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Effects of glyphosate and its commercial formulation, Roundup® Ultramax, on liver histology of tadpoles of the neotropical frog, *Leptodactylus latrans* (amphibia: Anura)

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HIGHLIGHTS

- Glyphosate increased the number of liver MMc and MMCs in *L. latrans* tadpoles.
- Liver damages were present on *L. latrans* larvae exposed to pure and formulated.
- This is the first report of adverse effects of glyphosate on anuran larvae liver.

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ABSTRACT

In the last years, the agricultural expansion has led to an increased use of pesticides, with glyphosate as the most widely used worldwide. This is also the situation in Argentina, where glyphosate formulations are the most commercialized herbicides. It is known that glyphosate formulations are much more toxic than the active ingredient, and this difference in toxicity can be attributed to the adjuvants present in the formula. In this context, the aim of the present study was to evaluate and compare sub-lethal histological effects of the glyphosate formulation Roundup Ultramax and glyphosate active ingredient on *Leptodactylus latrans* tadpoles at Gosner-stage 36. Semi-static bioassays were performed using 96 h of exposure with Roundup Ultramax formulation (RU; 0.37–5.25 mg a.e./L), glyphosate (GLY; 3–300 mg/L), and a control group. RU exposure showed an increment in the melanomacrophagic cells (MMc) and melanomacrophagic centers (MMCs) from 0.37 mg a.e./L. GLY exposure showed a significant increment in the number of MMc from 15 mg/L, and of MMCs from 3 mg/L. Also, histopathological lesions were observed in the liver of tadpoles exposed to both, GLY and RU. These lesions included: lipidosis and hepatic congestion, but only RU showed significant differences respect to control, with a LOEC value of 2.22 mg a.e./L for both effects. In sum, this study represents the first evidence of adverse effects of glyphosate and RU formulation on the liver of anuran larvae at concentrations frequently found in the environment.

1. Introduction

Agricultural practices underwent a paradigmatic shift with the advent of the "new green revolution" including the implementation of genetically modified seeds, zero tillage and direct seeding (Atlin et al., 2017; Bindraban et al., 2009; Evenson and Gollin, 2003). The use of transgenic seeds has led to the increase in the consumption of agrochemicals, and as a consequence in a rise of the concentration of agrochemicals into the different ecosystemic compartments (Etchegoyen et al., 2017; Li et al., 2014). These facts raised the question about environmental risk, and different hazard estimators were proposed (Solomon et al., 2000).

Amphibians are particularly sensitive to changes in the environment, they have high skin permeability, eggs with no shell, they

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are ectothermic, and they have a biphasic life cycle with aquatic and terrestrial life stages. These characteristics have made these animals as good bio-indicators of environmental quality (Blaustein et al., 2003; Blaustein and Kiesecker, 2002; Duellman and Trueb, 1994; Simon et al., 2011). Because of these facts, the herpetological scientific community has drawn its attention to the situation of amphibian populations around the world that were showing drastic reductions that, in certain cases, led to their disappearance (Alford et al., 2001; Houlahan et al., 2000; Stuart et al., 2004; Vaira et al., 2012, 2017; Young et al., 2004). In addition, recent studies have proposed the loss of habitat and the environmental pollution as the main contributors of the global amphibian decline (Arntzen et al., 2017; IUCN, 2017).

As said above, the agricultural expansion has led to the increased use of pesticides; being glyphosate the most widely used worldwide (Duke and Powles, 2008). This is also the case in Argentina, where glyphosate-based formulations represent the most commercialized herbicides in the country according to the Camara de Sanidad Agropecuaria y Fertilizantes (CASAFE, 2015). It is also interesting to note that the surface of the cultivated area of genetically modified soybean, engineered to be glyphosate resistant, has increased in the last years in Argentina (López et al., 2012; SAGyP, 2017), and currently, the country tops the list of major soybean producers worldwide, along with the United States and Brazil (Benbrook, 2016). Due to the growing concern about the possible contamination of resources because of the use of glyphosate-based formulations and other pesticides, several studies have been conducted in Argentina, aimed to determine their levels in different environmental matrices (Aparicio et al., 2013; Lupi et al., 2015; Mac Loughlin et al., 2017; Peruzzo et al., 2008; Primost et al., 2017; Ronco et al., 2016). Some of these studies revealed that the glyphosate concentrations ranged from 0.035 to 5.0 mg/kg in soils of Buenos Aires province, near to agricultural areas (Aparicio et al., 2013; Primost et al., 2017). Furthermore, values of glyphosate reported for surface waters and sediments ranged from 0.0005 to 0.7 mg/L and from 0.01 to 5.0 mg/kg, respectively, in agricultural areas from the provinces of Buenos Aires, Entre Ríos, Corrientes, Santa Fe, Chaco and Formosa (Aparicio et al., 2013; Lupi et al., 2015; Mac Loughlin et al., 2017; Peruzzo et al., 2008; Primost et al., 2017; Ronco et al., 2016).

