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Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water

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ABSTRACT

Although it is believed that glyphosate-based herbicides are relatively nontoxic to humans, its broad use in agriculture and consequent contamination of aquatic systems is a concern. In the present study, reproductive (sperm quality) and biochemical parameters (acetylcholinesterase and glutathione S-transferase activity, lipoperoxidation, and antioxidant capacity against peroxyl radicals) were evaluated in adult guppies (*Poecilia vivipara*) acclimated to fresh water and exposed (96 h) to environmentally realistic concentrations of glyphosate (130 and 700 $\mu g\,L^{-1}$) as the commercial formulation Roundup. Male guppies exposed to Roundup showed a poorer sperm quality, measured as reduced plasmatic membrane integrity, mitochondrial functionality, DNA integrity, motility, motility period and concentration of spermatic cells, than those kept under control condition (no Roundup addition to the water). Most of the spermatic parameters analyzed showed strong association to each other, which may help to understand the mechanisms underlying the observed reduction in sperm quality. Exposure to Roundup did not alter the biochemical parameters analyzed, though differences between genders were observed and deserve further investigations. Findings from the present study suggest that exposure to environmentally relevant concentrations of Roundup may negatively affect at long-term the reproduction of *P. vivipara*, with consequent changes in fish populations inhabiting environments contaminated with the herbicide.

1. Introduction

Roundup is a commercial formulation of glyphosate presented as the isopropylamine salt added by surfactants, usually polyoxyethylene amine (POEA), and inert compounds (WHO, 1994). Despite the potential effects of the other chemicals present in the Roundup formulation, glyphosate itself is a broad-spectrum post-emergent, systemic and non-selective herbicide. These characteristics led to a fast increase in the use of this herbicide in both agricultural and non-agricultural areas around the world (WHO, 1994, 2005). 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 have contributed to long-term biological effects (Jiraungkoorskul et al., 2002).

In a recent review, concentrations of Roundup, measured as glyphosate acid equivalents, in natural water bodies were reported to range between 0.01 and 0.7 $mg\,L^{-1}$, reaching the maximum value of 1.7 $mg\,L^{-1}$ in extreme situations after direct application of the herbicide into the water (Guilherme et al., 2010). Although it is believed that glyphosate-based herbicides are relatively nontoxic to humans (WHO, 1994; Zouaoui et al., 2013), the contamination of aquatic systems associated with the wide and indiscriminate use of these chemicals is now of great ecotoxicological concern (Lushchak et al., 2009).

In light of the described above, the identification of appropriated biomonitors and biomarkers related to the toxic effects

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of glyphosate-based herbicides is needed. In this context, fish species are described as suitable monitors of the effects of noxious compounds because of their ecological and economical relevance (Jiraungkoorskul et al., 2002). Additionally, changes at cellular and biochemical levels are among the most sensitive biological responses reported after fish exposure to aquatic pollutants (Glusczak et al., 2007; Sandrini et al., 2013). Therefore, studies focused on the biochemical and physiological effects of glyphosate-based herbicides in fish can provide not only useful toxicological information, but also help to select adequate biomonitors and biomarkers of glyphosate exposure and effect.

In Brazil, glyphosate has been used since 1978 (Galli and Montezuma, 2005). According to the National Health Surveillance Agency (ANVISA) (2010), since 2008 Brazil is the largest consumer of agrichemicals in the world. In fact, Silva et al. (2003) detected high concentrations of glyphosate in samples collected in water bodies near to areas of intense plantation in southern Brazil.

Although the intense use of glyphosate in Brazil, there is a lack of investigations using Brazilian native species to investigate the sublethal effects of this herbicide (Albinati et al., 2009).

With this background in mind, the sublethal effects of Roundup, a commercial formulation of glyphosate, were evaluated on a suite of physiological and biochemical parameters in the Brazilian guppy *Poecilia vivipara* (Bloch and Schneider, 1801). This fish species has been described as a promising biomonitor of aquatic pollution that has been employed recently in ecotoxicological studies with both inorganic and organic contaminants (Ferreira et al., 2012; Machado et al., 2013). Endpoints evaluated were selected based on their association with the short and long-term stability of fish populations as well as on the information they could provide to improve our knowledge on the mechanism involved in biological disturbances induced by glyphosate exposure and fish ability to counteract the effects and to detoxify the contaminant.

Considering the aspects above, the first endpoint evaluated in the present study was related to the response of sperm cell quality after fish exposure to sublethal concentrations of Roundup. In fact, reproduction is considered one of the most relevant biological functions related to long-term stability of fish populations. In this context, the effects of Roundup on several indicators of sperm quality, an early warning biomarker of reproductive disturbance, were evaluated. The potential impact of the herbicide exposure on fish behavior associated with change in neurotransmission function, measured through the brain and muscle acetylcholinesterase (AChE) activity, was also evaluated. Finally, some aspects related to the fish ability to detoxify the herbicide and its capacity to deal with the exposure to the contaminant were also considered. Therefore, the activity of glutathione S-transferase (GST), an enzyme involved in detoxification of organic contaminants, was analyzed as a measurement of detoxification ability. In turn, the total antioxidant capacity against peroxy-radicals (ACAP) and lipid peroxidation (LPO) were used as measurements of fish tissue capacity to protect against the oxidant effect of Roundup and the potential oxidative damage induced by exposure to the herbicide. The effects of Roundup on sperm quality and biochemical parameters (AChE, GST, ACAP and LPO) were evaluated in *P. vivipara* acclimated to fresh water and exposed to environmentally realistic concentrations of glyphosate (130 and 700 $\mu g\,L^{-1}$) as the commercial formulation Roundup.

