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Glyphosate and Roundup® alter morphology and behavior in zebrafish

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ABSTRACT

Glyphosate has become the most widely used herbicide in the world, due to the wide scale adoption of transgenic glyphosate resistant crops after its introduction in 1996. Glyphosate may be used alone, but it is commonly applied as an active ingredient of the herbicide Roundup®. This pesticide contains several adjuvants, which may promote an unknown toxicity. The indiscriminate application poses numerous problems, both for the health of the applicators and consumers, and for the environment, contaminating the soil, water and leading to the death of plants and animals. Zebrafish (*Danio rerio*) is quickly gaining popularity in behavioral research, because of physiological similarity to mammals, sensitivity to pharmacological factors, robust performance, low cost, short spawning intervals, external fertilization, transparency of embryos through larval stages, and rapid development. The aim of this study was evaluate the effects of glyphosate and Roundup® on behavioral and morphological parameters in zebrafish larvae and adults. Zebrafish larvae at 3 days post-fertilization and adults were exposed to glyphosate (0.01, 0.065, and 0.5 mg/L) or Roundup® (0.01, 0.065, and 0.5 mg/L) for 96 h. Immediately after the exposure, we performed the analysis of locomotor activity, aversive behavior, and morphology for the larvae and exploratory behavior, aggression and inhibitory avoidance memory for adult zebrafish. In zebrafish larvae, there were significant differences in the locomotor activity and aversive behavior after glyphosate or Roundup® exposure when compared to the control group. Our findings demonstrated that exposure to glyphosate at the concentration of 0.5 mg/L, Roundup® at 0.065 or 0.5 mg/L reduced the distance traveled, the mean speed and the line crossings in adult zebrafish. A decreased ocular distance was observed for larvae exposed at 0.5 mg/L of glyphosate. We verified that at 0.5 mg/L of Roundup®-treated adult zebrafish demonstrated a significant impairment in memory. Both glyphosate and Roundup® reduced aggressive behavior. Our data suggest that there are small differences between the effects induced by glyphosate and Roundup®, altering morphological and behavioral parameters in zebrafish, suggesting common mechanisms of toxicity and cellular response.

1. Introduction

Roundup® is the most popular and widely used herbicide in the majority of the world (Roberts et al., 2010; Wang et al., 2016; Gallardo et al., 2016). It is largely used in agriculture, forestry and horticulture (including domestic use) (Uren Webster et al., 2014). The indiscriminate use of Roundup® associated with careless handling, accidental spillage or discharge of untreated effluents into natural waterways has caused harmful effects on aquatic life and may promote long-term biological effects yet to be discovered (Moustafa et al., 2016; Gallardo et al., 2016).

Glyphosate is the primary active ingredient present in Roundup® (Monsanto Co., St. Louis, MO, USA). However, the actual application mixture also contains what is referred to as "inert" or "inactive" ingredients (Negga et al., 2011; Cox, 1998; Williams et al., 2000). Despite the classification of inocuous, the commercial formulation has greater side effects than glyphosate alone (Cavalli et al., 2013). The introduction of glyphosate-resistant crops in the late 1980s increased exponentially the use of glyphosate-containing herbicides (Araujo et al., 2014). In 1987, glyphosate was the 17th most used pesticide in the United States, and by 2001, it became the most applied herbicide (Negga et al., 2011).

The wide applications of glyphosate and its relatively long half-life in water (most commonly 45–60 days) will lead to its constant presence

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in coastal waters (Annett et al., 2014). Studies have characterized the effects of individual glyphosate-based herbicide formulations on a wide variety of aquatic organisms, including microorganisms (Arunakumara et al., 2013; Vendrell et al., 2009), invertebrates (Kreutzweiser et al., 1989; Folmar et al., 1979), amphibians (Edge et al., 2013; Relyea, 2005), and fish (Uren Webster and Santos, 2015; Mitchell et al., 1987), which indicated diverse physiological and behavioral effects depending on the dose and formulation.

Danio rerio, commonly known as zebrafish, is a tropical freshwater fish. It was previously a well-known domestic fish, which has rapidly become an indispensable animal model for scientists of today's world. The usage of zebrafish in scientific research could be seen playing significant roles in fundamental areas of research, such as toxicology (Zoupa and Machera, 2017; Alestrom et al., 2006; Beis and Stainier, 2006; Ingham, 2009). In addition, studies with other pesticides have already shown that behavioral changes in this animal are good markers for evaluating toxicological mechanisms triggered by exposure to these agents (Altenhofen et al., 2017; Pereira et al., 2012; Schmidel et al., 2014). The numerous advantages and characteristics of this small animal have contributed for the growing interest in this animal model for the biomedical research. Thus, the aim of this study is to evaluate the effects of exposure to glyphosate and Roundup® on morphological and behavioral parameters on zebrafish during the larval and adult stages, comparing the isolated active form and the commercial form.

