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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