2. Material and methods

2.1. Fish biology, collection and acclimation

P. vivipara is a guppy that belongs to the Poeciliidae family, being characterized as benthopelagic and non-migratory fish, behavior that allow its environmental exposure to several substances. It is euryhaline, being found in fresh water and estuarine environments along the coast of South America, from Venezuela to Argentina (Froese and Pauly, 2011). Indeed, it is one of the most common species of fish found in small ponds, rivers and coastal lagoon ecosystems of Brazil (Santos et al., 2011). For that reason, the National Institute of Science and Technology-Aquatic Toxicology (INCT-TA) recently has pointed *P. vivipara* as one of the priority species to access environmental health in Brazilian aquatic environments. Thus the findings of present work contribute simultaneously to better understand glyphosate effects on fishes as well as to establish standards biomonitors to South America.

Adults of *P. vivipara* were collected at the Gelo Creek (Cassino Beach, Rio Grande, RS, Southern Brazil) with nets and minnow traps. They were transferred to the animal care room of the Institute of Biological Sciences at the Federal University of Rio Grande (FURG) and acclimated for at least 7 days in continuously aerated and dechlorinated tap water. Room photoperiod (12L:12D) and temperature (28 °C) were fixed. Fish were daily fed with commercial food until apparent satiation. Feeding was stopped 24 h prior to the beginning of the experiments. Fish were fastening during the experimental period.

2.2. Fish exposure to Roundup

Due to the sexual dimorphism, 24 males [body length (mean ± standard deviation): 3.8 ± 1.2 cm; body weight: 0.54 ± 0.06 g; $n = 8$ fish per treatment] and 21 females (body length: 3.5 ± 0.9 cm; body weight: 0.41 ± 0.03 g; $n = 7$ fish per treatment) were individually kept under control condition (no Roundup addition into the water) or exposed (96 h) to Roundup (130 and 700 $\mu g\,L^{-1}$ of glyphosate). Both concentrations tested can be found in natural water bodies. According to Guilherme et al. (2010), the concentration of 700 $\mu g\,L^{-1}$ was the highest concentration detected in the environment. Considering that the lowest concentration found in natural water bodies (10 $\mu g\,L^{-1}$) was considered too low to induce a significant biological effect, an intermediary concentration (130 $\mu g\,L^{-1}$) potentially capable of inducing such effect, but considerably lower than the maximum concentration reported in the environment was tested. Other experimental conditions were kept as described above for the acclimation period (Section 2.1).

Every 24 h, exposure media were completely renewed. Before and after fish transfer to the experimental tank, water samples ($n = 24$) from control and treatments were collected, filtered (0.2 μm-mesh filter; Millipore, Merck; São Paulo, SP, Brazil), and stored at 4 °C in glass bottles until analysis.

After exposure, fish were euthanized by decapitation (AVMA, 2001) and tissue (brain, muscle, gills and liver) were dissected and stored at −80 °C for biochemical assays (Section 2.4). In males, testes were also dissected and immersed in Hanks balanced-salt solution (HBSS; 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$) for sperm analysis (Section 2.3).

The present work was approved by the Ethics Committee on Animal Use of Federal University of Rio Grande (CEUA – FURG; reference # P054/2011).

2.3. Sperm analysis

Testis samples were placed in 1.5 mL bullet tubes containing HBSS and shaken for the release of spermatozeugmatas (sperm bundles). Sperm was released by gently and repeatedly disrupting spermatozeugmatas with a 10-μL pipette tip (Sun et al., 2010). The sperm suspension was used for analyses described below.

For estimation of sperm motility and motility period, 10 μL of sperm suspension was placed on a glass microscope slide with a

cover slip. Sperm motility was estimated visually at 200× magnification using a phase contrast microscope (Olympus BX 51; América, São Paulo, SP, Brazil). Results of sperm motility were expressed as percentage of cells actively moving forward. Sperm vibration without effectively moving forward was not considered as being motile (Sun et al., 2010). The evaluation of the motility period, which is the period to achieve the complete lack of sperm motility, was performed in parallel with the sperm motility estimation. It was determined using a digital chronometer and expressed in seconds (Varela Junior et al., 2012). The sperm concentration was determined using a Neubauer chamber (Varela Junior et al., 2012).

Garner et al. (1986) have described the use of carboxyfluorescein diacetate (CFDA) and propidium iodide (PI), which are two fluorescent probes, to assess the plasma membrane integrity. A stock solution was prepared with 950 μL sodium citrate 3%, 20 μL PI (≥95%, Sigma–Aldrich, São Paulo, SP, Brazil), 20 μL CFDA (∼95%, Sigma–Aldrich, São Paulo, SP, Brazil) and 10 μL formaldehyde made up not more than 1 h before use. An aliquot (10 μL) of sperm suspension and 40 μL of stock solution were incubated for 10 min at 20 °C. After incubation, 10 μL of the mixture were placed on a glass microscope slide with a cover slip and the membrane integrity was verified under 400× magnification using an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil). For quantitative analysis of membrane integrity, 200 cells were counted and classified according to their color. Green cells indicated cells with intact plasma membrane while red or green/red cells were classified as injured cells (Harrison and Vickers, 1990).

Rhodamine 123 (Rh123) (≥95%, Sigma–Aldrich, São Paulo, SP, Brazil) was used to evaluate mitochondrial functionality. An aliquot (10 μL) of sperm suspension and 40 μL of Rh123 solution (13 μM) were incubated for 10 min at 20 °C. Evaluation was performed using an aliquot (1 μL) of the mixture placed on glass microscope slide with a cover slip. For quantitative assessment of mitochondrial functionality, 200 cells were counted under 400× magnification with an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil). Data were expressed as percentage. Cells exhibiting green fluorescence were classified as presenting functional mitochondria while sperm showing no fluorescence was classified as containing cells with dysfunctional mitochondria (He and Woods, 2004). The rate of mitochondrial functionality was determined by considering the proportion of sperm emitting green fluorescence compared with the total sperm analyzed (Varela Junior et al., 2012).