Although it is said that glyphosate is innocuous for non-target species, there is current evidence of adverse effects of high concentrations of both glyphosate and glyphosate-based herbicides on non-target organisms (Annett et al., 2014; de Brito Rodrigues et al., 2016; Uren Webster et al., 2014). Specifically, lethal effects were shown in anuran larvae after the exposure to several glyphosate-based formulations (Annett et al., 2014; Bach et al., 2016; Bernal et al., 2009; Fuentes et al., 2011; Güngördü, 2013; Howe et al., 2004; Mann and Bidwell, 1999; Moore et al., 2012; Relyea and Jones, 2009; Wagner et al., 2017a; b; Yadav et al., 2013). Also, different sub-lethal effects were observed in anuran larvae such as: growth, swimming activity, behavior, morphological abnormalities, DNA damage, alterations in enzyme activities, cardiac and respiratory functions, sex ratio, and histology of the respiratory tract (Bach et al., 2016; Baier et al., 2016; Clements et al., 1997; Costa et al., 2008; Edginton et al., 2004; Howe et al., 2004; Lajmanovich et al., 2003, 2011; 2013; Lanctôt et al., 2013, 2014; Relyea, 2004, 2012; Rissoli et al., 2016; Wagner et al., 2017a; b).

Although numerous biomarkers have been used to evaluate the effects of glyphosate on amphibians, new diagnostic tools are needed particularly in relation to the organs that play a vital role in the processes of detoxification such as the liver. In this sense, the effects of glyphosate on the histology of fish (Bawa et al., 2017; dos Santos Rezende et al., 2017; Hued et al., 2012; Jiraungkoorskul et al., 2003; Nešković et al., 1996; Shiogiri et al., 2012) and mammalian (Benedetti et al., 2004; Çağlar and Kolankaya, 2008; Larsen et al., 2012; Malatesta et al., 2008) liver were evaluated, but little information is available in anurans (Perez-Iglesias et al., 2016). The liver plays a fundamental role in the biotransformation processes of xenobiotics, which in ectotherms, involve both hepatocytes and melanomacrophagic cells (MMc) (Fenoglio et al., 2005; Steinel and Bolnick, 2017). MMc are macrophages that aggregate in melanomacrophagic centers (MMCs) that produce and store three pigments: melanin, hemosiderin, and lipofuscin (Agius, 1981; Franco-Belussi et al., 2012, 2013; Perez-Iglesias et al., 2016). MMc are involved in detoxification processes, due to a combination of enzymatic biotransformation and melanin scavenger action (Fenoglio et al., 2005). Also, because of their phagogicitic nature, MMc can engulf foreign material and participate in the immune defense (Loumbourdis and Vogiatzis, 2002; Sichel et al., 2002; Wolke, 1992). It has also been shown that xenobiotics can alter MMc and MMC's abundance in the liver (Cakici, 2015; de Gregorio et al., 2016; de Oliveira et al., 2016; Franco-Belussi et al., 2013; Loumbourdis and Vogiatzis, 2002; Paunescu et al., 2010; Perez-Iglesias et al., 2016; Zieri et al., 2015) and then they have been proposed as cytological and immunological biomarkers (de Oliveira et al., 2016; Perez-Iglesias et al., 2016; Steinel and Bolnick, 2017). However, to the best of our knowledge, and taking into account the importance of evaluating the effects of xenobiotics in larval stages because of the biological differences and major sensitivity with respect to amphibian terrestrial stages (Mann and Bidwell, 1999; McDiarmid and Altig, 1999), there are no reports on the effects of glyphosate on histological effects on anuran larvae liver.

Leptodactylus latrans (Leptodactylidae) is a common and widely distributed species in South America (Heyer et al., 2010). Its current conservation status is of "Not Threatened" (Vaira et al., 2012) and of "Least Concern" (IUCN, 2017). *L. latrans* has the peculiarity that eggs are laid into foam nests, it presents parental care and its larvae are gregarious, nektonic and move together in shoals (Cei, 1980). The species has been previously used in short duration (48 h) toxicity bioassays (Araújo et al., 2014a, 2014b; Lajmanovich et al., 2015), and it has recently been used in 96 h toxicity bioassays (Bach et al., 2016). Within this context, this study represents the second part of the work published by Bach et al. (2016), aiming to evaluate and compare sub-lethal histological effects of glyphosate and the commercial formulation Roundup Ultramax, on the liver of Gs-36 *Leptodactylus latrans* larvae.

2. Materials and methods

2.1. Chemicals

The solutions were prepared using the glyphosate-based formulation Roundup Ultramax® (RU; Monsanto Argentina S.A.I.C., Buenos Aires, Argentina), containing 74.7% of the monoammonium salt of *N*-(phosphonomethyl) glycine (equivalent to 67.9% of glyphosate acid [w/w]) and inert adjuvants *quantum satis*; and technical-grade glyphosate (GLY; 95.1% purity, GLEBA S.A., La Plata, Buenos Aires, Argentina). All dilutions were made from a 740 mg acid equivalents (a.e.)/L stock solution for RU and a 1500 mg/L stock solution for GLY with filtered dechlorinated tap water (pH 7.7; hardness 150 mg CaCO3/L). In the case of the stock solution of GLY, pH was adjusted ph 7 with 0.1 N NaOH. Samples of test solutions were taken at low, intermediate, and high concentrations, according to the experimental design, immediately after preparation (0 h) and after 24 h of exposure. The GLY concentrations in test solutions (in two water samples taken from a chamber with tadpoles) were determined by liquid-chromatography–mass

Table 1
Growth and development of *L. latrans* tadpoles exposed to Roundup Ultramax and glyphosate. Growth data are expressed as the mean snout-vent length (SVL; in mm) ± SD. Developmental data are presented as the mean Gosner stages ±SEM.

