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Exposure to a Commercial Glyphosate Formulation (Roundup®) Alters Normal Gill and Liver Histology and Affects Male Sexual Activity of *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes)

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Abstract Roundup is the most popular commercial glyphosate formulation applied in the cultivation of genetically modified glyphosate-resistant crops. The aim of this study was to evaluate the histological lesions of the neotropical native fish, *Jenynsia multidentata*, in response to acute and subchronic exposure to Roundup and to determine if subchronic exposure to the herbicide causes changes in male sexual activity of individuals exposed to a sublethal concentration (0.5 mg/l) for 7 and 28 days. The estimated 96-h LC_{50} was 19.02 mg/l for both male and female fish. Gill and liver histological lesions were evaluated through histopathological indices allowing quantification of the histological damages in fish exposed to different concentrations of the herbicide. Roundup induced different histological alterations in a concentration-dependent manner. In subchronic-exposure tests, Roundup also altered normal histology of the studied organs and caused a significant decrease in the number of copulations and mating success in male fish exposed to the herbicide. It is expected that in natural environments contaminated with Roundup, both general health condition and reproductive success of *J. multidenatata* could be seriously affected.

Freshwater ecosystems can be contaminated with agrochemicals by leaching, run-off, or direct or indirect spraying, this latter occurring by action of the wind (World Health Organization [WHO] 2005). Agrochemical products that get into natural waterways could exert detrimental effects on fish populations and other forms of aquatic life and may cause long-term effects in the environment (Martínez and Cólus 2002).

One of the most commonly applied herbicides in the world is the nonselective, postemergence herbicide, glyphosate (N-[phosphonomethyl] glycine). It is commercialized in >100 countries around the world, with Roundup (Monsanto) being the most popular formulation (Cox 2004). Roundup inhibits plant growth, through interference with the production of certain essential aromatic amino acids, by inhibiting the enzyme enolpyruvylshikimate phosphate synthase (Williams et al. 2000). Roundup shows high water solubility, varying from 10,000 to 15,700 mg/l at 25°C, and contains glyphosate as the active ingredient; polyethoxylene amine (POEA), a nonionic surfactant, is added to increase efficiency of the active ingredient by promoting penetration of the herbicide through plant cuticle (Burger and Fernández 2004; Brausch and Smith 2007). Due to cultivation of genetically modified glyphosate-resistant crops, the use of glyphosate has increased during the last years raising again concerns regarding the potential environmental impact of this herbicide (Giesy et al. 2000). In Argentina, soybean is the most important crop, with a planted surface that has increased by 11,000,000 hectares and a production of approximately 35,000,000 metric tons (95% correspond to glyphosate-resistant soybean) (Pengue 2005). Perusso et al. (2008), who determined the levels of glyphosate in water (0.1–0.7 mg/l), sediment (1.15–1.38 mg/l), and soil (0.53–4.45 mg/l) from transgenic soybean cultivations in Argentina, pointed out that rain events play a notable role in pollution, transporting the glyphosate present in the soil toward the water stream through the mechanisms of dilution or drift of to the surface material through run-off.

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It is well known that agrochemicals could affect the structure and function of biological systems by impacting them on different organization levels, such as molecular, subcellular, cellular, tissue, organ, organism, population, community, and ecosystem (van der Oost et al. 2003). At the tissue level, histological biomarkers provide powerful tools to detect and characterize the biological end points of a toxic compound. The utility of histological lesions as sensitive and reliable indicators of fish health has been reported in several studies (Stentiford et al. 2003; Bernet et al. 2004; Ramírez-Duarte et al. 2008). It has been shown that Roundup is toxic to fish and can cause histological changes in these animals. Several investigators have reported histological alterations, such as necrotic and proliferative lesions, aneurysms, and leukocyte infiltration in gills, whereas fatty degeneration, multifocal necrotic processes, and infiltration of leukocytes have been registered in liver of fishes exposed to commercial formulations of glyphosate (Neskovic et al. 1996; Szarek et al. 2000; Jiraungkoorskul et al. 2002, 2003; Ayoola 2008; Ramírez-Duarte et al. 2008).

Another successful end point for assessing the effects of chemical contaminants is sexual behavior. Different investigations have demonstrated that toxicant exposure disrupts the effective performance of reproductive behavior and could decrease reproductive success (Scott and Sloman 2004; Nakayama et al. 2004; Toft and Guillette 2005; Doyle and Lim 2005), and subsequently, population structure and dynamics could also be affected (Oshima et al. 2003).

The neotropical fish species *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes) presents a wide distribution in an extensive area of South America (Malabarba et al. 1998) and can be found in pristine as well as severely degraded habitats (Hued and Bistoni 2005). This fish species has been successfully used as a regional bioindicator model to evaluate the effects of different chemicals on different biological processes (Cazenave et al. 2005; Hued et al. 2006; Ballesteros et al. 2007; Amé et al. 2009), and it could also be used as an excellent model to evaluate male sexual activity because it is a sexually dimorphic, livebearing species in which the male anal fin differentiates into a complex structure called the "gonopodium," a tubular intromittent organ supported by anal fin rays (Ghedotti 2000; Meisner 2005; Jamieson 1991), which is used to transfer sperm during copulation.

Due to its high water solubility and extensive use in the environment, the exposure of nontarget aquatic organisms to Roundup is of concern to ecotoxicologists. The aim of this study was to evaluate the histological lesions of the native fish, *J. multidentata*, in response to acute and subchronic exposure to Roundup and to determine if subchronic exposure to the herbicide causes changes in male sexual activity of fish exposed to a sublethal concentration for 7 and 28 days.