The acridine orange (AO) fluorescence method described by Tejada et al. (1984) was used to assess the DNA integrity of spermatic cells. Sperm smears were dried in air and fixed in Carnoy solution (3 parts of methanol and 1 part of glacial acetic acid). Slides were rinsed several times with distilled water after being dipped in citric acid solution (0.1 M; pH 2.5) and stained with AO solution (0.2 mg mL^{-1} in distilled water) during 5 min. Smears were washed again with distilled water and covered with a cover slip (Gandini et al., 2006). For quantitative analysis, 200 cells were counted under 400× magnification using an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil) without exceeding 1 min of slide exposure. Data were expressed as percentage. Sperm with green fluorescence were considered with normal DNA (bicatenary DNA) and those showing red, orange or yellow fluorescence were considered as having damaged DNA (monocatenary DNA, denatured).

2.4. Biochemical analyses

Tissue (brain, muscle, gill and liver) samples were homogenized in phosphate buffer (0.1 M; pH 7.75) and divided into aliquots for AChE activity in muscle and brain samples, and for LPO, ACAP and GST activity in muscle, gill and liver samples. Protein content in tissue homogenates was determined using a commercial reagent kit (Sigma–Aldrich, São Paulo, SP, Brazil) based on the Bradford's method (Bradford, 1976).

AChE activity was measured in brain and muscle samples using a spectrophotometric method following the yellow color yielded by thiocholine after reaction with the dithiobisnitrobenzoate ion (Ellman et al., 1961). Data were normalized considering the protein content in the tissue homogenate.

LPO was determined based on the reaction between the malondialdehyde (MDA) resulting from damage caused to lipids by free radicals and the 2-thiobarbituric acid (TBA) under conditions of high temperature and acidity. The chromogen generated was measured by spectrofluorometry (Oakes and Van Der Kraak, 2003). Data were normalized considering the wet mass (mg) of the tissue sample employed for analysis.

ACAP determination and data expression were performed as described by Amado et al. (2009). The method employed is based on the fluorometric detection of reactive oxygen species using 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) as substrate.

GST activity was determined by the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) following procedures described by Keen et al. (1976). Data were normalized considered the protein content in the tissue homogenate.

2.5. Glyphosate concentration in water samples

Glyphosate concentration in filtered water samples was determined by ion chromatograph (IC Compact 881, Metrohm, Herisau, Switzerland) with conductometric detector, using an ion exchange column (Metrosep A Supp 5 150/4.0) and a chemical suppressor. The mobile phase was made with 9.6 mmol L^{-1} of Na_2CO_3 and 3 mmol L^{-1} of $NaHCO_3$ degassed for 30 min in an ultrasound bath. The solution for suppressor regeneration was prepared with ultrapure water and 0.1 mol L^{-1} of sulfuric acid. A calibration curve (0.05–2.0 mg L^{-1} glyphosate) was built and used for glyphosate concentration determination in water samples from the experimental media. All injections were performed with a loop injection of 20 μL. The quantification and detection limit were 0.05 and 0.01 mg L^{-1}, respectively. The method showed good linearity for the calibration curve in both ultrapure water ($r^2 = 0.999$) and the matrix (dechlorinated tap water) ($r^2 = 0.998$) (Amarante et al., 2002; Queiroz et al., 2011). The selectivity was determined by injecting major anions (fluoride, chloride, bromide, sulfate and phosphate) in water, none of them showing overlaping with the retention time of glyphosate. Data collection and treatment was performed using the Software MagicNet 2.3 (Metrohm, Herisau, Switzerland).

2.6. Statistical analyses

Data were expressed as mean ± standard error (SEM). All statistical analyses were done using the software BioEstat 5.0. Data normality was verified using the Shapiro–Wilk test. Parametric data were analyzed using analysis of variance (ANOVA) followed by the Tukey test. In turn, non-parametric data were analyzed using the Kruskal–Wallis ANOVA followed by the Dunn test. For all analyses, the significance level adopted was 95% ($p < 0.05$). Also, the Spearman correlation coefficient was used for semen data analysis in order to determine the level of association among the parameters analyzed (Ayres et al., 2007).

3. Results

Water analysis data showed that control water used to prepare the exposure media did not contain detectable levels of