2. Materials and methods

2.1. Animals and maintenance

Zebrafish wild-type (*Danio rerio*) adults (6–8 months, 0.2–0.4 g) were obtained from a local commercial supplier (Red Fish, Porto Alegre, Brazil) and acclimated for at least 2 weeks in the experimental room before the experiments. Animals were housed in a 30 L- thermostated aquarium filled with unchlorinated water constantly aerated at a targeted temperature of 26 ± 2 °C. Fish were kept under a 14-h light/10-h dark cycle photoperiod (lights on at 7:00 am) and were fed three times a day with commercial flake fish food (Alcon BASIC®, Alcon, Brazil), supplemented with brine shrimp (*Artemia* sp.).

At least one week prior to training, animals were transferred to 25 L temporary housing tanks in the task room to minimize further changes in context during the experiment. The housing tank mimicked the conditions mentioned above and had a glass partition that allowed manipulated and non-manipulated fish to be maintained separated during each experimental session and y*et al*lowed animals to be maintained among their original group during the investigation. This strategy was adopted to minimize animal stress due to isolation and its eventual impacts on behavioral responses. Feeding was not interrupted during the experimentation and all sessions were performed at morning. On each session animals were gently captured from the temporary housing tank using a 6 cm wide fine nylon mesh fish net.

Water used in the experiments was obtained from a reverse osmosis apparatus (18 MOhm/cm) and was reconstituted with marine salt (Crystal Sea, Maninemix, Baltimore, USA) at 0.4 ppt. The total organic carbono concentration was 0.33 mg/L. The total alkalinity (CO_3^{2-}) was 0.030 mEq/L. During fish maintenance, water parameters were monitored daily and maintained in the following ranges: pH: 6.5–7.5, conductivity: 400–600 S, ammonium concentration: < 0.004 ppm, and temperature: 25 to 28 °C. All protocols were approved by the Institutional Animal Care Committee (06/2016, CEUA-PUCRS).

2.2. Exposures

2.2.1. Larval exposure

Embryos were obtained from our breeding colony. For breeding, 1 female and 2 males per aquaria were placed in breeding tanks overnight in which the sexes were separated by a transparent barrier. After the lights went on in the following morning, the barrier was removed. Fertilized eggs were used for the experiments with larvae. Embryos were collected, sanitized and, 3 days after, they were subjected to the exposure.

After sanitized, embryos were kept for 3 days in six-well plates (10 embryos per well at a density of 1 embryo per 2 mL). Larvae (3 dpf) were placed in Petri dishes (10 larvae per dish at a density of 1 larvae per 2 mL), and subjected to Roundup® (Monsanto Company, Marysville, OH, USA) or glyphosate (Sigma-Aldrich, St. Louis, MO) exposure at nominal concentrations of 0 (control group), 0.01, 0.065 and 0.5 mg/L for 96 h (solutions were not changed during this period). The third day post-fertilization was chosen as it is the well described period during zebrafish development in which the majority of eggs hatch (Kalueff et al., 2014), allowing direct exposure to herbicides. Only larvae that hatched in the third dpf were used in the experiments. The three concentrations were chosen to represent concentrations that may occur in the environment (0.01 mg/L) or during occasional peak contamination events (0.5 mg/L) (Uren Webster and Santos, 2015). Animals were monitored daily for survival as determined by the lack of heartbeat visualized under a stereoscope.

2.2.2. Adult exposure

Adult animals, aged between 6 and 7 months, were exposed to Roundup® (Monsanto Company, Marysville, OH, USA) or glyphosate (Sigma-Aldrich, St. Louis, MO, USA) nominal concentrations of 0 (control groups), 0.01, 0.065 and 0.5 mg/L (Uren Webster and Santos, 2015) in 2 L aquarium (10 animals per tank) for 96 h (solutions were not changed during this period).

2.3. Morphological defects

Morphology evaluation was performed by monitoring morphological defects in larvae under a stereomicroscopy at 7 days post-fertilization (dpf). Body length (μm), ocular distance (μm), and surface área of the eyes (μm^2) were evaluted using NIS-Elements D software for Windows 3.2 (Nikon Instruments Inc., Melville, USA). Body length was estimated using the method described by Capiotti et al. (2011), with modifications; the distance from the larval mouth to the pigmented tip of the tail was measured. The ocular distance was evaluated by the distance between the inner edge of the two eyes (similar to the inner intercantal distance in humans), and the size of the eyes was determined by measuring the surface area of the eyes (Lutte et al., 2015).

2.4. Behavioral analyses

2.4.1. Exploratory behavior of larvae

The exploratory behavior of the larvae was based on Altenhofen et al. (2017) and evaluated at 7 dpf. The experiments were performed in a temperature-controlled room (27 ± 2 °C) between 1 p.m – 5 p.m. Each larva was individually placed in a 24-well cell culture plate containing 2 mL of water per well, and the total distance traveled of each animal were evaluated (N = 30). After a 60-s habituation, the sessions were recorded for five minutes for later analysis using ANY-Maze tracking software (Stoelting Co., Wood Dale, IL, USA).