GLY	**0**	**3**	**15**	**75**	**100**
Growth	13.15 ± 0.68	12.96 ± 0.74	12.05 ± 0.64	11.75 ± 0.68	11.66 ± 0.81
Development	36.13 ± 0.68	35.8 ± 0.86	35.20 ± 0.65	35.07 ± 0.89	35.63 ± 0.99
RU	**0**	**0.37**	**0.74**	**2.22**	**5.25**
Growth	13.56 ± 0.50	13.11 ± 0.56	13.70 ± 0.36	12.72 ± 0.61	11.48 ± 0.65
Development	36.40 ± 0.46	35.87 ± 0.66	36.67 ± 0.56	35.67 ± 0.66	35.20 ± 0.75

spectrometry (LC-MS; Agilent 1100 system, Agilent Technologies Inc., Miami, FL, USA) following pre-column derivatization with fluorenylmethyloxycarbonyl at pH = 9 chloride according to standardized methods (Meyer et al., 2009) using 13C, 15N-GLY as quality control in each samples analyzed.

2.2. Test species

As already described in Bach et al. (2016), we collected portions (about 10%) of *L. latrans*'s foam nests (N = 5), which were laid between 8 and 10 h earlier, from a fairly well-preserved area located (Demetrio, 2012) in El Pescado-Stream floodplain, La Plata (35° 1.262′ S, 57° 51.423′ W), Buenos Aires province, Argentina. The study site is located within a rural area, in a floodplain that receives water after prolonged rainfall and gives rise to the formation of temporary ponds, which serve as a breeding site for 14 species of amphibians throughout the year (Bach and Natale unpublished observation). All foam nests were collected with the permission of Dirección de Flora y Fauna from the Buenos Aires Province (code 22500-33442/16) and transported to the laboratory. Once there, the organisms were maintained in 500 L pools with dechlorinated tap water (pH 7.7; hardness 180–250 mg CaCO3/L), at 25 ± 1 °C and a photoperiod of 16L:8D, with continuous aeration. The larvae were fed *ad libitum* with blended lettuce until the individuals reached the Gosner-stage 36 (Gosner, 1960). All tadpoles were treated according to the Ethical Reference Framework for Biomedical