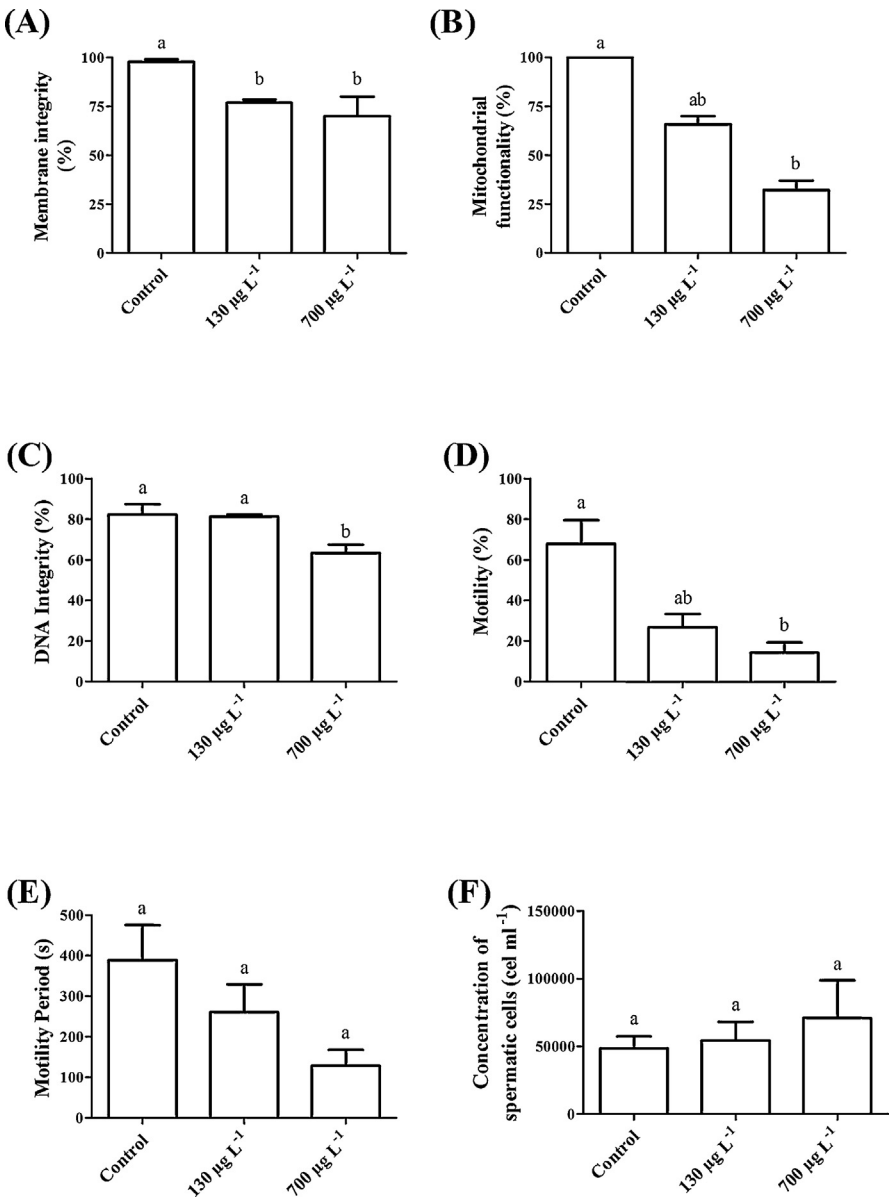

Fig. 1. Sperm quality in the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup. Values are means ± SEM. (A) Membrane integrity; (B) mitochondrial functionality; (C) DNA integrity; (D) motility; (E) motility period; and (F) cell density. Different letters represent significant difference among treatments ($p < 0.05$).

glyphosate (<0.05 mg L^{-1}). The concentration measured for the nominal 130 and 700 µg L^{-1} treatments were 144.8 ± 14.1 and 723.9 ± 21.8 µg L^{-1}, respectively. No fish mortality was observed over the experimental period.

Regarding spermatic responses, a decrease in the integrity of sperm plasma membrane was observed in fish exposed to any of the concentrations of Roundup tested when compared with fish from the control group. The effect observed was similar in both herbicide concentrations (Fig. 1A). Sperm mitochondrial functionality decreased with increasing concentrations of Roundup, being significantly lower in fish exposed to 700 µg L^{-1} of glyphosate than in control ones (Fig. 1B). Sperm DNA integrity was also reduced in male fish exposed to the highest concentration of Roundup tested (Fig. 1C). As observed for mitochondrial functionality, sperm motility was also reduced in fish exposed to 700 µg L^{-1} of glyphosate (Fig. 1D). Motility period showed a trend of reduction with increasing concentrations of Roundup, but no significant change was observed (Fig. 1E). Also, the spermatic cells density was not significantly altered by exposure to the concentrations of the herbicide and the experimental period tested (Fig. 1F).

Spearman correlation analysis showed an association between most of the sperm quality parameters analyzed, which may help to understand the mechanisms underlying the observed reduction in sperm quality. Roundup exposure was correlated with membrane integrity ($r^2 = 0.44$), mitochondrial functionality ($r^2 = 0.92$), DNA integrity ($r^2 = 0.38$), sperm motility ($r^2 = 0.66$) and motility period ($r^2 = 0.32$). In turn, membrane integrity was correlated with mitochondrial functionality ($r^2 = 0.37$) and sperm motility ($r^2 = 0.44$), while mitochondrial functionality showed association with DNA integrity ($r^2 = 0.39$), sperm motility ($r^2 = 0.56$) and motility period ($r^2 = 0.32$). Finally, sperm motility was correlated with the motility period ($r^2 = 0.42$) (Fig. 2).

With respect to biochemical parameters, no significant differences in AChE activity were observed in muscle (Fig. 3A) and brain

