TP	0.47 ± 0.16a	0.17 ± 0.03a	0.21 ± 0.06a	0.25 ± 0.04a
CA/NP	1.36 ± 0.45a	1.42 ± 0.22a	0.91 ± 0.23a	0.95 ± 0.14a
C/TP	0.14 ± 0.05a	0.05 ± 0.01a	0.05 ± 0.01a	0.07 ± 0.01a
C/NP	0.41 ± 0.06a	0.45 ± 0.06a	0.22 ± 0.04b	0.27 ± 0.04ab
C/CA	0.29 ± 0.02a	0.29 ± 0.04a	0.20 ± 0.05a	0.27 ± 0.05a

Time taken by pursuits (TP) is expressed in seconds. NP is the number of pursuits, CA is the number of attempts at copulation and C is the number of actual copulations

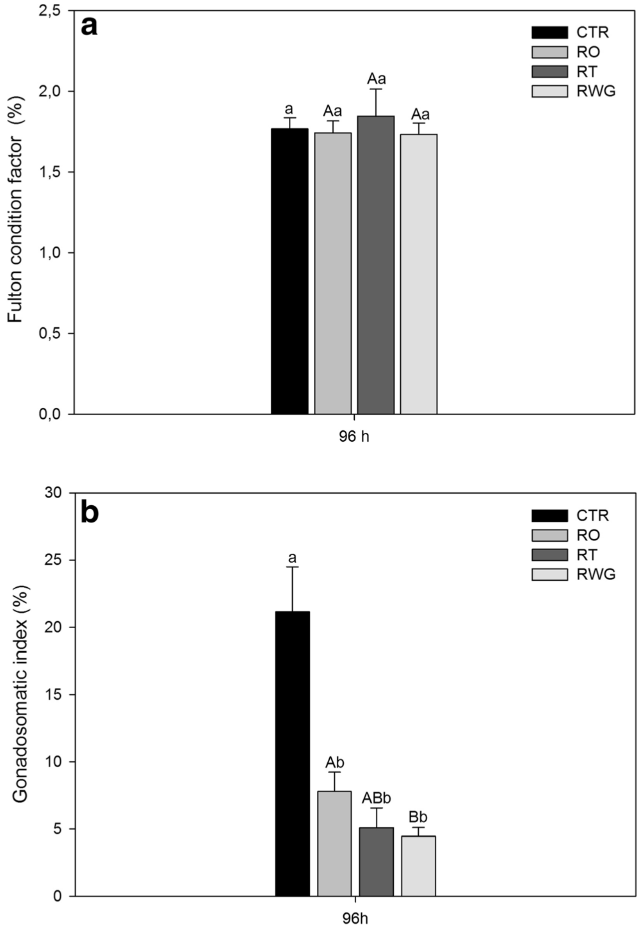

Fig. 3 Indices of **a** Fulton's condition factor, represented by *K* and **b** gonadosomatic index (IGS) in adult males of *Jenynsia multidentata* exposed (96 h) to the formulations Roundup Original® (RO), Roundup Transorb® (RT) and Roundup WG® at a concentration of 0.5 mg/L of glyphosate. Data are expressed as mean ± standard error. Lowercase letters represent significant differences between the treatments and the control group. Uppercase letters represent significant differences between the treatments. Differences were assessed by one-way ANOVA and Tukey test, $p < 0.05$

brain of *Gambusia affinis* and negatively affected its swimming performance. This evidence may indicate a relationship between swimming and AChE activity. Furthermore, previous results from Sánchez et al. (2017) showed an inhibition of the AChE activity in the muscle and brain of *J. multidentata* exposed to Roundup formulations (RO and RT) at 0.5 mg/L for 96 h, the same conditions as this study. Thus, it could be related to the decrease in the swimming ability of *J. multidentata* observed here.

There are several studies showing that glyphosate-based herbicides cause inhibition of AChE activity in the brain, muscle and gills of fish and other animals (Sánchez et al. 2017; Sandrini et al. 2013; Modesto and Martinez 2010; Glusczak et al. 2007). It is well known that AChE is an enzyme that catalyses the breakdown of the neurotransmitter ACh and some other cholines in order to stop synaptic transmission at the neuromuscular junctions and chemical synapses of the cholinergic type. ACh is a neurotransmitter linked to functions such as the visual response of optical circuits, as well as gustatory information during feeding, and the processing of motor information. The nicotinic acetylcholine receptors (nAChR) are related to neurotransmission, neuromodulation and olfactory mechanisms, while the muscarinic acetylcholine receptors (mAChR) are involved in glutamate release and memory construction. The inhibition of AChE can be related to both hypoactivity and hyperactivity in the behavioural responses of fish (Marigoudar et al. 2009).