Materials and Methods

Adult male and female individuals of the native widespread species *J. multidentata* were used for the experiments based on their suitability for laboratory studies. The selection criteria included their small size, ease of collection and maintenance in the laboratory, and wide distribution in South America (Malabarba et al. 1998). Individuals were captured with backpack electrofisher equipment from a site on Yuspe River (64°32′W; 31°17′S) (Córdoba, Argentina). This site has been used as a reference location for the collection of *J. multidentata* according to previous water-quality assessment studies (Hued and Bistoni 2005). Fish were transported to the laboratory in water tanks (20-l) and acclimated to laboratory conditions for 15 days before the experiments. They were maintained in a 120-l aerated glass aquarium containing dechlorinated tap water in a temperature-controlled room at 21 ± 1°C and under a light-to-dark cycle of 12 h:12 h. During the acclimatization period fish were fed twice a day with commercial fish food. Male and female fish standard lengths (means ± SDs) were 36.34 ± 4.16 and 43.71 ± 7.46 mm, respectively. The mean weight was 0.58 ± 0.21 g for male fish and 1.12 ± 0.5 g for female fish.

Short-Term Toxicity Testing

Short-term (96-h) static toxicity tests were performed to evaluate the toxicity of Roundup to *J. multidentata*. Commercial glyphosate formulation was Roundup Max granular (Monsanto, Argentina) which contains 74.70% of N-(phosphonomethyl) glycine as the active ingredient and 25.30% as surfactants.

Fish were exposed to the following nominal concentrations of Roundup: 5, 10, 20, 35, 60, and 100 mg/l for 96 h. Each control group and concentration tested was performed in duplicate with eight individuals (four male and four female fish) per group in 18-l aerated glass aquaria. Individuals were starved 24 h prior to the experiment and were not fed during the experiment. Fish without respiratory movements and no response to tactile stimulus were considered dead and were removed immediately. The median lethal concentrations (LC_{50}) at 96 hours were estimated separately for male and female fish by a probit transformation from the mortality dose curve using free Probit software (version 1.5) provided by the United States Environmental Protection Agency (2005).

Subchronic Toxicity Testing

For the subchronic toxicity test, two groups of nine individuals (five male and four female fish) were exposed to a 10% of the lowest concentration used in the acute toxicity test (0.5 mg/l of Roundup) for 7 and 28 days. Fish were fed twice a day with commercial fish food. Water of each aquarium was renewed partially every 2 days and completely removed once a week.

Male Sexual Activity

After the period of subchronic exposure, each male fish was introduced into a 10-l aquarium with an unexposed sexually mature female fish. Male sexual activity was registered using a digital camera (model no. DSC-W70; Sony) and by direct observation for 20 min with observations starting 10 min after the couple was introduced into the tank.

Based on the normal reproductive behavior described by Bisazza et al. (2000), the following parameters were estimated:

1. *Number of persecutions (NP)*: number of times that a male fish persecutes a female fish to make contact with the female gonopore.
2. *Copulation attempts (CA)*: number of times that a male fish enlarges its gonopodium to make contact with the female gonopore.
3. *Number of copulations (C)*: number of times that a male fish made direct contact through its gonopodium with the female gonopore.
4. Mating success (MS): estimated from the above-mentioned parameters through the following formula: $MS = \log C/(\log NP + \log CA)$; this estimate gives an idea of the effectiveness of a male fish to copulate after persecution or copulation attempts.

Histological Analysis

After 96 h of exposure, the surviving fish, as well as those male and female fish exposed to the sublethal concentration for 7 and 28 days, were killed with an overdose of tricaine methane sulfonate, MS-222 (Sigma-Aldrich) and dissected. Gills and liver from control and exposed fish were removed and fixed in 10% formaline. Tissue samples were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. Sections 4 to 6–μm thick were stained with hematoxylin and eosin (H&E). Tissue lesions were examined with a light microscope and photographed with a digital camera (model no. DSC-W70; Sony).

For gill histological analysis, 5 filaments were examined per individual fish. On each filament 10 secondary lamellae on the middlemost section of the filament were selected. The number of secondary lamellae possessing a particular alteration was recorded and divided by the total number of gill secondary lamellae examined to give the percentage of filaments affected.

To evaluate histological alterations of liver, tissue was divided into 5 equal areas (4.2 mm^2) to ensure there was no overlapping of in examinations of the studied areas. For each area, the extension and number of each alteration were recorded and divided by the total number of areas examined to give a percentage of liver areas affected.

Histopathological lesions were evaluated according to a standardized assessment tool by using a modified version of the protocol described by Bernet et al. (1999), which allows calculation of indices for every organ. Each histological alteration was classified into one of four reaction patterns: circulatory disturbances, regressive changes, progressive changes, and inflammation. Each reaction pattern includes alterations related to functional units of each organ. Circulatory disturbances (reaction pattern 1 = Rp1) correspond to pathological conditions of blood and tissue fluid flow (e.g., haemorrhage, blood congestion, aneurysm, etc.); regressive changes (reaction pattern 2 = Rp2) correspond to processes that cause functional decrease or loss of an organ (e.g., architectural and structural alterations of a tissue, atrophy, necrosis, etc.), progressive changes (reaction pattern 3 = Rp3) correspond to processes that lead to increased cell or tissue activity (e.g., hypertrophy and hyperplasia); and inflammation (reaction pattern 4 = Rp4) corresponds to inflammatoy processes associated with other reaction patterns (e.g., eodema, infiltration of leucocytes, etc.). Table 1 lists the reaction patterns and their specific alterations for gills and liver.