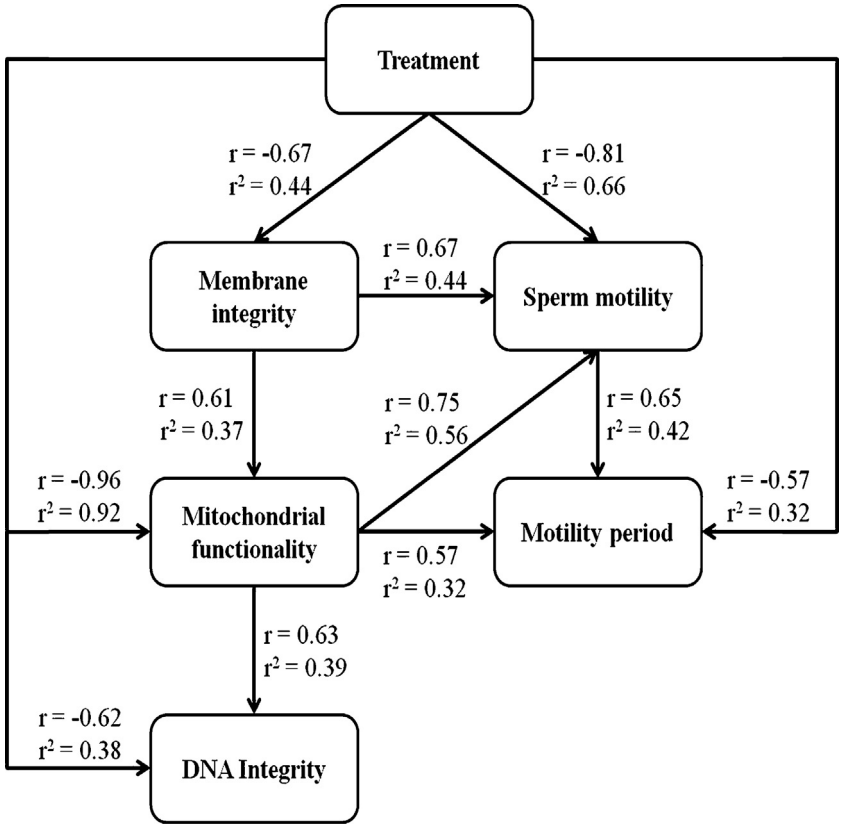

Fig. 2. Spearman correlation analysis for sperm quality data obtained with the guppy *Poecilia vivipara* exposed (96 h) to Roundup in fresh water. Only results showing statistical significance ($p < 0.05$) are shown.

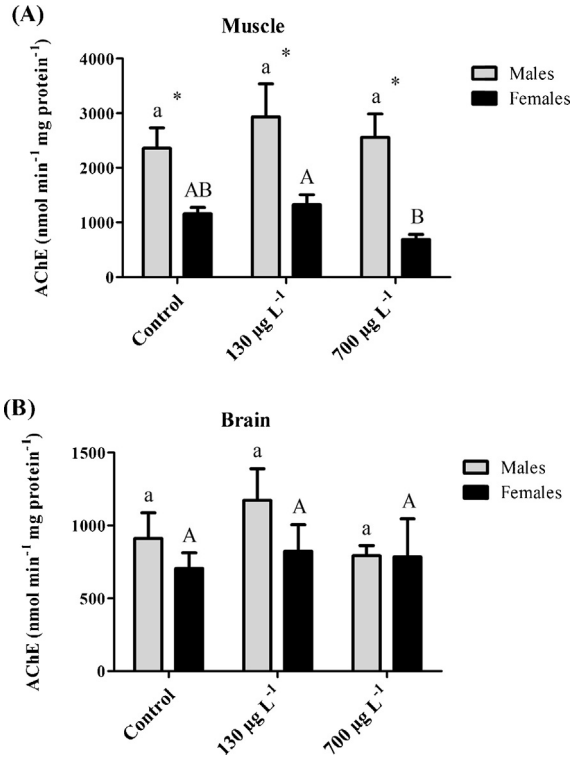

Fig. 3. AChE activity in (A) muscle and (B) brain of the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup in fresh water. Values are means ± SEM. Different lowercase and uppercase letters represent significant difference among treatments ($p < 0.05$) in males and females, respectively. *Significant difference ($p < 0.05$) between genders.

(Fig. 3B) of guppies kept under control conditions or exposed to Roundup. A similar result was observed for both male and female fish. However, the enzyme activity was lower in muscle of female fish exposed to 700 $\mu g\,L^{-1}$ of glyphosate than in those exposed to 130 $\mu g\,L^{-1}$ of glyphosate (Fig. 3A). Males showed higher levels of muscle AChE activity than females (Fig. 3A) while no significant difference in brain AChE activity was observed between genders (Fig. 3B).

No significant difference was observed in ACAP between control and Roundup-exposed fish. This lack of response was observed for muscle (Fig. 4A), gills (Fig. 4B), and liver (Fig. 4C) of both male and female fish. Control males showed higher gill ACAP than females (Fig. 4B). Females exposed to 130 $\mu g\,L^{-1}$ of glyphosate showed higher muscle ACAP than males (Fig. 4A), while males exposed to 700 $\mu g\,L^{-1}$ of glyphosate showed higher gill ACAP than females (Fig. 4B).

No significant effect of Roundup exposure was observed in muscle (Fig. 5A) and gill (Fig. 5B) LPO of male guppies. However, liver LPO was lower in male fish exposed to 700 $\mu g\,L^{-1}$ of glyphosate than in control male fish (Fig. 5C). In females, no significant difference was observed in tissue LPO between control and Roundup-exposed fish (Fig. 5). In all cases (control or Roundup-exposed fish), males showed higher liver LPO levels than females (Fig. 5C).

No significant effect of Roundup exposure was observed in GST activity in muscle (Fig. 6A), gills (Fig. 6B) and liver (Fig. 6C) of male and female guppies. In all groups (control and Roundup-exposed fish), females showed higher muscle GST activity than males (Fig. 6A). Also, control females showed higher gill GST activity than control males (Fig. 6B). No significant gender difference was observed in liver GST activity (Fig. 6C).