According to this study, *J. multidentata* is a social species; individuals prefer to be with their conspecifics than to be reclusive. The active preference for companions has been documented in a number of fish species, including *J. multidentata* (Calcagno et al. 2016; Croft et al. 2005; Griffiths 2003; Haro and Bistoni 2007; Soares et al. 2018; Ward et al. 2002). Social interaction between animals is often characterized by non-random partner selection (Whitehead and Dufault 1999), being influenced by a number of phenotypic, behavioural and ecological factors. Phenotypic characteristics that may influence social behaviour include body length, species, colour and parasite infestation (Krause et al. 2000). Such interactions may confer important adaptive benefits, including reduced predation risk through predator confusion, foraging efficiency (Krause et al. 2000; Ranta and Lindström 1990) and increasing food intake through reduced competition (Utne-Palm and Hart 2000). The significant effect of RWG on the period of reclusion can, therefore, affect *J. multidentata* shoal behaviour and also the ability of an individual fish to find a partner for breeding or to protect itself from predators. Mechanisms associated with the social behaviour of *J. multidentata* were not the goal of the present study. However, other studies have shown that the dopaminergic system is involved in social preference and shoaling of the zebrafish *D. rerio*. It has been proposed that proximity to conspecifics increases the levels of dopamine (DA) and its metabolite DOPAC in the brain of zebrafish (Saif et al. 2013). In this context, other experiments with *D. rerio* showed that D1-R antagonists (antagonists of DA receptors) decreased the social preference of this species (Scerbina et al. 2012). In addition, the hormone vasotocin (vasopressin like) seems to be implicated in shoaling and social preference since the administration of a vasotocin antagonist in *D. rerio* decreased these behaviours (Lindeyer et al. 2015).

Concerning the open field test, our results show that control individuals of *J. multidentata* prefer the peripheral area of the apparatus, indicating a natural behaviour of anxiety, perhaps because they were alone. Nevertheless, RO and RT affected this performance, increasing the time spent by the fish in the centre of the open field test apparatus. This may be associated with a state of depression in the fish and, in turn, could be related to a reduction in its "alert state" (Kalueff et al. 2014;

Schulte 2014). Anxiety can also be associated with fear; however, in the present study, the freezing of animals during the open field test was not observed.

The aggressiveness exhibited by *J. multidentata* is in accordance with the natural behaviour previously described for this species, and it is mainly associated with territorial defence and sexual behaviour (Ortubay et al. 2002; Garcia et al. 2004). RO, RI and RWG did not affect this behaviour. On the other hand, tests conducted in the inhibitory avoidance apparatus showed that fish preferred the dark area; this is to be expected for *J. multidentata* since it lives in association with riparian vegetation in natural environments, which is a protected habitat (Garcia et al. 2004; Quezada-Romegialli et al. 2009). Also, the present results showed that all fish consolidated their memory since the amount of time spent on the light side was significantly lower during training than during the test. The aversive situation generated by electric shocks made the fish associate the dark area with danger. However, exposure to RWG affected the performance of memory consolidation since the length of time spent in the light side was reduced when compared to the other treatments. The major mechanism involved in memory consolidation is the repeated excitation of hippocampal cells, through the stimulation of glutamate receptors AMPA (α-amino-3 receptors hydroxy-5-methylisoxazole-4-propionic) and NMDA (N-methyl-D-1aspartato) (Izquierdo 2011). It is known that these receptors regulate excitatory neurotransmission in the brain, playing an important role in neural plasticity, neural development and neurodegeneration (Nakanishi and Masu 1994). The hippocampus has been a focus of attention regarding the potential formation of LTM. Any change in the excitation or inhibition of these receptors may affect memory consolidation. Although we did not investigate the mechanism that underlies the negative effect of RWG on LTM in *J. multidentata*, we suspect that the herbicide might affect the glutamatergic pathway or glutamate receptors, as was evidenced by Cattani et al. (2014) in the hippocampus of rats exposed to Roundup.

J. multidentata shows sexual dimorphism and courting behaviour that facilitates the identification of males and females and also the investigation of reproductive activity. Adult males present a gonopodium (copulatory organ), constituted by a modification of the anal fin (Galindo-Villegas and Sosa-Lima 2002), which is introduced into the female genital pore (gonopore) to achieve copulation. The mating behaviour is coercive; males approach females from behind and try to introduce the gonopodium in the female gonopore (Bizarra et al. 2000). The energy invested by males in this process is necessary to transfer their genes and perpetuates the species. Because the reproductive behaviour of *J. multidentata* is well characterized and easy to view, this species has been indicated as a good model for the evaluation of contaminants on the reproductive success of fish (Cazenave et al. 2005; Amé et al. 2009; Hued et al. 2012; Sánchez et al. 2017).