Because the relevance of a lesion depends on its pathological importance and indicates how the lesion affects the function of an organ and the ability of the individual to survive, an importance factor (W) (range 1–3) was assigned to each alteration (Table 1). A high importance factor represents a greater potential of an alteration to impact fish health (Bernet et al. 1999).

For both gill and liver, the percentage of gill filaments or liver areas affected were assessed using a score ranging from 0 to 6 according the degree and extension for a particular alteration: 0 = unchanged, 2 = mild occurrence, 4 = moderate occurrence, and 6 = severe occurrence. The importance factors and the score values were multiplied to give an index for a particular alteration. The indices for each alteration within a reaction pattern were summed to give an index for each reaction pattern (RP_{Org} = reaction pattern index for an organ). The indices for each reaction pattern of an organ were summed to give an overall organ index (HI_{Gills} = gill histopathological index; HI_{Liv} = liver histopathological index). To determine the overall health

Table 1 Importance factor assigned to gill and liver histological alterations for each reaction pattern

Reaction pattern	Histological alteration	W
Gills		
Circulatory disturbances	Blood congestion	1
	Lamellar aneurysm	1
Regressive changes	Epithelial lifting	1
	Fusion of the distal end of secondary lamellae	2
	Secondary lamella shortening	2
	Necrosis	3
Progressive changes	Chloride cell hypertrophy	1
	Epithelial cell hypertrophy	1
	Mucous cell hyperplasia	2
	Chloride cell hyperplasia	2
	Epithelial cell hyperplasia	2
Inflammation	Leukocyte infiltration	2
Liver		
Circulatory disturbances	Haemorrage	1
	Sinusoid dilation	1
	Blood congestion	1
Regressive changes	Hydropic degeneration	1
	Lipid degeneration	2
	Hepatocyte picnosis	2
	Fibrosis	3
	Necrosis	3
Progressive changes	Hepatocyte hyperplasia	2
	Hepatocyte hypertrophy	2
Inflammation	Leukocyte infiltration	2

W = importance factor

status based on the histological lesions, a total histopathological index (HI_{Tot}) was calculated by adding gills and liver indices of an individual fish (Bernet et al. 1999). The greater the index values, the more severely the organs were affected.

Gill Morphometric Analysis

Morphometric analyses of gills were performed according to the criteria proposed by Nero et al. (2006) with some modifications. Five filaments were selected randomly for each fish and photographed. Digital images were analyzed using the Image J software (Rasband 2005). On the medium part of each filament 10 secondary lamellae (5 lamellae per side of each filament) were selected to measure the following parameters: secondary lamellar length (SLL) and width (SLW), interlamellar distance (ID), and basal epithelial thickness (BET) (Fig. 1a). To measured SWL and ID, three measurements were taken on each secondary lamella (points where secondary lamellae met the basal lamina, the middle, and at the distal edge of the secondary lamellae) to give an average value for each variable. For BET, three measurements were made along each filament: measurements at both ends and in the middle on both sides of the cartilaginous support. The measured parameters indicate changes in the gas diffusion distance in fish gills. The proportion of the secondary lamellae available for gas exchange (PAGE) was averaged for each filament of an individual and calculated as follows: PAGE (%) = 100 * (mean SLL/[mean BET + mean SLL]).

Statistical Analysis

Data distributions were analyzed using the Shapiro–Wilks index (Sokal and Rohlf 1999). The 96-h LC_{50} values between sexes were assessed with Student *t* test. To compare the biological parameters among different Roundup concentrations and time of exposure, Kruskal–Wallis test (Sokal and Rolhf 1999) was performd and followed by a Dunn's multiple comparison test. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using Infostat Software Package (Infostat 2002).

Results

Toxicity Tests: 96-h LC_{50} and Subchronic

The Roundup 96-h LC_{50} value for *J. multidentata* estimated was 19.02 mg/l and showed no differences between sexes. No mortality occurred in the control group or at 5 mg/l Roundup. All of the individuals exposed to 60 and 100 mg/l Roundup died during the exposure period. No mortalities were observed during the subchronic tests.

Male Sexual Activity

A significant low number of copulations (Cs) was registered in male fish exposed to 0.5 mg/l Roundup for 7 and 28 days with respect to the control group (Table 2). There were no differences in number of Cs between male fish exposed to 7 and 28 days to Roundup. MS differed significantly among treatment group and between treatment and control group, showing the lowest values after 28 days of exposure.

Gill Histopathological Analysis

No recognizable changes were observed in gills of control individuals of *J. multidentata* (Fig. 1a and b). However, treated groups exposed to different concentrations of Roundup showed several pathological changes. The severity and frequencies of histological alterations increased with increasing concentrations used.