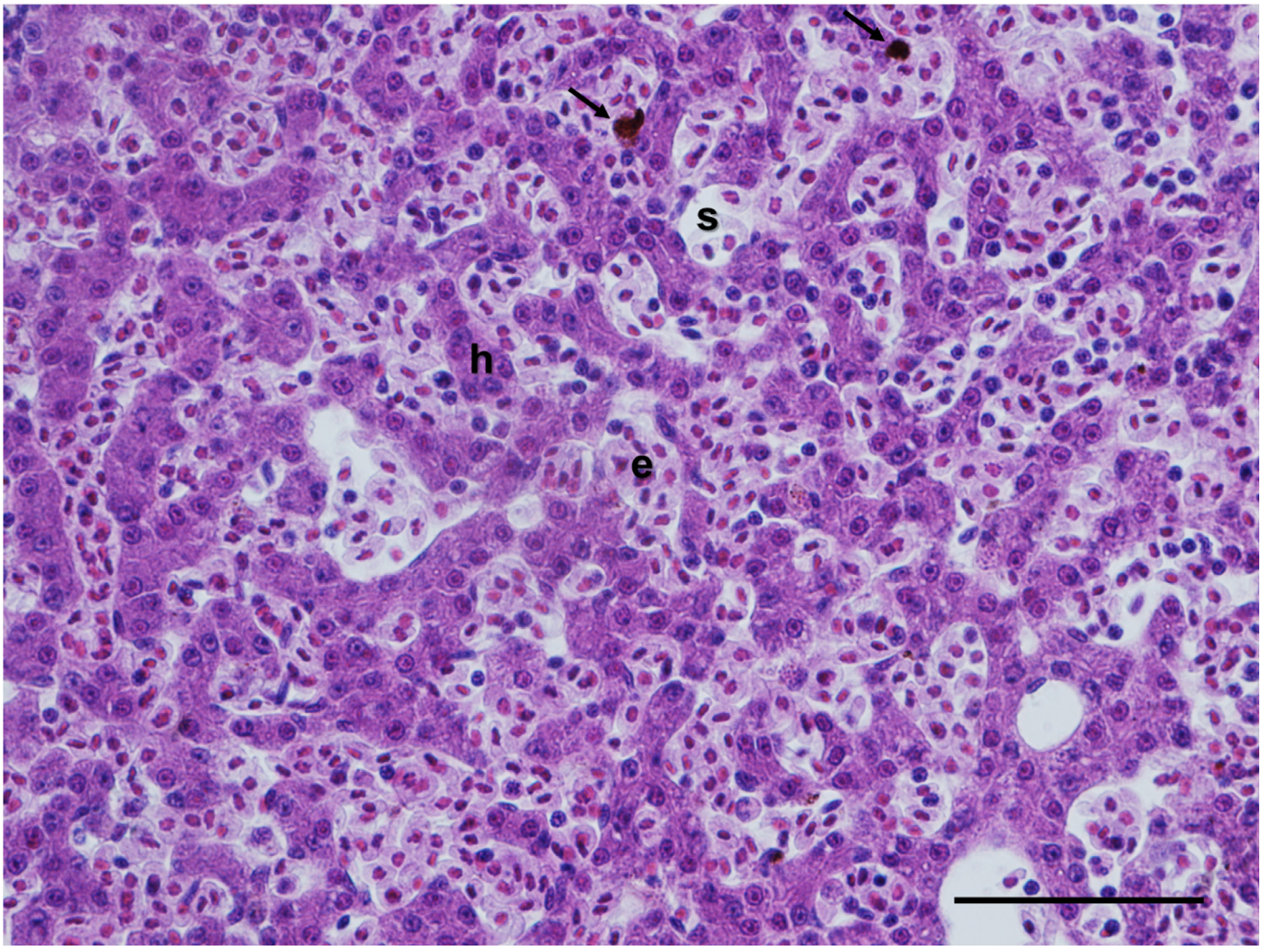

Fig. 1. Histological image of *L. latrans* larvae liver. h: hepatocytes, e: erythrocytes. The black arrows indicate the position of melanomachrophages. Scale bar represents 50 μm.

Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals (CONICET, 2005).

2.3. Toxicity bioassays

The bioassays were performed based on the results of previous studies involving lethal and sub-lethal effects of GLY and RU on Gosner-25 and 36 *L. latrans* tadpoles (Bach et al., 2016). In this case, we evaluated sub-lethal effects on tadpoles at Gosner-stage 36 ± 2 following standardized methods (ASTM, 2007; U.S.E.P.A., 1975) with minor modifications for native species (Bach et al., 2016; Natale et al., 2006). We evaluated four RU concentrations (0.37, 0.74, 2.22 and 5.25 mg a.e./L), four concentrations of GLY (3, 15, 75 and300 mg/L), and a control group with dechlorinated tap water. The concentrations of each compound were determined according to the sub-lethal concentrations reported by Bach et al. (2016). The bioassays were carried out in glass chambers with 500 mL of the corresponding test solution, with medium replacement every 24 h (semi-static conditions) by quadruplicate. Testing conditions were the same as the previously mentioned for larvae maintenance (Section 2.2). No Lethal effects were observed in tadpoles exposed to both GLY and RU. The bioassays began once 50% of the tadpoles reached Gosner-36, then 5 animals per 500 ml (density of 10 tadpoles/L; total n = 160) were randomly placed in the test chambers and they were not fed throughout the bioassays.

2.4. Sample processing for histological analysis

After 96 h of exposure, five tadpoles of each treatment (which did not show variations in the development stage or in the size between treatments; Bach et al., 2016, Table 1) were randomly selected, taking one tadpole from each tank (4 tanks per treatment) and the remaining tadpole was randomly selected from one of the four tanks. Once the larvae were selected, they were euthanized in benzocaine solution (250 mg/L), according to recommendations by Close et al. (1996) and national regulations (CONICET, 2005). Then they were fixed in Bouin's solution overnight at 4°C, and finally preserved in 70% (v/v) aqueous ethanol for subsequent histology evaluation. Larval body were dehydrated in an alcohol series, embedded in paraffin, serially sectioned at 6 μm and stained with hematoxylin and eosin. Ten non-overlapping sections, taken every 12 μm, were observed for each individual under an optical microscope (Nikon Eclipse E600), equipped with a digital camera (Nikon Digital Sight DSFi-Japan), using the program NIS-Elements F 3.0. The number of liver MMc and of MMCs per liver area, were determined according to de Oliveira et al. (2016) and Perez-Iglesias et al., 2016, with minor modifications. Briefly: we counted the number of MMc and MMCs in each liver section, and performed the quotient per liver area. The analyzed area of the liver was determined using Image-Pro Plus program. Histopathological endpoints were evaluated according to Cakici (2015) and were: infiltration, congestion and lipidosis.