2.4.2. Bouncing-ball avoidance behavior of larvae

Immediately after the exploratory behavior, larvae were placed in 6-well plate (5 larvae per well, N = 30) over a LCD monitor for cognitive ability and avoidance responses to a visual stimulus (a 1.35 cm diameter red bouncing ball) during a 5-min session after a 2-min acclimation (Pelkowski et al., 2011; Nery et al., 2014). The red bouncing ball travelled from left to right over a straight 2 cm trajectory on half of the well area (stimuli area), which animals avoided by swimming to the other non-stimuli half of the well. The number of larvae on the non-stimuli area during the 5-min session was considered indicative of their cognitive ability.

2.4.3. Adult exploratory behavior

The exploratory behavior of the adults was based on Gerlai et al. (2000) and Altenhofen et al. (2017). Adult exploration was evaluated at 96 h after the start of exposure. The experiments were performed in a temperature-controlled room (27 ± 1 °C) between 9 a.m – 1 p.m. Animals were placed individually in experimental tanks (30 cm length × 15 cm height × 10 cm width), and after 60 s of habituation, their locomotor behavior was recorded for five minutes. The videos were analyzed using the ANY-Maze software. The behavioral parameters analyzed were: distance traveled, mean speed, time mobile, line crossings and time spent in upper zone. The time spent in upper zone can indicate an anxiolytic-like behavior (Levin et al., 2007).

2.4.4. Adult aggressive behavior

The aggressive behavior was estimated using the method described by Gerlai et al. (2000) and Gerlai (2003), with modifications. Each fish was placed in an experimental tank (30 cm length × 15 cm height × 10 cm width). A mirror (45 cm × 38 cm) was placed at the side of the tank at an angle of 22.5° to the backwall of the tank so that the left vertical edge of the mirror touched the side of the tank and the right edge was further away. Thus, when the experimental fish swam to the left side of the tank, their mirror image appeared closer to them. A test fish was added to the tank and was allowed acclimate for 60 s; the aggressive behaviors that a fish conducted toward its mirror image were subsequently recorded over a period of 5 min. The vertical lines divided the tank into four equal sections and allowed the number of entries to each section made by the fish to be counted. Entry to the left-most segment indicated preference for proximity to the “opponent”, whereas entry to the right most segments implied avoidance. The amount of time the experimental fish spent in each segment was measured using ANY-Maze recording software.

2.4.5. Aversive memory in adults

The inhibitory avoidance test was evaluated using a glass tank (18 cm lenght × 9 cm width × 7 cm height), divided in two equally sized compartments, designated hereon as dark and white and divided by a sliding guillotine-type partition (9 cm × 7 cm) (Blank et al., 2009). Compartments were defined by opaque plastic self-adhesive films in black or white colors externally covering walls, floor and the corresponding sides of the partition. Two electrodes extending through the wall height and placed on each far side of the opposing side walls of the dark compartment were attached to an 8 V stimulator and administered a final 3 ± 0.2 V AC shock (intensity measured between electrodes and the center of the dark compartment) when manually activated. Zebrafish were trained and tested individually in the inhibitory avoidance apparatus. Animals were gently placed in the white side of the task tank while the partition between compartments was closed. After 1 min of familiarization with the new environment, the partition was raised, allowing fish to cross to the dark side of the tank through the 1 cm high opening. On training session, when animals entered the dark side with their entire body the sliding partition was closed and a pulsed electric shock administered for 5 s. Fish were then removed from the apparatus and placed in the dedicated compartment of the temporary housing tank. Animals were tested 24 h after training. The test session repeated the training protocol except that no shock was administered and animals immediately removed from the dark compartment. The latency to completely enter the dark compartment was measured on both sessions and the test latencies used as an index of retention.

2.4.6. Statistical analysis

All data were presented as mean ± S.E.M, except for larval survival that is presented as percentages. Larval survival during the four experimental days was analyzed by Kaplan-Meier analysis. Differences in locomotor parameters (larvae at 7 dpf and adults after 96 h of exposure) were evaluated by one-way analysis of variance (ANOVA) followed by post-hoc comparisons using Tukey corrections. Inhibitory avoidance training and test latencies for each group were compared by the Wilcoxon matched pairs test. For all comparisons, the significance level was set at $p < 0.05$.

3. Results

3.1. Glyphosate and Roundup® exposure in zebrafish larvae

3.1.1. Survival

We investigated the effect of glyphosate or Roundup® exposure on survival and morphology at 24, 48, and 72 h after the beginning of the exposure. Data for survival evaluation were analysed by Kaplan Meier survival test ($p = 0.6275$, N = 36). The results evaluated at 72 h demonstrated that animals exposed to concentrations of 0.01, 0.065, and 0.1 mg/L showed survival percentages of 92%, 96% and 91%, respectively. Animals exposed the same concentrations of Roundup® showed survival percentages of 91%, 90% and 91%, respectively. There were no differences between any group and control (survival of 93%). At 24 and 48 h there were no differences between groups (data not shown).

3.1.2. Exploratory behavior

The exploratory behavior of the larvae was examined at 7 dpf to determine whether glyphosate and Roundup® exposure could alter larvae locomotion and orientation. The distance traveled of animals exposed to glyphosate or Roundup® at concentration 0.01 and 0.5 mg/mL was decreased when compared to the control group ($F_{(6,197)} = 4.143$; $p = 0.0006$; Fig. 1a). The parameter of absolute turn angle ($F_{(6,197)} = 8.7662$; $p < 0.0001$; Fig. 1b) was decreased in the 0.01 mg/L glyphosate and Roundup® groups. The animals exposed to Roundup® concentration of 0.065 and 0.5 mg/L had increased time mobile ($F_{(6,197)} = 8.343$; $p < 0.0001$; Fig. 1c) compared with the control group.