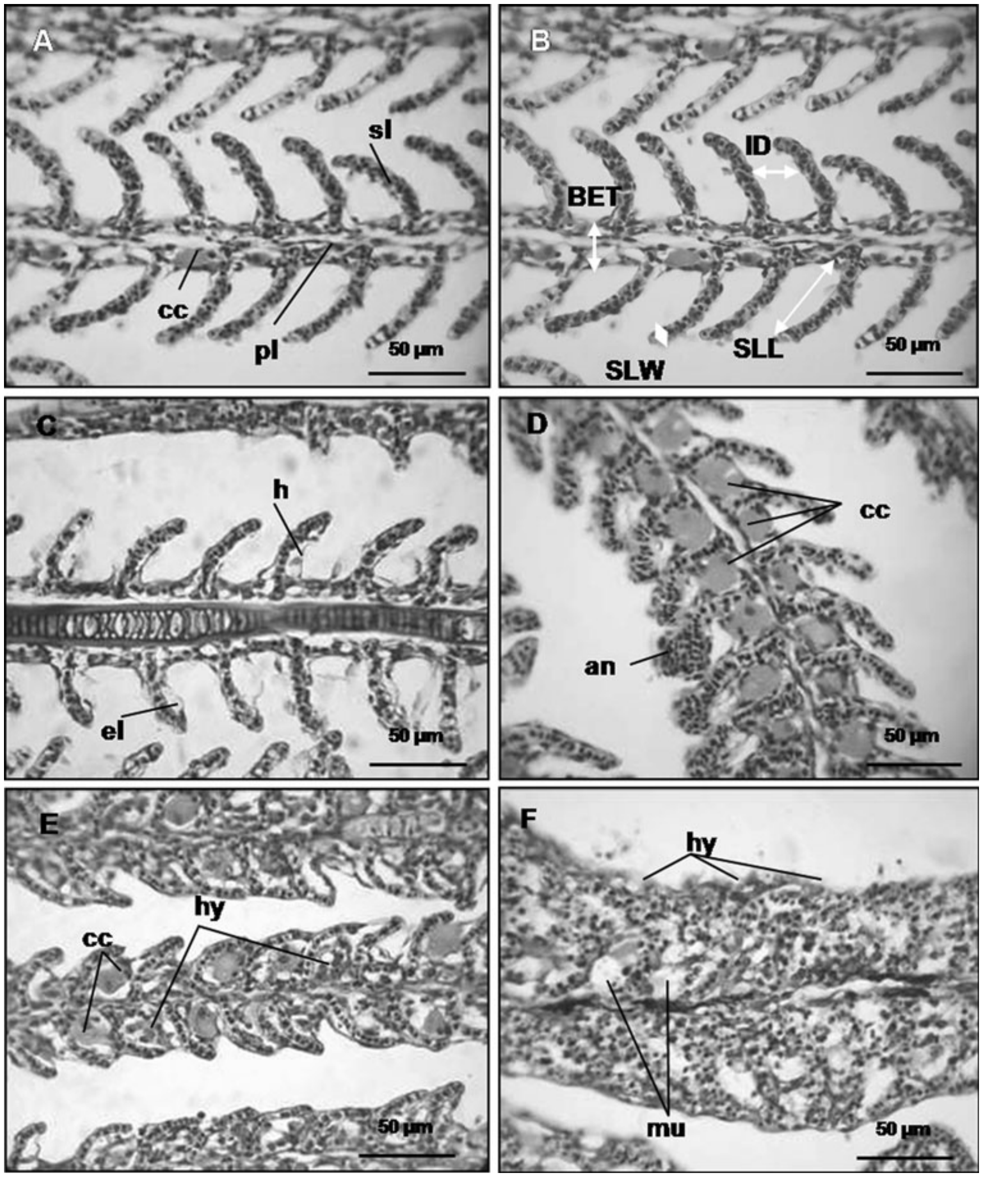

Fig. 1 Gill histological sections and morphometric parameters in individuals from the acute toxicity test. **a** and **b** Control, **c** 5 mg/l Roundup, **d** 10 mg/l Roundup, **e** 20 mg/l Roundup, **f** 35 mg/l Roundup. *An* aneurysm, *cc* chloride cell, *h* hypertrophy, *hy* hyperplasia, *el* epithelial cell lifting, *pl* primary lamella, *sl* secondary lamella, *mu* mucous cells. H&E stain 40×

Table 2 Parameters of male sexual activity of *J. multidentata* exposed to a sublethal concentration of Roundup for 7 and 28 days

Parameters of male sexual activity	Control	7 days	28 days
NP	74.00 ± 40.00	52.40 ± 28.90	56.16 ± 29.80
CA	30.00 ± 28.00	16.00 ± 10.00	19.00 ± 9.80
C	3.31 ± 1.50	1.40 ± 0.89 (a)	1.30 ± 0.81 (a)
MS	0.17 ± 0.03	0.13 ± 0.07 (a)	0.11 ± 0.07 (b)

Values are expressed as means ± SDs. Different letters indicate significant differences among treatments and between treatments and control group ($p < 0.05$)

NP number of persecutions, *CA* number of copulation attempts, *C* number of copulations, *MS* mating success

Individuals exposed to 5 mg/l Roundup presented lifting of secondary lamellar epithelium, edema formation, and hypertrophy of epithelial cells (Fig. 1c). Individuals treated with 10 mg/l Roundup presented lifting of secondary lamellar epithelium and hypertrophy of chloride cells. A severe lamellar aneurysm in the distal part of the secondary lamellae, giving it a “clubbing appearance,” was also noted (Fig. 1d). Fish exposed to 20 mg/l Roundup presented hypertrophy of chloride cells (Fig. 1e) and slight thickening of the secondary lamellae due to the hyperplasia of epithelial cells. Gills of individuals exposed to 35 mg/l of Roundup presented a pronounced mucous cell proliferation (Fig. 1f) and severe hyperplasia of epithelium cells, which led to secondary lamellar fusion due to proliferation of epithelial cells.

