





Acute toxicity and genotoxicity of the S-metolachlor-based herbicide Dual Gold® on *Leptodactylus luctator* (Hudson, 1892) tadpoles (Anura: Leptodactylidae).

Gisela Pereira ^{a,*},  Mariabelén Riero ^a , Rafael C. Lajmanovich ^{b,c}  and Raúl Maneyro ^a 

^a Laboratorio de Sistemática e Historia Natural de Vertebrados-Herpetología, Instituto de Ecología y Ciencias ambientales, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, Uruguay.

^b Laboratorio de Ecotoxicología, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje el Pozo s/n (3000), Santa Fe, Argentina.

^c Consejo Nacional de Investigaciones Científicas Técnicas (CONICET), Buenos Aires, Argentina.

* Corresponding author: gpereira@fcien.edu.uy

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ABSTRACT

Acute toxicity and genotoxicity of the S-metolachlor-based herbicide Dual Gold® on *Leptodactylus luctator* (Hudson, 1892) tadpoles (Anura: Leptodactylidae).

Herbicides used in agriculture and their metabolites are frequently detected in surface water bodies, where they can persist and cause adverse effects on aquatic organisms. The aim of this study was to evaluate the acute toxicity and genotoxic effects of the S-metolachlor (SM)-based herbicide Dual Gold® (DG®), on *Leptodactylus luctator* tadpoles (Anura: Leptodactylidae). To assess the toxicity of the herbicide, including the median lethal concentration (LC50) at 24h, the no-observed-effect concentration (NOEC), and the lowest-observed-effect concentration (LOEC), tadpoles were exposed to five nominal concentrations of DG® (5.0, 6.2, 7.8, 9.8, and 12.2 mg/L), and to dechlorinated water as a negative control (NC). The LC50_{24h} of DG® was 7.0 mg/L, the NOEC was 5.0 mg/L and the LOEC=6.2 mg/L. *L. luctator* tadpoles were sensitive to the herbicide, reaching 100% mortality after 24 h of exposure to the highest concentration tested (12.2 mg/L). To evaluate the potential genotoxicity of the herbicide, the frequencies of micronuclei (MN) and other erythrocyte nuclear abnormalities (ENA) were determined in larvae exposed to three nominal concentrations of DG® (1.0, 5.0, and 6.2 mg/L) for 48 and 96 h. The frequencies of MN and ENA were compared with a positive control (40 mg/L of Cyclophosphamide) and a negative control. The frequencies of MN and ENA in the erythrocytes of tadpoles exposed to the test concentrations of DG® and Cyclophosphamide were significantly higher than in the negative control group at both 48 and 96 h (with the only exception of MN at 1.0 mg/L at 48 h). Our results confirm the genotoxic and cytotoxic effects of this widely used herbicide in agriculture, a fact that represents a potential risk to amphibians that develop in ponds associated with or immersed in agroecosystems.

KEYWORDS: agrochemicals; lethality; micronuclei; erythrocyte nuclear abnormalities; commercial formulation; active ingredient; amphibians.

RESUMEN

Toxicidad aguda y genotoxicidad del herbicida Dual Gold®, a base de S-metolachloro sobre larvas de *Leptodactylus luctator* (Hudson, 1892) (Anura: Leptodactylidae).

Los herbicidas utilizados en agricultura y sus metabolitos, son frecuentemente detectados en cuerpos de agua superficiales, donde pueden persistir y provocar efectos adversos a organismos acuáticos. Evaluamos la toxicidad aguda y geno-

toxicidad del herbicida Dual Gold® (DG®), a base de S-metolachloro (SM), sobre larvas de *Leptodactylus luctator* (Anura: Leptodactylidae). Para determinar la toxicidad del herbicida, incluida la concentración letal media (CL50) 24h, la concentración sin efecto observado (NOEC); y con efecto observado más baja (LOEC); las larvas fueron expuestas a cinco concentraciones nominales de DG® (5.0; 6.2; 7.8; 9.8; y 12.2 mg/L); y a un control negativo con agua dechlorada. El valor de CL50_{24h} para DG® fue de 7.0 mg/L (NOEC=5.0 mg/L; LOEC=6.2 mg/L). Las larvas fueron sensibles al herbicida, alcanzando el 100% de mortalidad a las 24h, en la concentración más alta testada (12.2 mg/L). Para estudiar el potencial genotóxico del herbicida, se determinó la frecuencia de micronúcleos (MN) y de otras aberraciones nucleares de los eritrocitos (ENA), en larvas expuestas a tres concentraciones de DG® (1.0, 5.0 y 6.2 mg/L), durante 48 y 96h; y las mismas se compararon con un control positivo (40 mg/L de Ciclofosfamida) y negativo. Las frecuencias de MN y ENA en los eritrocitos de larvas expuestas a las concentraciones de prueba de DG® y Ciclofosfamida fueron significativamente mayores que en el grupo control negativo, a las 48 y 96 h (con la única excepción de MN a 1.0 mg/L a las 48 h). Nuestros resultados comprueban los efectos genotóxicos y citotóxicos de este herbicida ampliamente utilizado en la agricultura, lo cual representa un riesgo potencial para los anfibios que se desarrollan en cuerpos de agua asociados o inmersos en agroecosistemas.