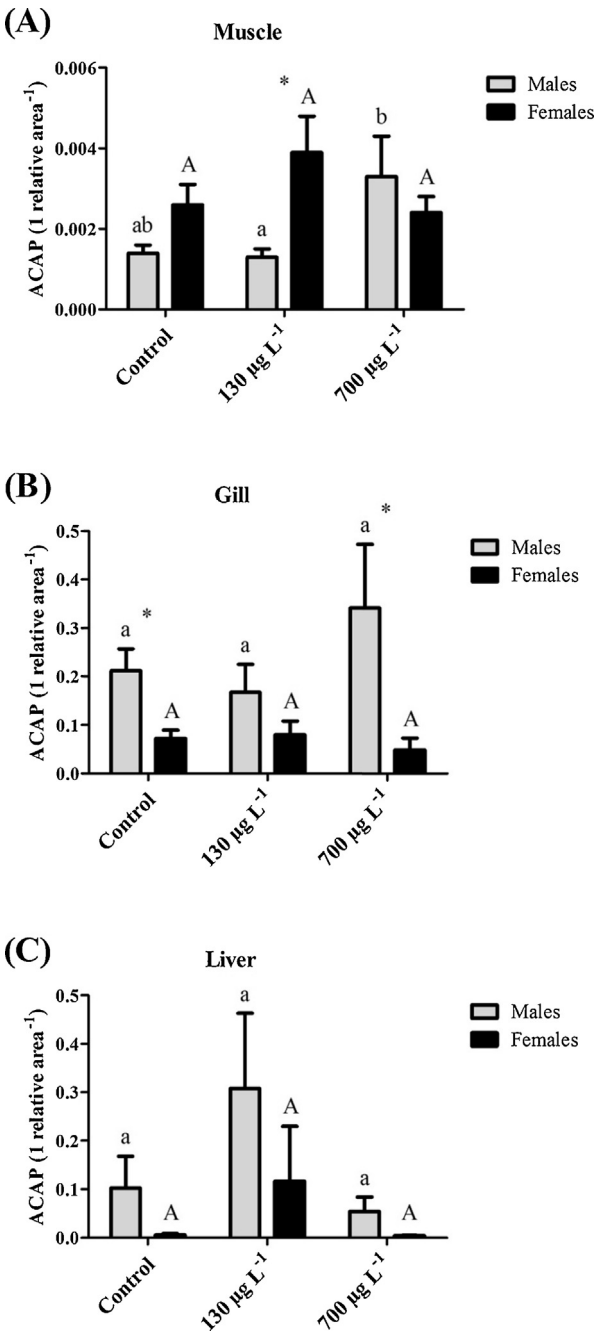

Fig. 4. ACAP in (A) muscle, (B) gill and (C) liver of the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup in fresh water. Values are means ± SEM. Different lowercase and uppercase letters represent significant difference among treatments ($p < 0.05$) in males and females, respectively. *Significant difference between genders.

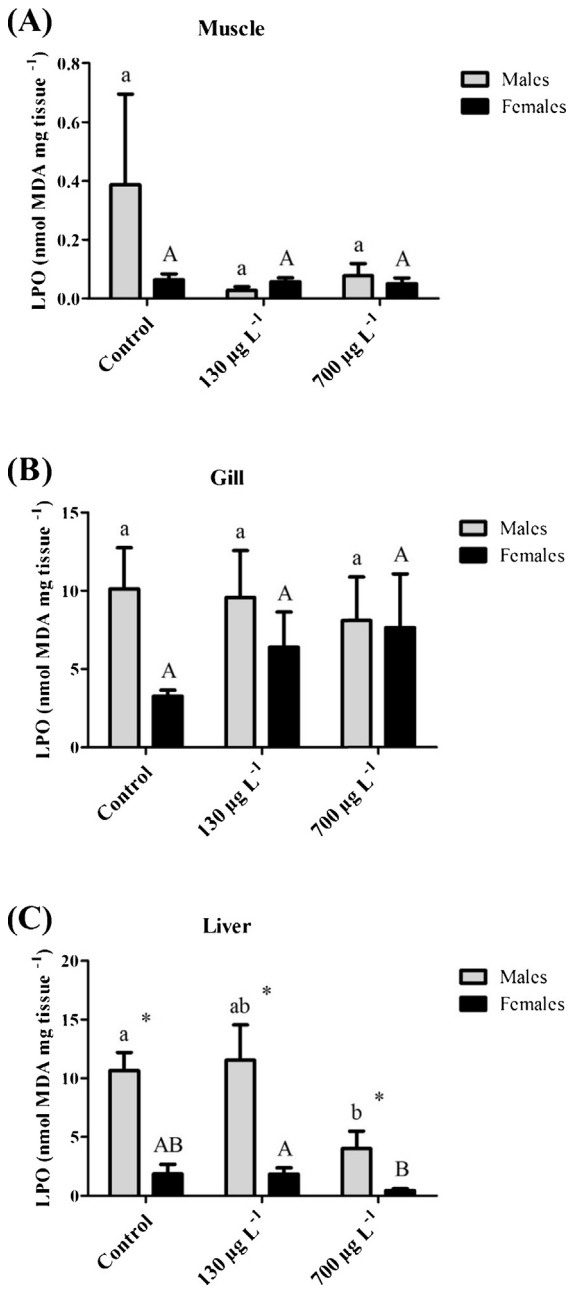

Fig. 5. LPO in (A) muscle, (B) gill and (C) liver of the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup in fresh water. Values are means ± SEM. Different lowercase and uppercase letters represent significant difference among treatments ($p < 0.05$) in males and females, respectively. *Significant difference between genders.

4. Discussion

As reviewed by Guilherme et al. (2010), the herbicide Roundup (measured as glyphosate acid equivalents) has been detected in natural water bodies at concentrations ranging from 0.01 to 0.7 mg L^{-1}, reaching up to 1.7 mg L^{-1} in extreme situations after direct application of the herbicide into the water. It is important to note that no fish mortality was observed in the present study after exposure of male and female guppies (*P. vivipara*) to Roundup at 130 and 700 μg L^{-1} for 96 h. Therefore, these concentrations can be considered as being sublethal to *P. vivipara* and of ecotoxicological interest.