2.5. Statistical analysis

GLY concentrations presented in this study were corrected by performing a regression analysis between measured and nominal concentrations of GLY in the water; the regression coefficient (b) was used to correct them. The measured concentrations at the initial time and after 24 h were compared by a paired Student *t*-test (Zar, 2010). Number of MMc or MMCs per section/mm^2 of all individuals studied per treatment were evaluated by one way-ANOVA test followed by Tuckey post-hoc test in order to detect differences between treatments. The averages were taken from the 10 sections per liver and the means of 5 individual tadpoles per treatment were tested for differences. ANOVA assumptions were corroborated by Kolmogorov-Smirnov test for normality and Bartlett's tests for homogeneity of variances (Zar, 2010). Data that failed to meet ANOVA assumptions were analyzed by a Kruskall-Wallis test followed by Dunn's post hoc test (Zar, 2010). Histopathological endpoints were expressed as the proportion of individuals with each histopathological endpoint and were angular transformed according to Zar (2010). One-way ANOVA was performed with Dunnett post-hoc test in order to detect differences in the histopathology with respect to control group. The level of significance used for all tests was $\alpha = 0.05$. All statistical analyses were conducted using Graph Pad Prism 5.00 Software.

3. Results

3.1. Chemical analysis

The GLY concentrations throughout this entire report are given after corrections made on the basis of the measurements on the test solutions of RU ($p < 0.01$, $b = 0.81$; $r^2 = 0.95$) and GLY ($p < 0.01$, $b = 0.32$, $r^2 = 0.99$). Moreover, the concentrations measured at the initial time (0 h) and after 24 h were not significantly different ($p = 0.507$), indicating that GLY concentrations remained relatively constant throughout the bioassays.

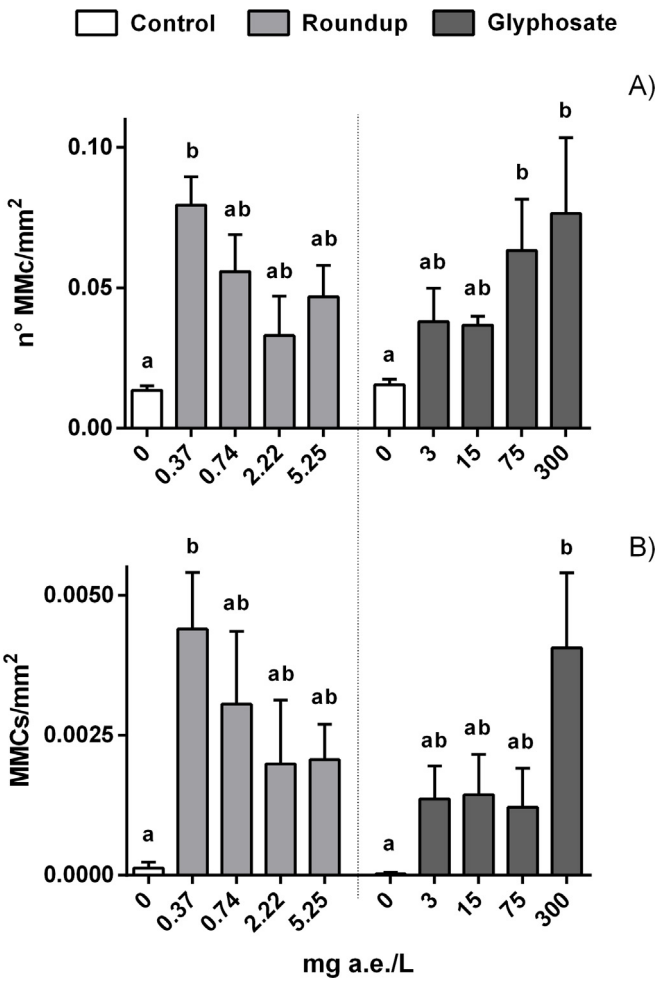

Fig. 2. Liver histological effects of Roundup and glyphosate technical grade exposure of *L. latrans* larvae. a) Number of melanomachrophage cells per mm^2. b) Number of melanomachrophage centers per mm^2. Data expressed as mean value ± SEM. Different letters represent significant differences between treatments.

3.2. Liver histology of Leptodactylus latrans

The liver of Gosner-36 tadpoles of *L. latrans* is characterized by a parenchyma formed by hepatocytes arranged in a simple-layer cordon. The parenchyma also presents hepatic sinusoids, with erythrocytes; and dispersed melanomacrophages. Also, large blood vessels can be distinguished through the parenchyma (Fig. 1).

3.3. Histopathological effects of RU and GLY

The liver of tadpoles exposed to RU presented a significant increase in the number of MMc/mm^2 ($H = 15.03$; *df*: 4; $p < 0.01$) and of MMCs/mm^2 ($H = 12.11$; *df*: 4; $p < 0.05$) in the lowest concentration of 0.37 mg a.e./L for both endpoints with respect to the control group (Dunn's post hoc test; $p < 0.01$ both; Fig. 2), with no