PALABRAS CLAVES: agroquímicos; letalidad; micronúcleos; anomalías nucleares de eritrocitos; formulación comercial; ingrediente activo, anfibios.

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INTRODUCTION

One of the primary causes of the massive global decline observed in amphibian populations is the environmental pollution resulting from the increased use of agrochemicals (IUCN, 2022). Pesticides used in agriculture and their metabolites are frequently detected in surface water bodies (e.g., Gilliom et al., 2006; De Liguoro et al., 2014; Nardo et al., 2015), where they can persist and cause adverse effects on non-target aquatic organisms such as fish and amphibians (Zhelev et al., 2018). Anurans amphibians are particularly vulnerable to aquatic contamination since this environment is where the amplexus, oviposition, and initial (and most critical) stages of their life cycle, such as embryonic and larval development, occur (Wells, 2007). Additionally, anurans have a thin and permeable skin, which allows the entry of water-soluble substances, and a high bioaccumulation rate (Unrine et al., 2007). Due to their high sensitivity, they are frequently used as bioindicators of environmental health (e.g., Attademo et al., 2005; Peltzer et al., 2013; Eterovick et al., 2016; Saccol et al., 2017; Bionda et al., 2018).

The use of bioassays to evaluate the exposure to agrochemicals is a common experimental approach to obtain dose/response information in amphibians (Pérez-Iglesias et al., 2019; Freitas et al., 2019; Lajmanovich et al., 2022). To detect the

biological consequences of exposure, biomarkers of toxic effects (Lajmanovich et al., 2021), such as alterations in the morphology of blood cells and enzymatic activity (Attademo et al., 2014; Lajmanovich et al., 2014; Gripp et al., 2017), are used. One of the most prominent indicators of damage to genetic material is the presence of micronuclei (MN) in red blood cells (Lajmanovich et al., 2005). Another nuclear abnormality considered as an indicator of genotoxicity is the presence of erythrocyte nuclear abnormalities (ENA) (Carrasco et al., 1990). In amphibians, evaluations of genotoxicity through the recognition of these biomarkers, using techniques such as the Micronucleus Test (Schmidt, 1975), are increasing (Pollo et al., 2015; Pérez-Iglesias et al., 2015; Lajmanovich et al., 2018; Benvindo-Souza et al., 2020).

S-metolachlor (SM) (CAS 87392-12-9) is a selective herbicide belonging to the chloroacetamide group (Lewis et al., 2016; PAN Pesticide Database, 2022). It is absorbed by roots and shoots, and inhibits cell division and growth (Valotton et al., 2008; Lewis et al., 2016). SM has a high potential for bioaccumulation, is moderately mobile in the environment, has low volatility, and has moderate solubility in water and persistence in the soil (Lewis et al., 2016; Observatorio Ambiental Nacional, 2022), all of which increases the likelihood of runoff into surface water bodies.

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SM is also considered an important contaminant of groundwater (PAN Pesticide Database, 2022). The effects of SM have been evaluated in fish (Quintaneiro et al., 2017; Liu et al., 2022), crabs (Velisek et al., 2019; Stara et al., 2019), crustaceans (Liu et al., 2006), and bivalve mollusks (Mai et al., 2013). In amphibians, studies on the toxic effects of Metolachlor have been conducted on *Xenopus laevis* (Daudin, 1802) embryos (Osano et al., 2002) and *Lithobates catesbeianus* (Shaw, 1802) larvae (Wan et al., 2006), and endocrine alterations have also been evaluated in adult individuals of *Lithobates pipiens* and *L. clamitans* (McDaniel et al., 2008). Previous studies have also evaluated the chronic effects of SM on larvae of *Anaxyrus americanus* (Holbrook, 1836), *Pseudacris triseriata* (Wied-Neuwied, 1838), and *Dryophytes versicolor* (LeConte, 1825) (Williams et al., 2010), as well as its effects on *Pelophylax perezii* (López-Seoane, 1885) embryos (Quintaneiro et al., 2018). Paunescu et al. (2018) assessed the effects of the SM-based herbicide DG®, and reported different alterations, such as hepatic lesions, hyperglycemia, and a decrease in the number of erythrocytes, in *Pelophylax ridibundus* (Pallas, 1771) adults exposed to the herbicide. More recently, de Arcaute et al. (2020) studied the acute toxicity and the interactions between equitoxic and non-equitoxic mixtures of the herbicides Credit® (based on glyphosate) and Twin Pack Gold® (formulated with flurochloridone and SM) on *Rhinella arenarum* (Hensel, 1867) tadpoles and found that the toxicity of the mixtures varied from being additive to being slightly antagonistic.