3.1.3. Aversive behavior

The cognitive escaping responses from an aversive stimulus was evaluated and it was observed a significant effect of groups exposure ($F_{(6,308)} = 8.925$; $p < 0.0001$; Fig. 2) with glyphosate and Roundup®. The findings demonstrated an increase in the number of animals in non-stimuli area in the concentrations of 0.1 (89%), 0.065 (86%) and 0.5 mg/L (88%) glyphosate and 0.065 (95%) and 0.5 mg/L (90%) Roundup®, when compared with the control (76%).

3.1.4. Morphological evaluation

The teratogenic effects of glyphosate and Roundup® on larvae morphology were evaluated at 7 dpf. There were significant decreases in body length with all concentrations of Roundup® ($F_{(6,197)} = 9.301$; $p < 0.0001$; Fig. 3a) and only a significant reduction in ocular distance when compared with the control group at concentration 0.5 mg/L of glyphosate ($F_{(6,197)} = 2.582$; $p = 0.0198$; Fig. 3b). There were no differences in surface area of the eyes between the control and either glyphosate or Roundup® exposed groups at all concentrations ($F_{(6,197)} = 3.367$; $p = 0.0035$; Fig. 3c).

3.2. Glyphosate and Roundup® exposure in zebrafish adults

3.2.1. Exploratory behavior

The behavior pattern of adult animals was analyzed after 96 h of exposure to glyphosate and Roundup®. Expsosure to 0.5 mg/L glyphosate and 0.065 and 0.5 mg/L Roundup® decreased the distance traveled ($F_{(6,105)} = 5.728$; $p < 0.0001$; Fig. 4a), mean speed ($F_{(6,105)} = 6.042$; $p < 0.0001$; Fig. 4b) and the number of line crossings ($F_{(6,105)} = 4.769$; $p = 0.0002$; Fig. 4d) when compared to the control group. There were no differences in time mobile between the control and the glyphosate or Roundup® exposed groups at all concentrations. ($F_{(6,105)} = 0.9546$; $p = 0.4597$; Fig. 4c).

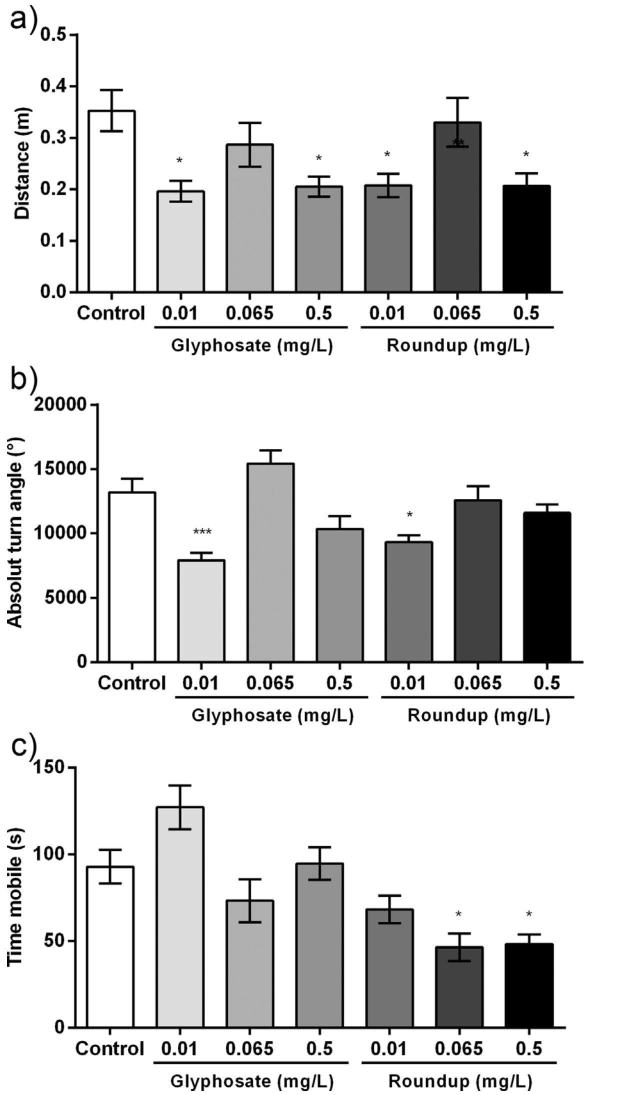

Fig. 1. Exploratory behavior of glyphosate and Roundup®-treated zebrafish larvae. Distance traveled (a), absolute turn angle (b), time mobile (c). Data are expressed as the mean ± S.E.M. from 36 animals analyzed individually for each group and were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc comparisons using Tukey corrections. (* to $p < 0.05$, *** to $p < 0.001$).