Findings from the present study showed that sperm of *P. vivipara* acutely (96 h) exposed to Roundup showed a lower quality, measured as a reduction in the following parameters: integrity of sperm plasma membrane, mitochondrial functionality, DNA integrity, sperm motility and motility period. An effect of the herbicide on all of these parameters was homogeneously observed in male fish exposed to the highest concentration of Roundup tested (700 μg L^{-1} of glyphosate). This herbicide concentration also induced a slight but not significant increase in spermatic cells density, which could be a response to the poorer sperm quality observed in fish exposed to this condition.

Concerning the association among the different sperm parameters analyzed, there was a correlation between the reduced

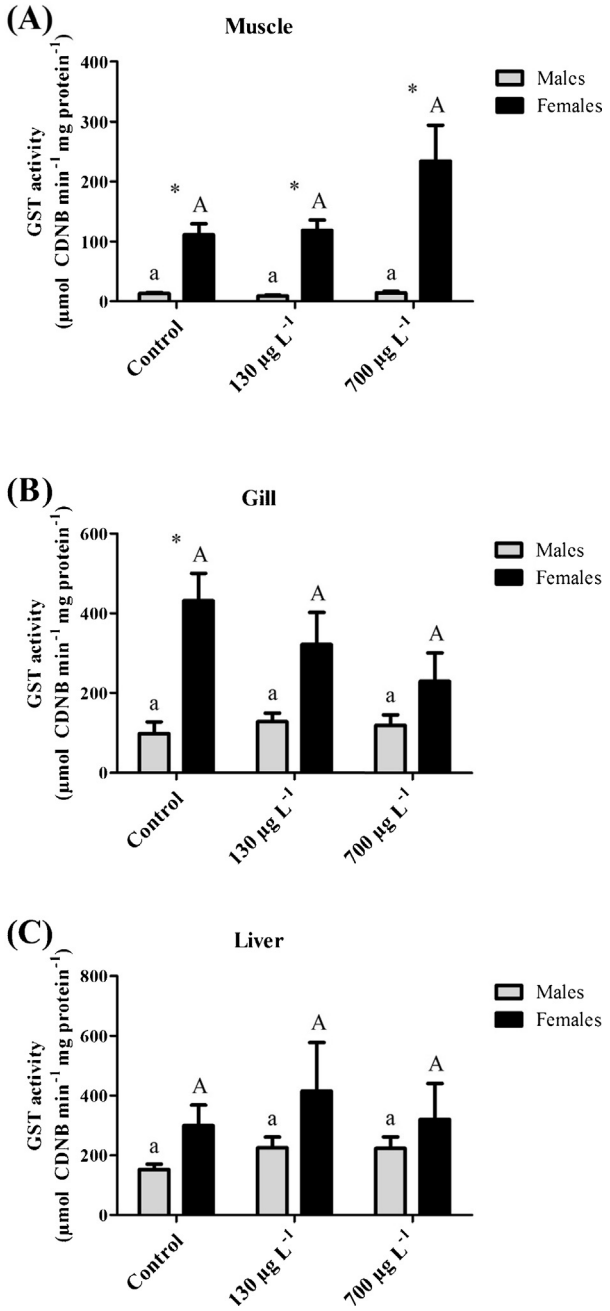

Fig. 6. GST activity in (A) muscle, (B) gill and (C) liver of the guppy *Poecilia vivipara* kept under control conditions or exposed (96 h) to Roundup in fresh water. Values are means ± SEM. Different lowercase and uppercase letters represent difference among treatments ($p < 0.05$) in males and females, respectively. *Significant difference between genders.

membrane integrity and the reduced mitochondrial functionality. This is consistent with a possible damage induced by exposure to Roundup and/or its metabolites on sperm membrane, which would likely imply the impairment of the mitochondria functionality. In turn, the reduced mitochondrial functionality paralleled with the reduced membrane integrity could explain the observed decrease in sperm motility. Additionally, the observed damage to the mitochondria induced by Roundup exposure can be also responsible for the increased DNA damage and reduced motility period of fish sperm. In fact it is reported that mitochondrial dysfunction can lead simultaneously to production of reactive oxygen species and lower energy availability in mammalian cancer cells (Pelicano et al., 2009). It is important to note that changes in the structural and functional integrity of plasma membrane and mitochondria, as well as reduced sperm motility, are critical endpoints of the fertilization process in teleost fish eggs (He and Woods, 2004).

At our best knowledge, there is no report on the direct effect of Roundup on sperm plasma membrane in fish. It is important to consider that this herbicide is made of organic compounds, which could interact with the sperm plasma membrane and directly affects its integrity and functions. If these compounds enter the cell after crossing the cell membrane, they could potentially interact with intracellular elements and affect the normal cell functioning. For example, DNA damage was observed in sperm cells of fish exposed to Roundup in the present study. This condition could compromise the expression of protein and others biomolecules, thus indirectly contributing for the reduced cell membrane integrity observed in fish exposed to Roundup. Considering that no significant correlation between DNA damage and membrane integrity was observed in the present study, the first hypothesis is more likely to occur.

Finally, the strong correlation observed between the exposure concentration of Roundup and fish sperm quality reinforces the idea that realistic environmental levels of Roundup could cause relevant damages to fish reproduction. It is worth to mention that fish sperm quality can be indirectly affected by disruption of steroid hormones regulation after a longer period of exposure than that employed in the present study. For example, Walsh et al. (2000) reported that Roundup decreases steroidogenesis in Leydig cells by reducing the level of Steroidogenic Acute Regulatory Substances (StARS), thus contributing for the development of a reproductive dysfunction.