Given that, due to its higher herbicidal activity (Shaner et al., 2006), the S-isomer of Metolachlor is one of the most widely used compounds in agriculture worldwide (Atwood & Paisley-Jones, 2017), further studies are needed to evaluate its effects on amphibian tadpoles. In Uruguay, SM is mainly used in fallow crops such as soybean, eucalyptus, corn, sorghum, and peanuts. The quantity of imported SM active ingredient increased from 222 336 kg in 2014 to 538 369 kg in 2020. It is classified as a priority pesticide for environmental management (Observatorio Ambiental Nacional, 2022). Regionally, the presence of Metolachlor and SM has been detected in sediments,

as well as in surface and groundwater, in areas with agricultural activity (Andrade et al., 2021; Vera-Candiotti et al., 2021; Lajmanovich et al., 2023; Van Opstal et al., 2023). Despite the central role of agricultural activity in the Pampas biome (Baeza et al., 2022), only one study evaluating the effect of herbicide mixtures, including a formulation with SM and another active ingredient, on regional amphibians is known (de Arcaute et al., 2020). However, there are no studies assessing the effects of commercial formulations based solely on SM on Pampean amphibians.

Thus, the aim of this study was to evaluate the lethality and genotoxic effects of the SM-based herbicide DG® on *Leptodactylus luctator* tadpoles (Anura: Leptodactylidae), using acute toxicity bioassays. Our specific objectives were to determine the mean lethal concentration (LC50_{24h}) of DG® on *L. luctator* tadpoles and to evaluate the frequency of MN and ENA in peripheral blood erythrocytes of *L. luctator* tadpoles.

MATERIALS AND METHODS

Chemicals

The commercial formulation of the herbicide Dual Gold® 960 EC (DG®) (87.3% S-metolachlor ((S)-2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide, CAS# 87392-12-9), marketed by Syngenta Uruguay S.A., was used. Cyclophosphamide (CP) (ENDOXAN, 50 mg from the Baxter laboratory in Germany) was used as a positive control. The test concentrations reported throughout the study represent the nominal concentrations of the active ingredient SM contained in the commercial formulation (DG®).

Test organisms

Leptodactylus luctator (Anura: Leptodactylidae) has a wide distribution in the southern cone of South America (Magalhaes et al., 2020). It has a broad habitat use and can be found in natural field areas as well as in environments affected by agricultural and forestry activities (Maneyro & Carreira, 2012). It is considered a very common species and is not listed as threatened in regional

red lists (e.g., Carreira & Maneyro, 2015) or at a global scale (Heyer *et al.*, 2010). Several previous toxicological studies have used this species as a test organism (Araujo *et al.*, 2014; Josende *et al.*, 2015; Lajmanovich *et al.*, 2015; Bach *et al.*, 2016; 2018; Samojeden *et al.*, 2022).

Collection and maintenance of *Leptodactylus luctator* tadpoles

Tadpoles were manually collected from a site without agricultural activities located in Melilla, Montevideo, Uruguay. They were then transported to an animal facility located in the “Facultad de Ciencias, de la Universidad de la República”, Montevideo, with water from the collection site. The tadpoles ($n=300$) were collected with the permission of the “Dirección Nacional de Biodiversidad y Servicios Ecosistémicos” (code EM2022/36001/003604), of the “Ministerio de Ambiente” (Montevideo, Uruguay). Their average body length was 9.7 ± 0.7 (8.4–11.5) mm and their weight was 0.13 ± 0.02 (0.09–0.18) g, and they were in stage GS 25–28 (Gosner, 1960). Tadpoles were acclimated for 48 h in 20-L aquaria containing previously filtered (PSA filter, senior 3 model) and dechlorinated tap water ($\text{pH}=7.6\pm 0.4$; conductivity= 374 ± 22 $\mu\text{S/s}$; dissolved oxygen= 8.4 ± 0.9 mg/L; temperature= 19 ± 1.3 °C), with constant artificial aeration, a 12:12 hour light/dark photoperiod, and an ambient temperature of 22 ± 2 °C. They were fed *ad libitum* with boiled lettuce (*Lactuca sativa*) until the beginning of the bioassays (Lajmanovich *et al.*, 2014).