3.2.2. Memory

There was no impairment in memory at all concentrations of

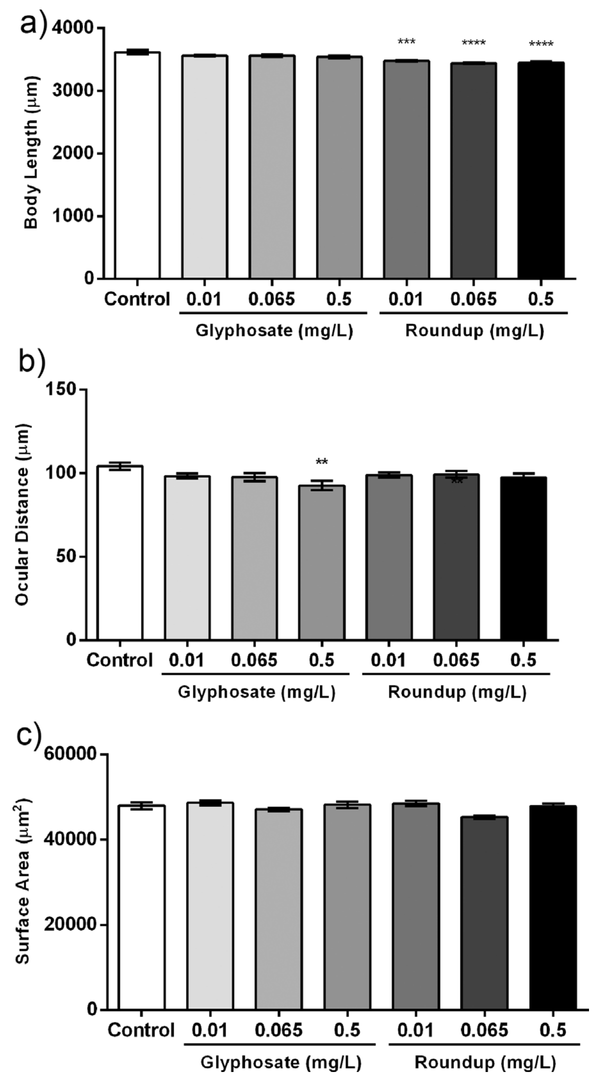

Fig. 3. Morphological parameters of control, glyphosate and Roundup®- treated zebrafish larvae. Surface area (a), body length (b) and ocular distance (c). Data are expressed as mean ± S.E.M. from 30 animals analyzed individually for each group and were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc comparisons using Tukey corrections. (** to $p < 0.01$, *** to $p < 0.001$, **** to $p < 0.0001$).

animals exposed to glyphosate (0.01, 0.065, and 0.5 mg/L) ($U = 0.50$, $p < 0.0001$; $U = 9.00$, $p < 0.0001$; $U = 29.00$, $p < 0.0002$, respectively) and animals exposed to Roundup® at the lower

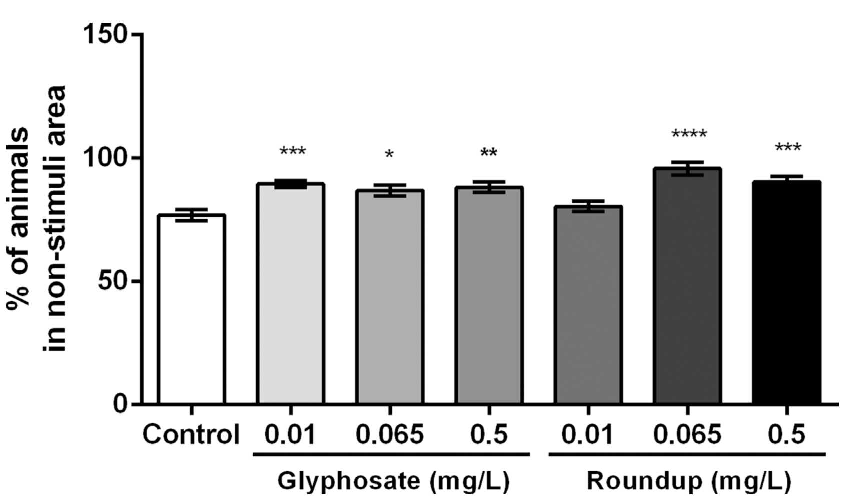

Fig. 2. 7 dpf larvae escape behavior from an aversive stimulus (charts were plotted with means ± S.E.M. (n = 45 per group), escape responses to a non-stimuli area). Data analyzed by one-way analysis of variance (ANOVA) followed by post-hoc comparisons using Tukey corrections. The animals exposed to glyphosate and Roundup® showed diminished escape responses when compared to control group (* to $p < 0.05$, ** to $p < 0.01$, *** to $p < 0.001$, **** to $p < 0.0001$).