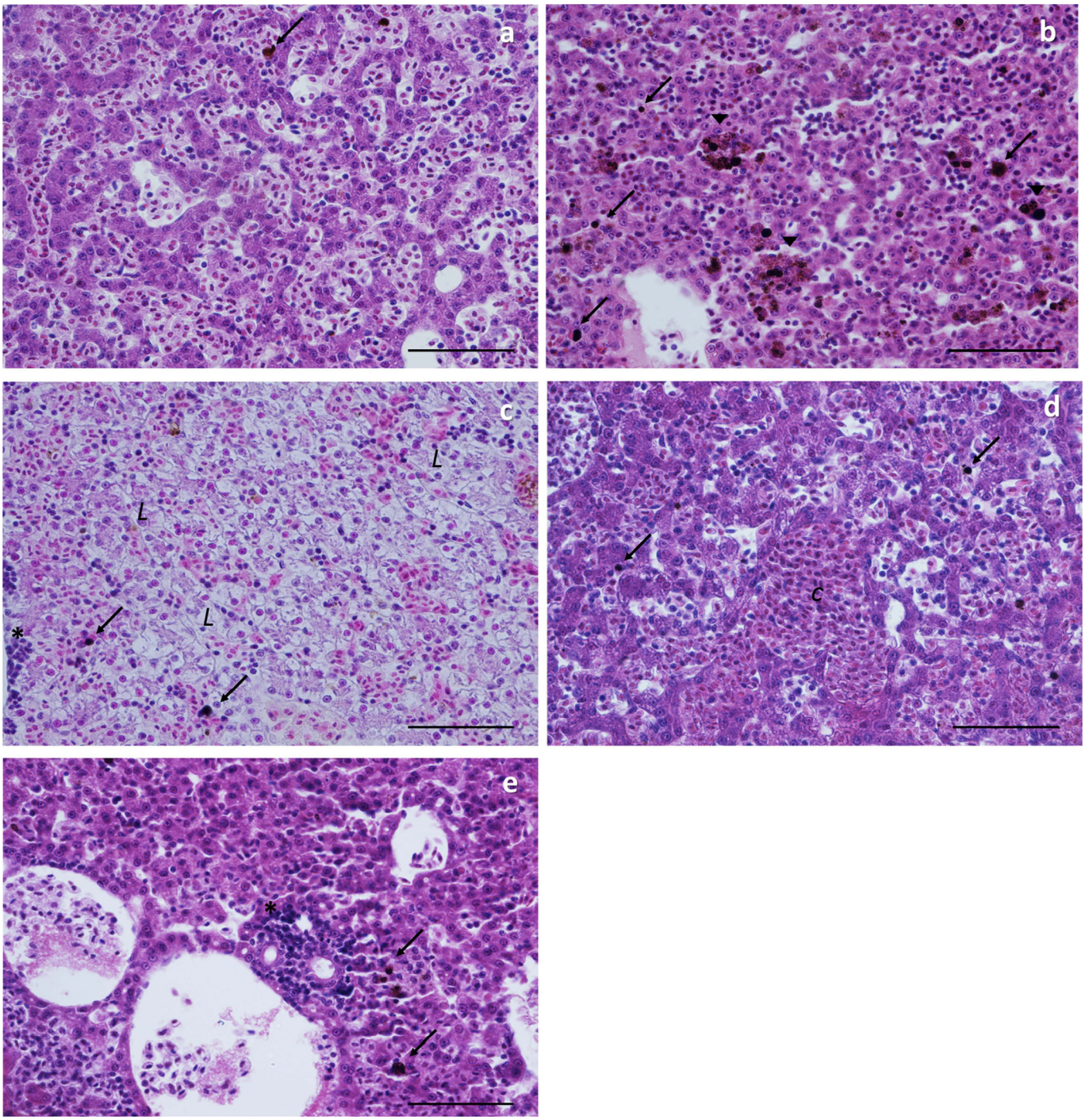

Fig. 3. Liver histopatological effects of Roundup and glyphosate technical grade exposure of *L. latrans* larvae. a) control image. Arrow is pointing a melanomacrophage cell. b) liver of a tadpole exposed to GLY (300 mg/L) showing an incremented number of isolated melanomachrophagic cells (arrows) and melanomachrophagic centers (arrowheads). c) liver of a tadpole exposed to RU (2.22 mg a.e./L) showing lipidosis (L), isolated melanomachrophagic cells (arrows), and infiltration of mononuclear cells (*). d) liver of a tadpole exposed to RU (2.22 mg a.e./L) showing congestion (c) and isolated MMc (arrows). e) liver of a tadpole exposed to RU (2.22 mg a.e./L) showing infiltration of mononuclear cells (*) and isolated MMc (arrows). Scale bars represent 50 μm.

Table 2
Histopathologic lesions detected in the liver of *L. latrans* larvae exposed to Roundup Ultramax and glyphosate. [1]Concentrations of RU are expressed in mg a.e./L. [2]Concentrations of GLY expressed as mg/L. Data are expressed as the mean proportion of affected tadpoles (number of tadpoles showing the histopathological endpoint/total of tadpoles in the test chamber) ± SEM.