Experimental design

All the procedures were conducted following the experimentation protocols approved by the “Comisión de Ética en el uso de Animales (CEUA) de la Facultad de Ciencias de la Universidad de la República” (protocols number 474 and 684).

LC50 bioassays

The concentrations to be tested in 24-h acute toxicity tests were determined based on the concentration range obtained in preliminary tests,

using a dilution factor of 1.25. To determine the mean lethal concentration ($\text{LC}_{50_{24\text{h}}}$), the no-observed-effect concentration (NOEC), and the lowest observed-effect concentration (LOEC), tadpoles were exposed to five nominal concentrations of SM: T1=5.0 mg/L, T2=6.2 mg/L, T3=7.8 mg/L, T4=9.8 mg/L, and T5=12.2 mg/L, and to dechlorinated filtered water as a negative control. The experiments were conducted using 1-L glass containers containing 500 mL of the test solution. Three replicates per treatment were conducted, with five tadpoles per replicate. The containers were randomly distributed. Mortality was recorded at 24 h, and determined by the absence of movement after touching the tadpoles with a plastic rod, as well as by changes in their color and appearance (Bach *et al.*, 2016). Dead individuals were immediately removed and fixed in 10% formalin.

Genotoxicity

To determine the genotoxic potential of the herbicide, 96-h acute experiments were performed. Tadpoles were exposed to three different nominal concentrations of SM ($\text{NOEC}/5=\text{Ta}$; $\text{NOEC}=\text{Tb}$ and $\text{LOEC}=\text{Tc}$), as well as to dechlorinated tap water as a negative control and 40 mg/L of CP as a positive control (Lajmanovich *et al.*, 2005; Pérez-Iglesias *et al.*, 2015). All test solutions and controls were prepared in triplicate immediately before use and replaced every 48h, in accordance with the guidelines established by USEPA (1975). The experiments were conducted using 3-L glass containers containing 2 L of the test solution. Ten tadpoles were placed in each container/replicate ($n=150$). Mortality was recorded every 24 h.

Throughout both the LC_{50} bioassays and genotoxicity experiments, the tadpoles were not fed, and the pH, dissolved oxygen (mg/L), conductivity, and temperature of the water were monitored in accordance with the recommendations of USEPA (1975).

Genotoxicity analysis: Micronucleus test

Blood was extracted from twelve randomly selected tadpoles per treatment (four per replicate) at 48 and 96 h of exposure and duplicate smears

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were prepared on clean slides. The tadpoles were euthanized using topical lidocaine, then decapitated, and blood was extracted using heparinized capillaries, following the experimental protocols approved by the CEUA (protocols numbers 474 and 684), as stated above.

The smears were fixed with absolute alcohol and stained using the May-Grünwald Giemsa staining method (Barni et al., 2007; Lajmanovich et al., 2014). The frequencies of MN and ENA were determined in 1000 erythrocytes from each tadpole, using an optical microscope (Olympus BX41 model) under 1000x magnification (Lajmanovich et al., 2005). Coded and randomized slides were examined blind by single operator. Blood smears were photographed using an Evolution vf cooled color camera, and the images were processed using ImageJ software (National Institute of Health, USA).

To correctly identify MN, we utilized the criteria established by Fenech (2000) and Meintières et al. (2001). These criteria emphasize that MN are morphologically identical to the main cell nucleus, but smaller in size (with a diameter less than 1/3 of the main nucleus), have similar staining intensity, are not connected, or are overlapped with the main nucleus, and are located within the cytoplasm. ENA were classified as: lobed nuclei (LN), kidney-shaped nuclei (K), pyknotic nuclei (PN), notched nuclei (NN), blebbed nuclei (BbN), budded nuclei (BN), erythroplastids or anucleated erythrocytes (EP), and binucleated erythrocytes (BE) (Carrasco et al., 1990; Guilherme et al., 2008; Lajmanovich et al., 2014; 2015). The frequency of ENA was expressed as the mean value (‰) of the sum of all individual aberration types observed (Lajmanovich et al., 2014). The frequencies of MN and ENA were evaluated and compared to a positive control (40 mg/L of CP) and a negative control (filtered and dechlorinated water).