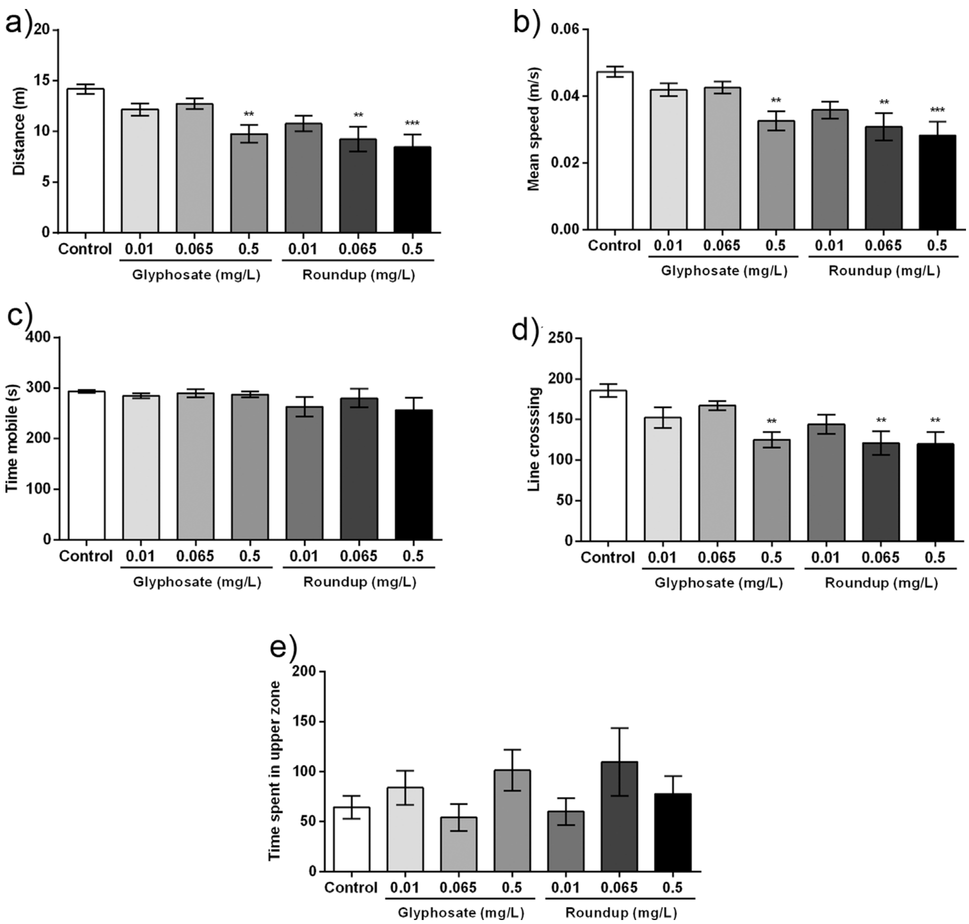

Fig. 4. Exploratory behavior of control and glyphosate and Roundup®-treated adult zebrafish. Distance traveled (a), mean speed (b), time mobile (c), line crossing (d) and time spent in upper zone (e). Data are expressed as the mean ± S.E.M. from 16 animals analyzed individually for each group and were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc comparisons using Tukey corrections. (** to $p < 0.01$, *** to $p < 0.001$).

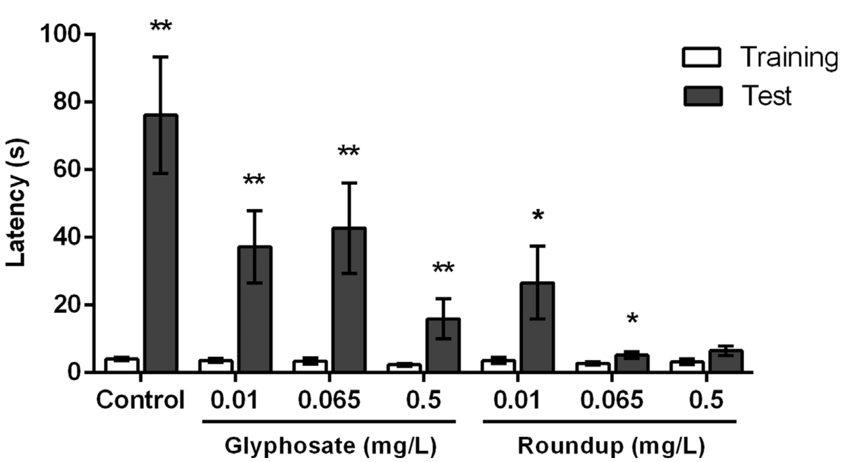

Fig. 5. Inhibitory avoidance task performance on training and long-term memory test sessions of control and glyphosate and Roundup®-treated adult zebrafish after 96 h of exposure. Data are presented as mean ± S.E.M from 17 animals analyzed individually for each group. (* to $p < 0.05$, ** to $p < 0.01$). No differences were found between training performance among in 0.065 and 0.5 Roundup® treated groups as evaluated by Kruskal–Wallis test.

concentration tested (0.01 mg/L) and intermediate concentration showed no memory impairment ($U = 50.00$, $p < 0.001$; $U = 65.00$, $p < 0.05$, respectively), while animals exposed to the higher concentration showed memory impairment ($U = 67.50$, $p > 0.05$) compared to the control group ($U = 15.00$, $p < 0.0001$) (Fig. 5).