Treatment		Infiltration		Congestion		Lipidosis	
		Mean	*p*	Mean	*p*	Mean	*p*
Roundup Ultramax[1]							
0	0.00 ± 0.00		–	0.08 ± 0.05	–	0.00 ± 0.00	–
0.37	0.19 ± 0.11		0.585	0.10 ± 0.10	0.999	0.00 ± 0.00	>0.999
0.74	0.02 ± 0.02		0,999	0.13 ± 0.08	0.982	0.00 ± 0.00	>0.999
2.22	0.30 ± 0.16		0,163	0.46 ± 0.08	0.009	0.98 ± 0.02	0.001
5.25	0.36 ± 0.11		0,111	0.38 ± 0.06	0.048	0.32 ± 0.17	0.450
Glyphosate[2]							
0	0.00 ± 0.00		–	0.14 ± 0.12	–	0.00 ± 0.00	–
3	0.34 ± 0.14		0.181	0.32 ± 0.12	0.916	0.56 ± 0.20	0.139
15	0.36 ± 0.14		0.157	0.28 ± 0.10	0.968	0.74 ± 0.15	0.043
75	0.34 ± 0.14		0.182	0.46 ± 0.16	0.403	0.56 ± 0.23	0.128
300	0.28 ± 0.10		0.364	0.42 ± 0.22	0.415	0.60 ± 0.17	0.212

differences between RU concentrations. Larvae exposed to GLY, also showed a significant increment in the number of MMc/mm^2 ($H = 12.39$; *df*: 4; $p < 0.05$) and of MMCs/mm^2 ($H = 9.88$; *df*: 4; $p < 0.05$), at 75 and 300 mg/L in the first case (Dunn's post hoc test; $p < 0.05$ and $p < 0.01$ respectively; Fig. 2) and 300 mg/L in the second (Dunn's post hoc test; $p < 0.01$; Fig. 2). No effects were detected between GLY concentrations for neither MMc nor MMCs.

The results of the histopathologic analysis revealed that tadpoles exposed to both, RU and GLY, showed lesions in the liver (Fig. 3). As said, the histopathological endpoints observed after 96 h of exposure were infiltration, congestion and lipidosis (Fig. 3; Table 2). Although an increase in the occurrence of histopathological endpoints in larvae exposed to both forms of the herbicide was observed, significant differences in the occurrence of congestion ($F = 5.10$; *df*: 4; $p < 0.01$) and lipidosis ($F = 39.12$; *df*: 4; $p < 0.0001$) of larvae exposed to RU, but not to those exposed to GLY were observed. Congestion and lipidosis resulted significantly incremented from 2.22 mg a.e./L both (Dunnet post-hoc test; $p < 0.01$ and $p < 0.0001$, respectively).

4. Discussion

The organization of the liver of *L. latrans* tadpoles resulted similar to those described for other anuran species with hepatocytes forming cords, sinusoids, large blood vessels and isolated melanomacrophages (Franco-Belussi et al., 2012; Honrubia et al., 1993; McDiarmid and Altig, 1999). However, to the best of our knowledge this is the first description of the structure of the liver in *L. latrans* tadpoles.

Previous studies evaluating the effects of GLY in *L. latinasus* (Perez-Iglesias et al., 2016), revealed that this herbicide, in its pure form, increased the pigmented area, corresponding to melanomacrophages, in the liver of adult frogs. In the present study, not only GLY, but also the commercial formulation RU, were able to induce an increment in the number of MMc and MMCs in the liver of *L. latrans* tadpoles, revealing that the protective activity of melanomacrophages is also present in larval livers. Nevertheless, there are few reports on the effects of pesticides on the hepatic melanomacrophages of anuran larvae and adults, De Oliveira et al. (2016) found similar effects in anuran larvae by the exposition of *Lithobates catesbeianus* tadpoles to Clomazone. In addition, Cakici (2015) found an increment in the number of MMc after the exposure of adult individuals of *Bufotes viridis* to the insecticide carbaryl and Paunescu et al. (2010) found similar results after the exposure of adults of *Pelophylax ridibundus* to chlorpyrifos. Other studies revealed that heavy metals (*Pelophylax ridibundus,* Loumbourdis and Vogiatzis, 2002) and pharmaceuticals found in the environment, like Flutamide -an anti-androgenic drug- (*Rhinella schneideri,* De Gregorio et al., 2016) or 17β-estradiol and testosterone cypionate (*Physalaemus nattereri,* Zieri et al., 2015), could induce the same effects on hepatic melanomacrophages on adult anurans. Then, the increase in the number of MMc and MMCs can be explained by their role in the detoxification processes of xenobiotics, and their relation with the immune response due to its phagocytic activity (Fenoglio et al., 2005; Loumbourdis and Vogiatzis, 2002; Sichel et al., 2002; Wolke, 1992). In this regard, it is interesting to note that the increment of MMc and MMCs can be taken as a generalist and protective response of the liver produced by sub-lethal exposure to xenobiotics in both adult anurans and larvae, and for this reason, it have been proposed as a good cytological biomarker of exposure (de Oliveira et al., 2016; Perez-Iglesias et al., 2016).