Data analyses

The lethal concentration (LC_{50}) value and its respective 95 % confidence limits were determined using the Trimmed Spearman-Kärber method (Hamilton et al., 1977). The NOEC and LOEC values were calculated using the “mixtox” pack-

age in R software (Zhu & Cheen, 2016). Linear regression analyses were performed to confirm the association between mortality and test concentrations. The frequencies of MN and ENA were determined at 48 and 96 h after initial treatment and expressed as mean \pm standard error. Data of MN and other ENA were analyzed using the binomial proportion test (Margolin et al., 1983). To verify changes in genotoxic responses to the same herbicide concentrations as a function of exposure time, the frequencies of MN and ENA were compared between treatments at 48 and 96 h using the non-parametric Mann-Whitney U test. Statistical analyses were performed using PAST (Paleontological STatistics) software version 2.17c (Hammer et al., 2001); BioEstat 5.0 (Ayres et al., 2007); and R software version 4.2.2 (R Core Team, 2022). The Levene test was used to test for homogeneity of variances, and the Shapiro-Wilk test was used to test for normality of data. The significance level was set at $\alpha = 0.05$.

RESULTS

No mortality was observed in the negative controls in either of the two assays ($LC_{50_{24h}}$ and genotoxicity). The $LC_{50_{24h}}$ of the SM-based herbicide DG® (95 % confidence limits) was 7.0 mg/L (6.6-7.4), the NOEC was 5.0 mg/L, and the LOEC was 6.2 mg/L. A positive and significant correlation was found between mortality and the concentration of the herbicide ($R^2=0.74$; $p<0.05$). The highest concentration (12.2 mg/L of SM) resulted in the death of all tadpoles after 24 h of exposure. No mortality was recorded in the positive controls or treatments Ta (1 mg/L of SM) and Tb (5.0 mg/L of SM) in the genotoxicity assay, both at 48 and 96 h of exposure. No mortality of individuals was recorded in the Tc treatment (6.2 mg/L of SM) at 48 h. However, mortality at the highest concentration was 89 % at 96 h, and therefore the Tc treatment was not included in the genotoxicity analysis at this exposure time.

Leptodactylus luctator tadpoles presented mature oval-shaped erythrocytes, with a central nucleus and a well-defined boundary (Fig. 1A). MN were easily identified in the cellular cytoplasm as small spherical fragments, smaller than the main nucleus, and always separated from it (Fig. 1G).