Fish exposed to 10% of the 96-h LC_{50} of Roundup (0.5 mg/l) presented similar gill histological alterations as seen in the acute toxicity test. However, only those individuals exposed to subchronic conditions presented leukocyte infiltration. Gills showed a pronounced mucous cell proliferation and shortening of the secondary lamellae after 28 days of Roundup exposure. These alterations were not registered in fish exposed for 7 days.

Liver Histopathological Analyses

Histology of control fish liver showed a normal typical paranchymatous appearance. The hepatocytes were homogeneous both in size and cytoplasm density (Fig. 2a), whereas livers of exposed fish presented different alterations, which varied in severity and frequency in a concentration-dependent manner.

Livers from individuals exposed to 5 mg/l Roundup only presented hydropic degeneration (Fig. 2b), whereas those treated with 10 mg/l Roundup showed hydropic degeneration, blood sinusoid dilation, and foci of leukocyte infiltration (Fig. 2c). In individuals treated with 20 mg/l Roundup areas with focal necrosis and infiltration of leukocytes were noted. These alterations were accompanied by blood sinusoid dilation and vascular congestion (Fig. 2d). Livers from fish exposed to 35 mg/l Roundup presented all of the alterations mentioned previously. However, they showed the highest frequency and extension of focal necrosis with respect to individuals exposed to 20 mg/l Roundup accompanied by leukocyte infiltration as a result of inflammatory processes (Fig. 2e).

Liver histological alterations registered in individuals exposed to 10% of the 96-h LC_{50} of Roundup (0.5 mg/l) were similar to those registered in fishes from the acute toxicity test. However, at 28 days of exposure, livers presented severe hepatocyte picnosis. This alteration was not registered after 7 days of exposure.

Histopathological Indices

Because histopathological indices (HIs) did not differ significantly between male and female fish, the analyses were performed all together. The gill HIs from individuals exposed to acute concentrations of Roundup varied according to the concentrations tested (Table 3). $HI_{Gill.Rp1}$ values (circulatory disturbances) did not differ significantly among the treatment and control group, whereas the values obtained for $HI_{Gill.Rpr2}$ (regressive changes) and $HI_{Gill.Rp3}$, (progressive changes) were significantly different with respect to the control group at $\geq$10 mg/l Roundup. Because inflammatory changes were not observed (Rp4), $HI_{Gill.Rpr4}$ values could not be calculated.

Liver histological analyses showed $HI_{Liv.Rp1}$ values significantly different among the Roundup concentrations tested and with respect to the control group (Table 3), indicating relevant circulatory changes. $HI_{Liv.Rp2}$ values (regressive changes) presented significant differences with respect to the control group but did not vary among treatments. Progressive changes were not observed (Rp3); therefore $HI_{Liv.Rpr3}$ values could not be calculated.

Histopathological indices for gill and liver reaction patterns of individuals exposed to 10% of the 96-h LC_{50} of Roundup (0.5 mg/l) for 7 and 28 days did not present significant differences between treatment groups and the control group (Table 3). Total gill and liver histopathological indices (HI_{Gill} and HI_{Liv}, respectively) were significantly different among treatments and between the treatment and control group, changing in a concentration-dependent manner or according to the duration of exposure. Branchial and hepatic injuries of greater severity and extension were observed at the highest concentrations of Roundup for individuals from the acute toxicity test. Therefore, the highest index values corresponded to the highest Roundup concentrations. HI_{Gill} values for subchronic exposure varied with respect to the control group but did not show differences between treatments (Table 3). In contrast, HI_{Liv} values presented significant differences, showing the highest mean values at 28 days of exposure.

To determine overall fish health status through histological analyses, a total pathological index (HI_T) was calculated by adding gill and liver indices of an individual fish. HI_T values were significantly different among the Roundup concentrations tested, and between treatments and the control group, and increased in a concentration-dependent manner, showing the highest values at the highest concentration tested (Table 3). HI_T values from individuals exposed in the subchronic toxicity test presented significant differences, showing the highest values after 28 days of exposure.