3.2.3. Aggression

Glyphosate- exposed groups to 0.01 (91%), 0.065 (93%) and 0.5 mg/L (93%) and Roundup® -exposed groups at all concentrations 0.01 (85%), 0.065 (86%) and 0.5 mg/L (86%) remained less time in the segment nearest to the mirror ($F_{(6,169)} = 4.7108$; $p = 0.0007$; Fig. 6a) when compared with the control group (%). There was a significant decrease on the number of entries into the mirror contact zone, when compared with the control group. ($F_{(6,203)} = 7.438$; $p < 0.0001$; Fig. 6b).

4. Discussion

Aquatic contamination by herbicides can occur as result of direct spraying, or during heavy rainfall or leaching of agricultural fields (Roy et al., 2016; Benachour and Seralini, 2009). Glyphosate has been regularly detected in a diversity of water bodies (Mercurio et al., 2014), and its presence in surface waters has been found 60 days after the formulation was applied, which indicates that this compound can persist in the environment (Roy et al., 2016). Moreover, up to approximately 0.04 mg/L glyphosate has been reported to occur in rivers near

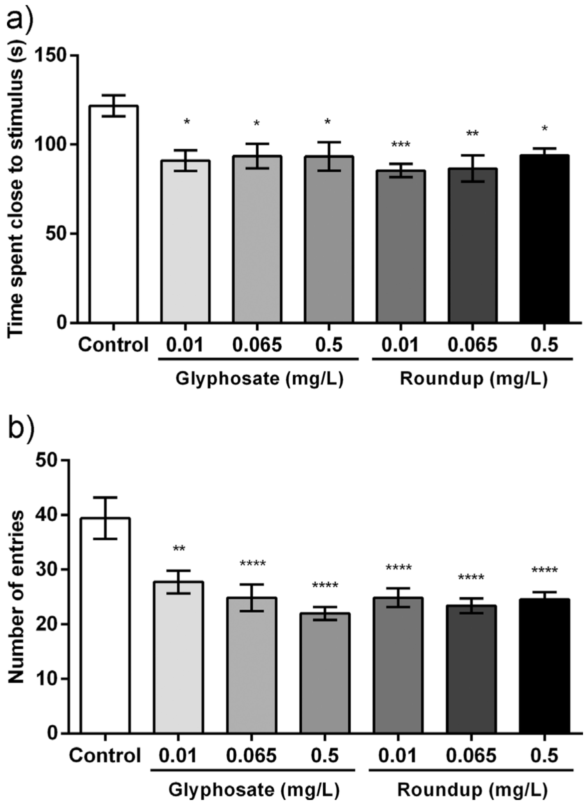

Fig. 6. Effects of glyphosate and Roundup®-induced aggression deficits in zebrafish. The data are expressed as the mean ± S.E.M. (n = 26 per group), and were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc comparisons using Tukey corrections. (* to $p < 0.05$, ** to $p < 0.01$, *** to $p < 0.001$, **** to $p < 0.0001$).

urban run off and waste water treatment effluent. Therefore, exposure to non-target organisms is inevitable and is related to glyphosate's high water solubility, contaminating the aquatic microbiota, animals and fish. (Roy et al., 2016).

This study demonstrated that glyphosate or Roundup® exposure induces behavioral and morphological changes in different developmental stages of zebrafish. The exposure to different glyphosate and Roundup® concentrations, starting at 3 dpf during 96 h can cause small morphological alterations in zebrafish larvae. The findings showed that concentrations of glyphosate did not alter body length and surface area of the eyes. However, the exposure to highest concentration (0.5 mg/L) induced decrease in the ocular distance and this effect is probably maintained during the animal lifespan. The exposure to all concentrations of Roundup® (0.01, 0.065 and 0.5 mg/L) in early stages of development decreased body lenght. This study has shown that 96-h exposure to these herbicides causes morphological alterations in zebrafish larvae. A study by Zhang et al. (2017) also shows that from 100 to 400 mg/L of glyphosate exposure to zebrafish larvae (4 dpf) resulted in shorter body lengths, smaller eyes and heads, especially in 400 mg/L.

Studies also have shown that other neurotoxic agents can cause numerous morphological changes in zebrafish larvae. It has been observed that exposure during 120 h to the fungicide tebuconazole was able to increase the ocular distance in larvae that were exposed to 4 mg/L (Altenhofen et al., 2017). A similar effect was also verified where zebrafish larvae were exposed to concentrations up to 10 ppm (near the solubility limit in water) of atrazine during its development (from 1 to 120 hpf). The results showed that larvae in all atrazine exposures had a significant increase in head length compared with the control (Weber et al., 2013).