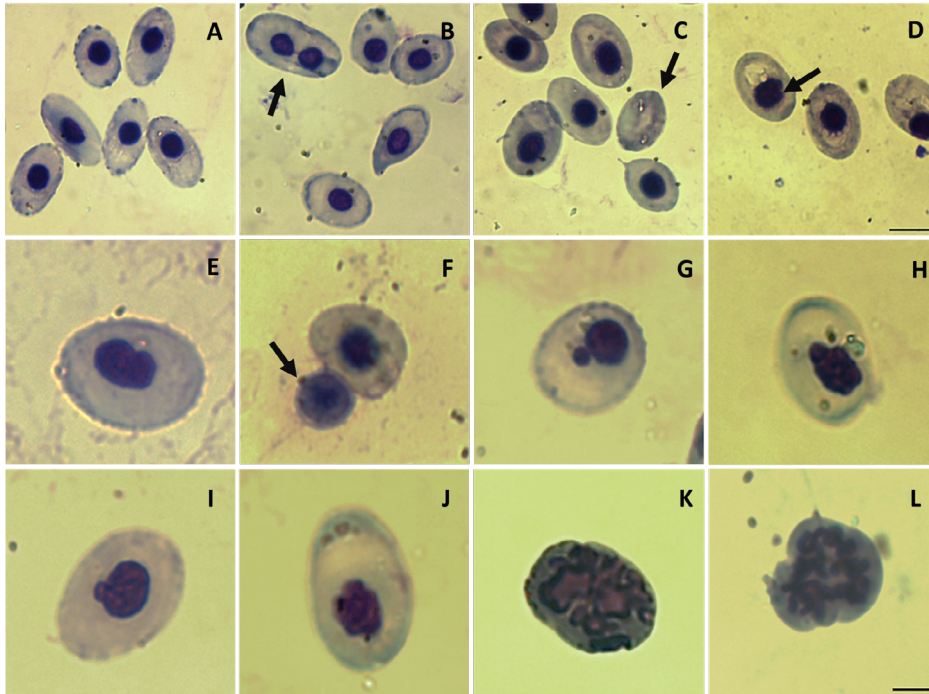


Figure 1. Details of erythrocytes observed in *Leptodactylus luctator* tadpoles exposed to the SM-based herbicide DG[®]. (A) Normal erythrocytes; (B) Binucleated erythrocyte (BE); (C) Erythroplastid or anucleated erythrocyte (EP); (D) Notched nucleus (NN); (E) Kidney-shaped nucleus (K); (F) Pyknotic nucleus (PN); (G) Micronucleus (MN); (H) Lobed nucleus (LN); (I) Budded nucleus (BN); (J) Blebbed nucleus (BbN); (K) Apoptotic cell (AP); (L) Erythrocyte in mitosis (M). May Grünwald-Giemsa staining, 100x. Black bar in image D represents a scale of 10 µm (for images A-D). Black bar in image L represents a scale of 5 µm (for images E-L). *Detalle de eritrocitos observados en larvas de Leptodactylus luctator expuestas al herbicida DG[®], a base de SM. (A) eritrocitos normales; (B) eritrocito binucleado (BE); (C) eritoplástido o eritrocito anucleado (EP); (D) núcleo con muesca (NN); (E) núcleo en forma de riñón (K); (F) núcleo picnótico (PN); (G) Micronúcleo (MN); (H) núcleo lobado (LN); (I) núcleo con brote (BN); (J); núcleo ampollado (BbN); (K) célula apoptótica (AP); (L) eritrocito en mitosis (M). May Grünwald-Giemsa, 100x. La barra negra en la imagen D representa una escala de 10 µm (imágenes A-D). La barra negra en la imagen L representa una escala de 5 µm (imágenes E-L).*

Apoptotic cells (AP) (Fig. 1K) and cells in mitosis (Fig. 1L) were also observed in tadpoles exposed to the herbicide. Figure 2 shows the frequencies of MN and responses as a function of exposure time to the different concentrations of SM evaluated. The frequency of micronucleated erythrocytes in *L. luctator* tadpoles increased with herbicide concentration at both 48 and 96 h of exposure (Fig. 2). At 48 h, the frequency of MN in treatment Ta (1 mg/L of SM) was not significantly different from that in the negative control group (Fig. 2). However, the differences between these two treatments were significant at 96 h. For the rest of the test concentrations, including the positive control (40 mg/L of CP), the frequencies of MN were significantly higher than

those in the negative control group, both at 48 and 96 h (Fig. 2).

In addition to the MN, we identified the presence of different ENA in tadpoles exposed to the herbicide (Fig. 1). We also observed an increase in the frequency of ENA at higher concentrations of SM, both at 48 and 96 h of exposure (Fig. 3). All test treatments exhibited statistically significant differences in the frequency of ENA compared to the negative control group, at both evaluation times (Fig. 3). Additionally, significant differences were observed in the frequency of ENA at 48 and 96 h between tadpoles exposed to CP and the negative control group. The frequencies of each type of ENA in each treatment at 48 and 96 h are presented in Table 1. At 48 h of ex-

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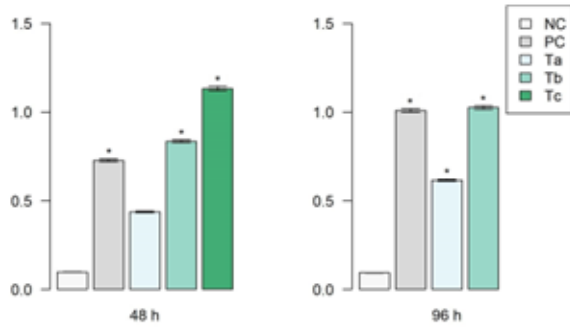


Figure 2. Frequency of micronuclei (MN) (per-1000 cells) in *Leptodactylus luctator* tadpoles exposed to different concentrations of the SM-based herbicide DG®. NC: negative control (0 mg/L of SM); PC: positive control (40 mg/L of cyclophosphamide); Ta=1 mg/L of SM; Tb=5.0 of mg/L SM; Tc=6.2 mg/L of SM. RBCs= red blood cells. * $p < 0.05$: significant differences compared to the negative control (binomial proportion test). *Frecuencia de micronúcleos (MN) (por 1000 células) en larvas de Leptodactylus luctator expuestas a diferentes concentraciones del herbicida DG®, a base de SM. NC: control negativo (0 mg/L de SM); CP: control positivo (40 mg/L de ciclofosfamida); Ta=1 mg/L de SM; Tb=5.0 de mg/L SM; Tc=6.2 mg/L de SM. * $p < 0.05$: diferencias significativas con respecto al control negativo (Test de proporción binomial).*

posure, the frequencies of ENA were as follows: NN>EP>K>LN>AP>BbN>BE>BN>PN; whereas at 96 h, the order was as follows: EP>BE>LN>NN>BN>K>BbN>PN>AP.