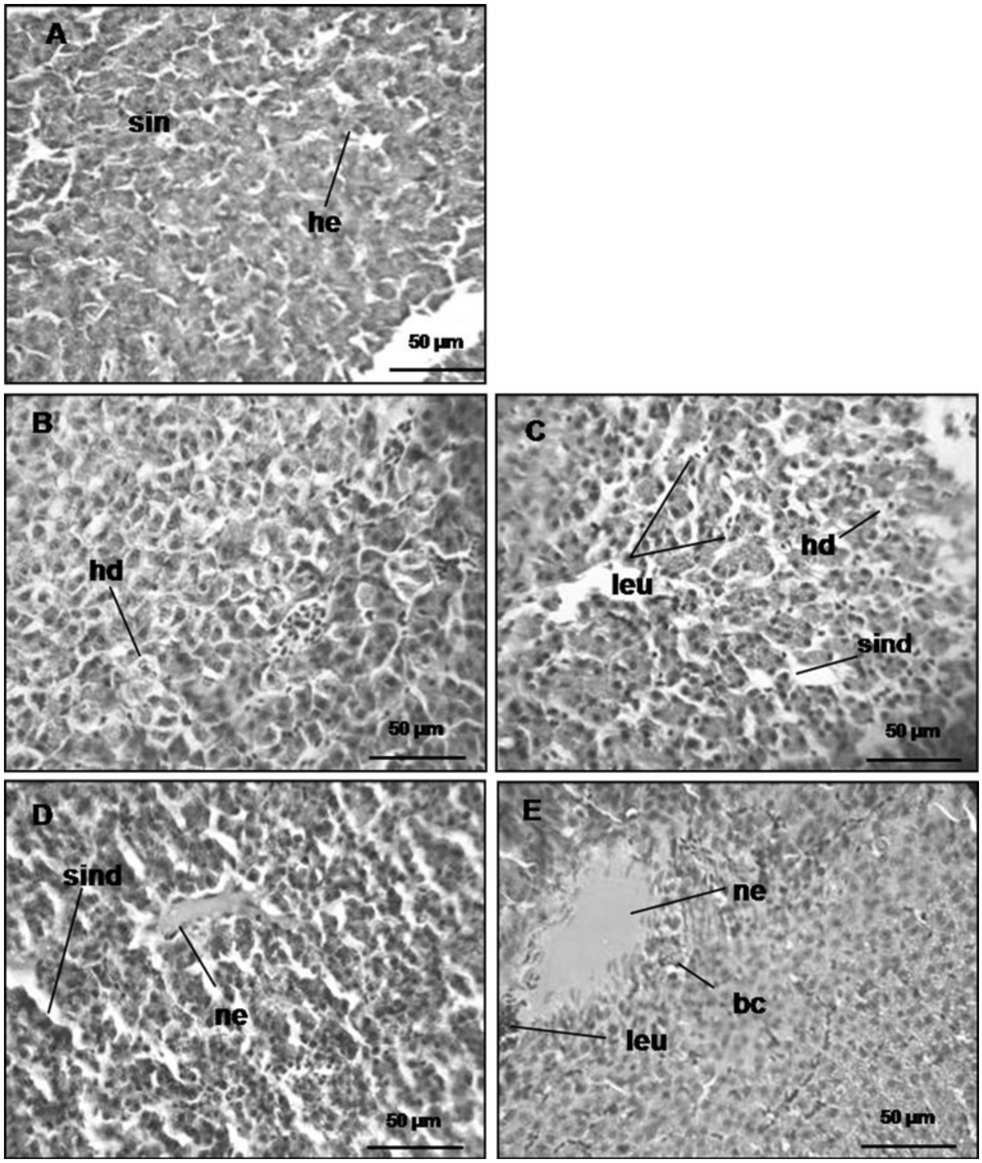

Fig. 2 Liver histological sections in individuals from the acute toxicity test. **a** Control, **b** 5 mg/l Roundup, **c** 10 mg/l Roundup, **d** 20 mg/l Roundup, **e** 35 mg/l Roundup. *Bc* blood congestion, *hd* hydropic degeneration, *he* hepatocytes, *leu* leukocyte infiltration, *ne* necrosis, *sin* sinusoides, *sind* sinusoid dilation. H&E stain 40×

Gill Morphometric Analyses

Gill morphometrics measured presented different patterns of variation according to Roundup concentrations tested (Table 4). The increased severity of gill alterations was measured through increased SLW and decreased ID and SLL values.

Individuals exposed to Roundup had gills with significantly greater SLW values with respect to the control group, whereas ID and SLL values decreased at the highest concentrations of Roundup used. Although BET did not present significant differences, there was a tendency for values to increase at the highest concentrations.

SLL and BET values were used to calculate the PAGE index, which represents the gill surface area available for gas exchange. This index presented significant differences from the acute toxicity test and showed the lowest values at the highest Roundup concentrations (Table 4). Although the morphometric parameters were not significant different between 7 and 28 days of exposure, the PAGE index presented significant differences respect to the control group, showing the lowest values in fish exposed to Roundup.

Table 3 Histopathological indices from acute and subchronic exposure to Roundup

HIs	Acute toxicity test					Subchronic toxicity test (0.5 mg/l)	
	Control	5 mg/l	10 mg/l	20 mg/l	35 mg/l	7 days	28 days
$HI_{Gill.Rp1}$	2.00 ± 1.63	5.50 ± 1.91	5.50 ± 1.91	4 ± 2.31	4.00 ± 0	4.00 ± 1.63	2.67 ± 1.15
$HI_{Gill.Rp2}$	0	0	4.00 ± 3.27 (a)	6 ± 2.31(a)	10.00 ± 2.31 (b)	2.50 ± 1.91	1.33 ± 1.15
$HI_{Gill.Rp3}$	4.00 ± 0	8.50 ± 3.42	7.50 ± 3.42 (a)	17 ± 7.39 (a)	19.00 ± 5.77 (a)	8.50 ± 3.42	8.00 ± 2
$HI_{Gill.Rp4}$	0	0	0	0	0	2.00 ± 0	2.00 ± 0
$HI_{Liv.Rp1}$	3.50 ± 1	6.00 ± 0 (a)	6.00 ± 0 (a)	7 ± 1.15 (b)	7.00 ± 1.15 (b)	4.00 ± 2.31	3.00 ± 1.41
$HI_{Liv.Rp2}$	0	10.00 ± 0 (a)	15.00 ± 1.41 (a)	12 ± 3.4 (a)	14.00 ± 0 (a)	7.50 ± 5.74	10.00 ± 5.6
$HI_{Liv.Rp4}$	0	2.00 ± 2.31	1.00 ± 2	5 ± 5.03	4.00 ± 0	2.00 ± 2.31	0
HI_{Gill}	6.00 ± 1.63	14.00 ± 2.31 (a)	17.00 ± 7.75 (a)	27.00 ± 7.75 (b)	33.00 ± 3.46 (b)	15.50 ± 5.26 (a)	12.67 ± 3.06 (a)
HI_{Liv}	3.50 ± 1.00	20.00 ± 3.27 (a)	24.00 ± 5.26 (a)	23.00 ± 4.76 (a)	25.00 ± 1.15 (a)	13.50 ± 5.26 (b)	24.00 ± 2.83 (a)
HI_T	10.00 ± 3.27	34.00 ± 4.00 (a)	41,50 ± 0.57 (a)	50.00 ± 11.78 (b)	58.00 ± 4.62 (b)	29.00 ± 3.83 (a)	38.00 ± 0.01 (a)