Our study demonstrated that exposure to glyphosate and Roundup® in the highest and lowest concentration (0.01 and 0.5 mg/mL) were able to alter the swimming behavior of zebrafish larvae, reducing the distance traveled. In addition, it is not possible to exclude that morphological changes may be affected the locomotion. This effect was also observed in the absolute turn angle of the animals in the lowest concentration (0.01 mg/L) tested, which indicates that both glyphosate and Roundup® are able to change the swimming pattern of larvae. Bortolotto et al. (2014) have associated absolute turn angle alterations with parkinson-associated symptoms in adult zebrafish exposed to paraquat. This parameter has been suggested as a sensitive measure of motor coordination (Blazina et al., 2013). In addition, the glyphosate causes behavioral changes in animals that were submitted to exposure in the larval stage (Uren Webster et al., 2014). Pesticides can alter exploratory parameters in zebrafish larvae. Andrade et al. (2016) showed that the fungicide carbendazim induced changes in the locomotor activity of zebrafish larvae at 120 hpf, with significant decrease in the distance moved was observed at concentrations above 0.8 g/L during the light period (Andrade et al., 2016). Imazalil fungicide exposure after 96 h, especialy at high concentrations, resulted in decreased locomotor activity in zebrafish larvae. Both distance and swimming speed were significantly lower in the 100 and 300 mg/L imazalil-treated groups than in the control group (Jin et al., 2016). Studies also showed that the distances of groups treated with 100 and 300 mg/L after 120 h exposure herbicide atrazine was significant lower than those in the control group (Liu et al., 2016; Pérez et al., 2013). Therefore, as observed for other pesticides, glyphosate and Roundup® are able to alter the locomotor pattern of zebrafish at early stages of development.

The aversive behavior showed a significant effect of glyphosate and Roundup® exposure. All groups, except for the lowest Roundup® concentration (0.01 mg/L) showed an increase in the time swimming in the unstimulated area when compared to control. This result is probably related to the decrease of the exploratory capability observed in the larvae; however, it cannot be excluded that the herbicide increased the ability to perceive danger and, therefore, the time spent away from the aversive stimulus. Considering this altered behavior, the larvae may have escaped the stimulus and remained longer in the unstimulated area of the plate. These results suggest that the decrease in the exploratory behavior of zebrafish larvae exposed to glyphosate or Roundup® may cause animals to be more susceptible to predation. In addition, exposure to glyphosate may alter the normal morphology and behavior of the larvae even at the concentration that is close to the environmental concentration of glyphosate (0.01 mg/L), indicating that environmental glyphosate may alter the morphology and decrease spontaneous movement of the larvae.

In adults, our findings demonstrated that glyphosate or Roundup® exposure reduced the distance traveled, the mean speed and the line crossings in fish exposed to the concentrations of 0.5 mg/L glyphosate and 0.065 and 0.5 mg/L of Roundup®. Other studies observed the change of this parameter with other toxic agents. Bortolotto et al. (2014) showed that the pesticide paraquat can alter locomotion parameters in zebrafish adults, resulting in decreased of locomotion and distance traveled 24 h after injection of this herbicide. Pereira et al. (2012) described that exposure to endosulfan, a broad spectrum organochlorine pesticide, decreased line crossings, distance traveled, mean speed, and body turn angle in adults zebrafish when compared with the control groups. Tilton et al. (2011) observed that chlorpyrifos, an organophosphate pesticide, significantly reduced the treated adult animal's swimming rate for 24 h. Our findings indicate that isolated or commercial forms of glyphosate are also able to modulate locomotion in adult zebrafish.

In addition, we found a significant impairment in long-term memory in the inhibitory prevention task for the highest concentration of Roundup®, suggesting that this pesticide induces memory impairment. In contrast to our findings, Pereira et al. (2012) observed that the exposure to 2.4 μg endosulfan/L for 96 h neither altered training session nor test session. The study of Balbuena et al. (2015) showed that after ingesting food contaminated with glyphosate impaired navigational memory in foraging honeybees. In this study, our results have shown that exposure to glyphosate or Roundup® decreases the time fish spent in the segment nearest to the mirror, indicating impairment in agressive behavior. Aggressive behavior and memory are very important regarding resources dispute, nesting sites dispute and mate dispute and this impairment could be very harmful to fish living in the wild.

The significant alterations observed concerns for the potential toxicity of this herbicide to fish populations inhabiting contaminated rivers. Our data raises concerns about the potential for environmental relevant concentrations of glyphosate and Roundup® to affect wild fish populations, even at concentrations that are found in the environment. Our data suggest that there are small differences between glyphosate and Roundup®, both altering morphological and behavioral parameters in zebrafish larvae whereas only behavior was impaired in adults.

Our results showed that both glyphosate and Roundup® are toxic to larvae and adult zebrafish. Several authors have suggested that the toxicity of Roundup® may be derived from synergistic effects between glyphosate and other formulation products, such as a surfactant that enhances the penetration of glyphosate through the plant cuticle (Cuhra et al., 2013). Folmar et al. (1979) compared the acute toxicity of technical-grade glyphosate acid, Roundup®, isopropylamine saltof glyphosate, and surfactant to several freshwater invertebrates and fishes. The authors observed that acute toxicity of the surfactant and Roundup® formulation were similar, which corroborates the findings of this study. An interesting perspective from this work is to investigate the neurobiological basis of the observed effects.

Our results indicate that the current toxicity levels allowed in water bodies causes zebrafish larvae to decrease its exploratory behavior. This could unbalancing fish population (e.g. increase predation or alter resources dispute). Therefore, it is necessary that more studies evaluate if the tolerable glyphosate levels in the water should be changed.

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