No significant differences were observed in the frequencies of MN between the same test concentrations at 48 and 96 h of exposure. However, significant differences were found in the frequency of ENA at 48 and 96 h in the Ta treatment (5.0 mg/L of SM) ($U=28$; $p < 0.05$), and in the positive control (40 mg/L of CP) ($U=22$; $p < 0.01$).

DISCUSSION

As mentioned previously, *Leptodactylus luctator* is a common and widely distributed species in the southern cone of South America. However, still few studies have used this anuran amphibian as a test organism in ecotoxicological research (e.g., Araujo et al., 2014; Josende et al., 2015;

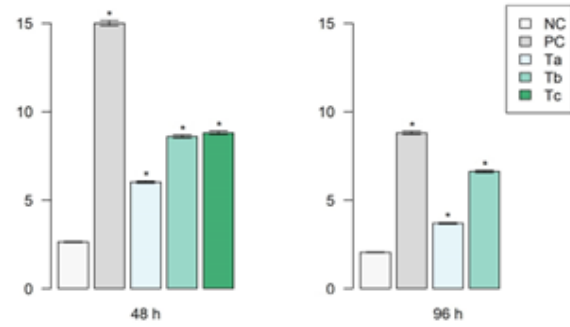


Figure 3. Frequency of erythrocytes nuclear abnormalities (ENA) (per-1000 cells) in *Leptodactylus luctator* tadpoles exposed to different concentrations of SM-based herbicide DG®. NC: negative control (0 mg/L of SM); PC: positive control (40 mg/L of cyclophosphamide); Ta=1 mg/L of SM; Tb=5.0 mg/L of SM; Tc=6.2 mg/L of SM. RBCs= red blood cells. * $p < 0.05$: significant differences compared to the negative control (binomial proportion test). *Frecuencia de Aberraciones nucleares en los eritrocitos (ANE) (por 1000 células) en larvas de Leptodactylus luctator expuestas a diferentes concentraciones del herbicida DG®, a base de SM. NC: control negativo (0 mg/L de SM); CP: control positivo (40 mg/L de ciclofosfamida); Ta=1 mg/L de SM; Tb=5.0 mg/L de SM; Tc=6.2 mg/L de SM. * $p < 0.05$: diferencias significativas con respecto al control negativo (Test de proporción binomial).*

Lajmanovich et al., 2015; Bach et al., 2016; 2018; Samojeden et al., 2022). Although SM is one of the most commonly used herbicides in agriculture (Atwood & Paisley-Jones, 2017), with frequent use in Uruguay (Observatorio Ambiental Nacional, 2022) and the region (Andrade et al., 2021; Vera-Candiotti et al., 2021; Lajmanovich et al., 2023; Van Opstal et al., 2023), its toxicity and genotoxic effects on *L. luctator* tadpoles have not yet been studied.

The LC_{50}_{24h} value for the herbicide DG®, determined in this study for *L. luctator* tadpoles, was 7.0 mg/L. Studies on the acute toxicity of SM and SM-based commercial formulations on amphibian tadpoles are scarce. A LC_{50}_{24h} value of 56 mg/L has been reported for the commercial formulation Primextra® II (35.8 % SM, 28.7% of atrazine) on *Lithobates catesbeianus* tadpoles (Wan et al., 2006), and a LC_{50}_{192h} value of 37.5

Table 1. Frequency (%) of different types of erythrocytes nuclear abnormalities (ENA) in *L. luctator* tadpoles exposed to different concentrations of SM (mean \pm SE). NC: negative control (0 mg/L of SM); PC: positive control (40 mg/L of cyclophosphamide); Ta=1 mg/L of SM; Tb=5.0 mg/L of SM; Tc=6.2 mg/L of SM; BE: binucleated erythrocytes; K: kidney-shaped nuclei; EP: erythroplastids or anucleated erythrocytes; BN: budded nuclei; LN: lobed nuclei; BbN: blebbed nuclei; NN: notched nuclei; PN: pyknotic nuclei; AP: apoptotic cells. * $p < 0.05$: significant differences compared to the negative control (binomial proportion test). *Frecuencia (%) de distintos tipos de aberraciones nucleares en eritrocitos (ANE) de larvas de L. luctator expuestas a diferentes concentraciones de SM (media \pm SE). NC: control negativo (0 mg/L de SM); CP: control positivo (40 mg/L de ciclofosfamida); Ta=1 mg/L de SM; Tb=5.0 mg/L de SM; Tc=6.2 mg/L de SM; BE: eritrocitos binucleados; K: núcleos en forma de riñón; EP: eritoplastidos o eritrocitos anucleados; BN: núcleos con brotes; LN: núcleos lobados; BbN: núcleos ampollados; NN: núcleos con muescas; PN: núcleos picnóticos; AP: célula apoptótica. * $p < 0.05$: diferencias significativas con respecto al control negativo (Test de proporción binomial).*