Values are expressed as means ± SDs. Different letters indicate significant differences among treatments and between treatments and control group ($p < 0.05$)

Table 4 Gill morphometric parameters and PAGE index from acute and subchronic exposure to Roundup

Test	Treatment	SLL	SLW	ID	BET	PAGE
Acute toxicity test	Control	53.48 ± 3.67	8.73 ± 0.6	18.14 ± 0.53	30.18 ± 7.91	64.91 ± 6.53
	5 mg/l	59.46 ± 6.51	11.98 ± 1.22 (a)	9.16 ± 2.35 (a)	37.57 ± 7.65	60.33 ± 5.57
	10 mg/l	56.36 ± 3.81	12.89 ± 1.12 (a)	8.47 ± 2.24 (a)	38.03 ± 6.62	59.93 ± 2.56
	20 mg/l	44.48 ± 8.26 (a)	14.27 ± 0.82 (b)	7.67 ± 2.41 (a)	40.99 ± 7.5	52.02 ± 3.74 (a)
	35 mg/l	46.52 ± 0.81 (a)	13.69 ± 0.18 (b)	5.41 ± 2.42 (b)	47.84 ± 16.11 (a)	50.59 ± 8.22 (a)
Subchronic toxicity test (0.5 mg/l)	7 days	45.64 ± 11.24	10.80 ± 4.86	18.91 ± 5.54	38.13 ± 8.29	54.00 ± 6.06 (a)
	28 days	45.96 ± 9.07	8.18 ± 2.43	15.20 ± 5.48	41.20 ± 6.38	52.33 ± 2.89 (a)

Values are expressed as means ± SDs. Different letters indicate significant differences among treatments and between treatments and control group ($p < 0.05$)

SLL secondary lamellar length, *SLW* secondary lamellar width, *ID* interlamellar distance, *BET* basal epithelial thickness

These results indicate that exposure to 0.5 mg/l Roundup for 7 and 28 days causes a significant decrease in PAGE.

Discussion

The present study shows that Roundup induces changes in the organs of *J. multidentata* by way of histopathological alterations as well as altered male sexual activity in this species. The toxicity of glyphosate, the active ingredient, is considered to be low by the World Health Organization (WHO 2005). It has been indicated that its relatively low toxicity contributes little to the total toxicity of formulated products. Therefore, formulated products containing the active ingredient together with the nonionic surfactant (POEA) are more toxic than those containing the active ingredient only, particularly to aquatic nontarget organisms, which could be more sensitive to surface-active substances (Amarante et al. 2002).

The 96-h LC_{50} values of Roundup in teleost fish vary widely from 2 to 50 mg/l (Folmar et al. 1979; WHO 1994; Mitchell et al. 1987; Abdelghani et al. 1997; Jiraungkoorskul et al. 2002). Because the 96-h LC_{50} registered from the present work was 19.02 mg/l, and according to the range of values mentioned in the literature, *J. multidentata* could be classified as a moderately sensitive species to Roundup exposure. A similar value (17.79 mg/l) was obtained for another cyprinodontiform species, *Gambusia affinis* (Poecilidae) (Rendón-von Osten et al. 2005).

Changes in gill epithelia have been considered good indicators of the effects of xenobiotics on fish (Alazemi et al. 1996) because gills are the major site of toxic impact for many compounds because of their large surface area and external location (Ballesteros et al. 2007; Ayoola 2008; Albinati et al. 2009). The severity of gill alterations in *J. multidentata* was concentration dependent, and the main damages registered were epithelial lifting, hyperplasia, and hypertrophy, which implies increased diffusion respiratory distance. These morphological alterations are in agreement with previous reports indicating different degrees of gill damages in fish species exposed to glyphosate (Jiraungkoorskul et al. 2003; Ramírez Duarte et al. 2008). The histological modifications registered here are considered responses by the organisms to protect internal tissues from diffusion of the toxicant through the gills. One of the most frequent responses was hyperplasia, which led to thickened epithelial layers and fusion of adjacent secondary lamellae. This alteration serves as a defense mechanism, leading to decreased respiratory surface and increased toxicant–blood diffusion distance (Cengiz and Ünlü 2003). However, gill hyperplasia also leads to decreased PAGE and increased diffusion respiratory distance. Thus, these defensive responses impair the normal physiology of the organism, preventing the normal diffusion of oxygen (Ballesteros et al. 2007). As a result, fish could suffer asphyxiation and finally death.