All the herbicides affected the positive response of males to the presence of females. However, RT was identified as the most toxic formulation since it was the only formulation that significantly reduced TP, NP, CA and C and also C/NP. Results suggest that male fish exposed to RT were less effective at the time of copulation since they invested much more energy in pursuing and attempting to copulate with the female and that effort did not always end successfully. Our data corroborates the data from Hued et al. (2012) that reported a decrease in capacity and the number of copulations of male *J. multidentata* exposed to sublethal concentrations of 0.5 mg/L of Roundup Max® Granular. The success of the male sexual activity is directly related to levels of testosterone. In this connection, it is known that cytochrome P450 aromatase is the only enzyme that converts testosterone (T) into 17β-oestradiol, playing a pivotal role in testosterone levels. The aromatase activity in the brain of teleost fish is higher than in other vertebrates, indicating its importance not only for the maintenance of testosterone levels but also for all the physiological processes involving testosterone (T) and 17 β-oestradiol (Diotel et al. 2010). In addition, other studies have shown that exposure to glyphosate-based formulations and co-adjuvants alters aromatase expression in fish as a result of toxicity (Defarge et al. 2016; Gasnier et al. 2009; Romano et al. 2010). Taken together, these pieces of evidence suggest it is possible that some component of RT's formulation influences aromatase expression or activity, affecting the sexual activity of *J. multidentata* males. However, it was not measured in this study. In addition, Cortés et al. (2016) reported that Roundup affected the olfactory bulb of the fish *Piaractus brachypomus* and they proposed that the olfactory system is a route of herbicide contamination. The olfactory system is directly involved in vital processes such as social interaction, feeding, predator defence and reproduction (Hamdani and Doving 2007). Another hypothesis is that perhaps the olfactory bulb of *J. multidentata* males is damaged by exposure to Roundup, affecting the ability of males to identify female olfactory signals (pheromones).

Finally, all formulations decreased the GSI in relation to the control group. This effect was greater in fish exposed to RWG. The GSI is a morphological biomarker that has been considered by several authors as an indicator of gonadal development and maturation in fish (Isaac-Nahum and Vazzoler 1987; Vazzoler et al. 1989; Grier and Taylor 1998). The results regarding the GSI may be associated with the sperm quality profile report by Sánchez et al. (2017), who showed a decrease in motility and sperm concentration in *J. multidentata* exposed to RO, RT and RWG. Other studies focusing on aspects of sperm quality have shown that glyphosate and Roundup® in different concentrations, including some that are environmentally relevant, cause negative effects on the quality of spermatozoa of *D. rerio*, *Poecilia vivipara* and *J. multidentata* (Harayashiki et al. 2013; Lopes et al. 2014; Sánchez et al. 2017). Such effects on the gonads may be reflected in males' sexual behaviour since they produce hormones that control sexual performance and success.

Conclusions

Roundup formulations greatly affect the behavioural patterns of *J. multidentata*, which can seriously influence their long-term survival, considering that this species inhabits areas where these herbicides are often released into the environment. Comparison between herbicides indicates that RWG is the most damaging formulation, taking into account fish social interaction, space exploration, swimming performance and consolidation of LTM, while findings regarding sexual behaviour in adult males showed that exposure to RT has much more severe effects. These differences reinforce the idea that the adjuvants present in the formulations have different targets of toxicity. Ultimately, this study presents sensitive and ecologically relevant endpoints to access the effects of Roundup exposure.

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Authors' contributions Jessica Andrea Albañil Sanchéz and Camila De Martinez Gaspar Martins conceived the study and were in charge of the overall outline and planning. Camila De Martinez Gaspar Martins was responsible for obtaining financial support and managing it. Jessica Andrea Albañil Sanchéz performed all the experiments and analysed the results. Daniela Martí Barros, Maria de los Angeles Bistoni, Maria Laura Ballesteros and María Angelina Roggio provided critical feedback and helped shape the research. Jessica Andrea Albañil Sanchéz drafted this manuscript and designed the figures, and Camila De Martinez Gaspar Martins and Maria de los Angeles Bistoni contributed to finalizing the manuscript and its revisions.

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Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethical approval All procedures with animals presented here complied with ethical standards (CEUA-FURG, reference Pq015/2013).

Consent to publish Not applicable.

Conflict of interest The authors declare that they have no conflict of interest.

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