In addition, the present data showed that the effects of RU on MMc and MMCs were observed only at the lowest concentrations tested, and the opposite occurred with GLY, where an increase of MMc and MMCs was observed at high concentrations. These data showed a difference in the toxicity of two orders magnitude between both forms of the herbicide (RU formulation and glyphosate alone), taking into account the lowest concentrations that induced an increase in MMc (0,37 mg a.e./L of RU and 75 mg/L of GLY). The differences in toxicity could be explained by the action of the adjuvants in the formulation, either because they are toxic, or because they facilitate the penetration of glyphosate into the organism (Annett et al., 2014; Bonfanti et al., 2018; Edginton et al., 2004; Giesy et al., 2000; Howe et al., 2004; Moore et al., 2012).

Furthermore, we observed that the increase in MMc and MMCs due to Roundup exposure was only significant at low concentrations. This fact could be due to a hormetic response based on a detoxification mechanism of the organism itself, in which a certain degree of toxic exposure triggered physiological responses that were not induced at higher toxic exposure (Calabrese, 2008; Vandenberg et al., 2012). It is important to note, that MMCs responded at lower RU concentrations (0.37 mg a.e./L), while other effects involving hepatic damage, as lipidosis and congestion, were observed at a higher concentration (2.22 mg a.e./L), indicating that glyphosate induces other types of responses at these concentrations. These results are in agreement with the non-monotonic dose-response effects on melanomacrophages of *Rhinella schneideri* exposed to flutamide (De Gregorio et al., 2016), and with the observations of degenerative conditions in the liver when exposed *L. latinasus* to high concentrations of glyphosate, and a predominant phagocytic activity of MMCs at low concentrations (Perez-Iglesias et al., 2016).

On the other hand, we have also found histopathologic lesions,

such as congestion, infiltration and lipidosis, in the liver of *L. latrans* larvae exposed to both, GLY and RU. Previously, Perez-Iglesias et al., 2016 observed similar hepatic lesions after exposure to GLY in adult individuals of *L. latinasus*. These authors observed that pure GLY induced an increment in the proportion of individuals with hepatic congestion and vacuolization of the hepatocyte's cytoplasm (hepatic lipidosis). Our study reveals the same kind of hepatic lesions after GLY and RU exposure of *L. latrans* larvae, but only those exposed to RU showed significant differences with respect to control ones. These difference in the toxicity between both treatments have been already described, and the different authors ascribed these differences to the adjuvants in the formulation that favor the bioavailability of the active ingredient, or else, to additives that contribute to toxicity (Bach et al., 2016; Giesy et al., 2000; Moore et al., 2012). In addition, de Oliveira et al. (2016) found that the herbicide clomazone induced hepatic lipidosis in *L. catesbeianus* tadpoles. Insecticides have also been shown to produce these effects in adult anurans; for example, Paunescu et al. (2010) reported mild karyomegalia, polyploidy, infiltration and fibrosis in the liver of *Pelophylax ridibundus* after exposure to Reldan 40EC© (chlorpyrifos-methyl) insecticide. Other study by the same group (Paunescu et al., 2012) evaluated liver histopathology caused by the insecticide Talstar 10EC© (Bifenthrin) in *Pelophylax ridibundus* showing an induction of lipidosis, nuclear pyknosis, peri-sinusoidal and periportal fibrosis, dilatation of sinusoids and presence of cellular infiltrates. Cakici (2015) found lipidois, necrosis, infiltration, enlargement of sinusoids, hemorrhage and congestion in the liver of exposed toads of *Bufotes viridis* to carbaryl. In addition, it was also found hepatic lipidosis after exposure of the Italian newt (*Lissotriton italicus*) to nonylphenol, a surfactant that might be used in combination to pesticides in commercial formulations (Bernabò et al., 2014).

The hepatic histopathology observed after exposure to RU and GLY, indicate that the herbicide produces hepatic lesions in anuran tadpoles. The infiltration of mononuclear cells (lymphocytes) is related to inflammatory processes and to the immune system, and leads to the increase of melanomacrophages (Crawshaw and Weinkle, 2000; Howerth, 1984; Silva et al., 2013). Lipidosis (the cytoplasm of hepatocytes is covered with lipidic vacuoles) could be explained by the disruption of lipid oxidation, or also by damage in the rough endoplasmic reticulum, that induces the reduction of protein synthesis, resulting in triglyceride accumulation in hepatocytes (Bernabò et al., 2014; Greenfield et al., 2008). Finally, congestion or hypervascularization (Perez-Iglesias et al., 2016) is an increase in the vascularization of the liver that could be related to the increase in melanomacrophages and the infiltration of mononuclear cells.

In sum, this study represents the first evidence of adverse effects of pure glyphosate and the formulation RU on the liver histopathology of anuran larvae at environmental concentrations previously reported in the literature. This study can also contribute to the understanding of the toxicological physiology of GLY and RU, demonstrating that, despite the fact that lethal effects would indicate an absence of toxicity of glyphosate with respect to commercial formulations, sub-lethal effects indicate similarities in the responses produced, thus reducing the toxicity gap between the two compounds. Finally, we would like to emphasize the importance of using native species, such as *L. latrans*, when evaluating local and regional problems.

Disclosure statement

None of the authors have any disclosure to make.

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