HI_{Gill} values increased in individuals exposed to greater concentrations of glyphosate. Although $HI_{Gill.Rp1}$ values (circulatory disturbances) did not present significant differences, it was observed that the high frequency and extension of vascular congestion and aneurysm increased the index values. Shortening of the secondary lamella and the great extension and high frequency of epithelial lifting were the main alterations that caused the increased $HI_{GillRp2}$ values (regressive changes). In contrast, $HI_{GillRp3}$ values were increased by the severity of hypertrophy and hyperplasia of epithelial, chloride, and mucous cells at the highest Roundup concentrations. These responses reflect increased cell activity cell in an attempt to protect internal tissue exposure to the toxicant and could lead to decreased normal gill function (Hinton et al. 2001).

For the acute toxicity test, morphometric gill analyses showed significantly decreased SLL and ID values and increased SLW values and PAGE indices. These results coincide with those obtained through the HI and demonstrate changes in gill dimensions due to progressive changes (mucous cell proliferation, epithelial cell hyperplasia, and chloride cell hypertrophy), inflammatory processes (epithelial lifting), and architectural and structural changes (secondary lamellar fusion). All of these changes make gas exchange more difficult by increasing the distance of branchial diffusion (Bols et al. 2001). For the subchronic toxicity test, only the PAGE index showed significant differences between treatment groups and the control group. Although treated individuals showed marked histological damage compared with controls, the exposure time did not cause variations in the levels of damage.

It is noteworthy that PAGE indices in fish exposed to 0.5 mg/l Roundup showed similar values to those recorded in fish exposed to 20 and 35 mg/l Roundup in acute toxicity tests. This suggests that exposure to low concentrations of toxicant for prolonged periods cause histological damage levels similar to those that occur in individuals exposed to high concentrations for short periods of exposure.

Liver histological alterations observed in *J. multidentata* exposed to Roundup also changed in a concentration-dependent manner, varying from hydropic degeneration at low concentrations to focal necrosis at the highest concentrations. HI_{Liv} values and those indices based on reaction patterns increased in a concentration-dependent manner. The great extension and high frequency of vascular congestion and blood sinusoid dilation, as well as processes that might decrease liver function such as hydropic degeneration and necrosis cause increased $HI_{Liv.Rp1}$ (circulatory disturbances) and $HI_{Liv.Rp2}$ values (regressive changes), respectively.

Liver is considered the target organ of chemically induced tissue injuries due to its function as a xenobiotic biotransformer and its central role in the circulatory system (Hinton et al. 2001). When toxic compounds exceed the detoxification level of the liver, high concentrations of a toxicant induce liver biotransformer activity and provoke modification of the normal hepatic structure. Several investigators have reported degenerative changes, lipidic vacuolization, and hyaline droplets in hepatocytes (Szarek et al. 2000; Jiraungkoorskul et al. 2002, 2003; Langiano and Martinez 2008; Albinati et al. 2007, 2009) in fishes exposed to commercial formulations of glyphosate. These alterations could impair the normal function of the organ and appear to be commonly developed in fish exposed to Roundup. Livers from individuals exposed to low Roundup concentrations presented hydropic degeneration, blood sinusoid dilation, and some areas with leukocyte infiltration. To ensure the biodetoxification activity of hepatocytes, a greater blood flow to the liver occurs and causes blood congestion and sinusoid dilation (Neskovic et al. 1996). These alterations became more severe at greater concentrations and were frequently accompanied by necrotic foci surrounded by leukocytes. Leukocyte infiltration is a common process that occurs to remove dead cells of necrotic areas (Crawford 2000). All of the histological alterations we noted have been frequently associated with agrochemical toxicity (Ballesteros et al. 2007; Albinati et al. 2009).

Bisazza et al. (2000) described the coercive mating behavior in *J. multidentata*. Male fish approach female fish from behind and try to thrust their copulatory organ at the female genital pore. These investigators mentioned that courtship display was never observed in this species, which is consistent with our observations. Our results indicated that male sexual activity is affected by Roundup exposure. Although all of the male fish (treated and control groups) were sexually active, the individuals exposed to Roundup showed the lowest sexual activity as measured by decreased number of Cs and MS. This last parameter relates the number of Cs with the number of times that a male fish persecutes a female and attempts to copulate. The significant decrease showed by MS suggests that male fish exposed to Roundup are less effective at the time of copulation because they invest much more effort in persecuting and attempting to copulate with the female fish with respect to nonexposed male fish, and that effort does not always end with the completion of copulation.

Roundup concentrations tested in the subchronic experiments could be considered environmentally realistic considering that values between 0.1 and 0.7 mg/l Roundup have been recorded in streams of Argentina (Perusso et al. 2008). Therefore, if fish exposed to sublethal concentrations for extended periods decreased their frequency of mating in laboratory bioassays, it is expected that in the natural environment the reproductive success of *J. multidenatata* would be seriously affected. According to our results, the number of copulations and MS are sensitive and ecologically relevant end points in assessment of the effects of Roundup exposure.

In summary, the present research showed that acute and subchronic exposures to Roundup induced a diversity of gill and liver histological alterations in the neotropical fish *J. multidentata* that might impair normal organ functioning and also caused decreased sexual activity in male fish exposed to the herbicide for 7 and 28 days. The use of histopathological indices allowed quantification of histopathological lesions and thereby determination of the degree of damage in fish exposed to different concentrations of the herbicide. The registered responses could be useful indicators of toxicity by glyphosate in the studied species.

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