The findings described above point out to the potential effects of Roundup on male fish reproduction. In this context, it is important to note that Soso et al. (2007) reported that females of the catfish *Rhamdia quelen* exposed to Roundup showed a disruption in steroidogenesis characterized by a decreased level of 17β-estradiol. This hormone is produced by the ovarian follicular layer and stimulates vitellogenin production and secretion. Besides the impairment in ovarian follicle function, these authors also reported a reduction in egg viability, since a low number of viable swim-up fries were obtained. Moreover, Hued et al. (2012) reported a lower sexual activity, measured by a decreased number of copulations and matching success in the guppy *Jenynsia multidentata*, another viviparous fish, after exposure to Roundup. It is worth to note that this guppy species also show internal fertilization, as observed in *P. vivipara*.

In summary, findings from the present study along with those already described in the literature support the idea that fish reproduction could be impaired in aquatic environments contaminated with Roundup. Furthermore, parameters analyzed in the present study to assess the semen quality can be considered as potential biomarkers of fish exposure to this herbicide.

Male and female *P. vivipara* showed differential levels of some of the biochemical parameters analyzed. In viviparous fish, males tend to be more mobile than females (Magurran and Maciás Garcia, 2000). The higher swimming activity increases the metabolic rate, which could explain the higher muscle AChE activity found in males of *P. vivipara* in the present study. In turn, an elevated metabolic rate stimulates the production of reactive oxygen species (ROS), demanding a higher tissue ACAP to protect cells against the oxidative damage induced to macromolecules. The higher levels of ACAP in gills and LPO in liver of male *P. vivipara* reported in the present study are consistent with this idea. Similarly, Vega-López et al. (2007) reported higher levels of LPO in liver of male *Girardinichthys viviparous*, another viviparous fish. They explained the observed gender difference considering a higher cytochrome P450 content and CYP1A catalytic activity in males than in females. This would

lead to an increased generation of ROS and consequent higher LPO levels. In fact, a positive response of the enzymatic antioxidant defenses (superoxide dismutase and catalase activities) in response to the increased LPO level was observed in liver of males *G. viviparous*, indicating that they were more subjected to oxidative stress than females. In turn, GSTs are detoxifying enzymes of phase II that catalyze the conjugation of GSH with a variety of electrophilic compounds (Ferreira et al., 2010). The higher GST activity found in female *P. vivipara* can be associated with its viviparous reproduction, once during gestation the maternal system must provide oxygen to and remove metabolic wastes from the embryo (Timmerman and Chapman, 2003).

In the present study, none of the biochemical parameters analyzed was significantly affected by Roundup exposure, except for a reduced LPO level observed in liver of male *P. vivipara* exposed to the highest concentration of Roundup. This response would indicate that LPO level, as discussed below for biochemical responses in general, is quite dependent on fish species sensitivity and the concentration of herbicide tested. The lower LPO level observed in liver of Roundup-exposed fish could be explained considering an stimulation of the total antioxidant scavenging capacity of the tissue induced by the herbicide exposure without a significant increase in the generation of reactive oxygen species, thus favoring the antioxidant system and leading to a reduced LPO. Regarding the other biochemical parameters, similar result was reported by Rendón-von Osten et al. (2005) for the mosquitofish *Gambusia yucatana* after exposure to Rival, a commercial formulation of a glyphosate-based herbicide like Roundup. These authors reported no significant effects of the herbicide on GST and AChE activity, and suggested that GSTs are not involved in the detoxification of the agrichemical.

Biochemical responses in fish exposed to Roundup were already described and were shown to vary depending on the species and the tissue analyzed (Glusczak et al., 2007, 2006; Langiano and Martinez, 2008; Ferreira et al., 2010; Modesto and Martinez, 2010; Salbego et al., 2010; Cattaneo et al., 2011; Menezes et al., 2011). It is important to note that concentrations tested in these studies were higher (ranging from 0.95 to 20 mg L^{-1}) than those employed in the present study (0.13 and 0.70 mg L^{-1}), except for that tested by Menezes et al. (2011) who using an intermediate concentration (0.45 mg L^{-1}) were able to demonstrate significant changes in the response of biochemical parameters in the catfish *R. quelen* exposed to Roundup.

Based on the discussed above, it is possible that concentrations employed in the present study were not high enough to induce significant increases in the response of the biochemical parameters analyzed in *P. vivipara*. This suggests that this guppy is likely more tolerant to Roundup than the catfish *R. quelen*. In fact, the 96-h LC_{50} value of Roundup for *R. quelen* is 10 mg L^{-1} (Albinati et al., 2007), being lower than that described for *G. yucatana* exposed to Rival (17.79 mg L^{-1}). It is important to note that *G yucatana* is a fish species belonging to the Poecillidae family like *P. vivipara*. Therefore, the lack of biochemical responses in *P. vivipara* after exposure to Roundup could be due to the comparatively low concentrations tested in the present study. Nevertheless, it is also possible that the herbicide was not effectively absorbed by guppies or they were able to detoxify it quickly before it could cause deleterious effects.

5. Conclusion

Findings reported in the present study show that exposure to Roundup reduces the sperm quality in the guppy *P. vivipara*, an effect likely associated with changes in plasma membrane integrity and mitochondrial functionality in spermatic cells. They suggest that exposure to environmentally realistic concentrations of this herbicide may negatively affect at long-term the reproduction of *P. vivipara*, with consequent changes in fish populations inhabiting environments contaminated with Roundup.

Finally, although both concentrations of Roundup tested did not affect the biochemical parameters analyzed in adults of *P. vivipara*, data from the present study indicated gender-related differences in the response of these parameters. These different biochemical patterns certainly deserve further future investigation, especially in the context of the increasing use of *P. vivipara* in ecotoxicological studies.

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