ENA	h	Treatments				
		NC	PC	Ta	Tb	Tc
BE		0.78 \pm 0.01	2.72 \pm 0.02*	1.05 \pm 0.01	0.83 \pm 0.01	0.38 \pm 0.004
K		0.58 \pm 0.01	2.18 \pm 0.02*	1.14 \pm 0.01	1.67 \pm 0.02*	0.50 \pm 0.01
EP		0.39 \pm 0.004	2.72 \pm 0.02*	0.61 \pm 0.01	1.42 \pm 0.01*	1.89 \pm 0.02*
BN		0	0.54 \pm 0.005	0.44 \pm 0.004	0.33 \pm 0.003	0.25 \pm 0.003
LN	48	0.39 \pm 0.004	1.64 \pm 0.02*	0.96 \pm 0.01	0.92 \pm 0.01	1.26 \pm 0.01*
BbN		0.19 \pm 0.002	1.09 \pm 0.01*	0.09 \pm 0.001	0.50 \pm 0.01	0.50 \pm 0.01
NN		0.29 \pm 0.003	3.27 \pm 0.03*	1.14 \pm 0.01*	1.92 \pm 0.02*	1.76 \pm 0.02*
PN		0	0.45 \pm 0.004	0.09 \pm 0.001	0.33 \pm 0.003	0.38 \pm 0.004
AP		0	1.27 \pm 0.01	0.52 \pm 0.01	0.67 \pm 0.01	1.89 \pm 0.02
BE		0.37 \pm 0.004	1.93 \pm 0.02*	0.97 \pm 0.01*	1.03 \pm 0.01*	-
K		0.28 \pm 0.003	1.10 \pm 0.01*	0.09 \pm 0.001	0.47 \pm 0.004	-
EP		0.93 \pm 0.01	1.65 \pm 0.02	1.49 \pm 0.01	2.24 \pm 0.02*	-
BN		0.19 \pm 0.002	0.73 \pm 0.01*	0.35 \pm 0.003	0.47 \pm 0.004	-
LN	96	0	0.92 \pm 0.01	0.53 \pm 0.005	0.84 \pm 0.01	-
BbN		0	0.55 \pm 0.01	0	0.47 \pm 0.004	-
NN		0.19 \pm 0.002	1.65 \pm 0.02*	0.26 \pm 0.002	0.75 \pm 0.01*	-
PN		0	0.18 \pm 0.002	0	0.28 \pm 0.003	-
AP		0.09 \pm 0.001	0.09 \pm 0.001	0	0.09 \pm 0.001	-

mg/L has been reported for SM on embryos of the common frog *Pelophylax perezi* (Quintaneiro et al., 2018). Our results suggest that *L. luctator* tadpoles are less tolerant to SM than *P. perezi* embryos. Differences in sensitivity could be attributed to intrinsic differences between species, as well as to the different developmental stages evaluated, and to the commercial formulation used in this study, which, in addition to the active ingredient contains

excipients such as surfactants and solvents, which may influence its toxicity. Previous studies have shown that a commercial formulation of various agrochemicals may be more toxic than the active ingredient itself (Köner & Çava, 2008; Nikoloff et al., 2013; Lajmanovich et al., 2014; Bach et al., 2016). While DG[®] contains more than twice the amount of SM in its formulation than Primextra[®] II, it is not possible to directly compare the sensi-

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tivity of *L. luctator* tadpoles with that of *L. catesbeianus*. This is because Primextra® II, in addition to SM, contains another active ingredient, atrazine, which can also influence the toxicity level of the formulation. Our results demonstrate the acute toxicity of the herbicide DG® on exposed *L. luctator* tadpoles. Given that, in the field, pesticides are applied in commercial formulations, it is recommended to use these compounds to assess the real risk of agrochemical products.

Previous studies have reported a LC50_{24h} value of 7.13 (6.80-7.55) mg/L for Roundup ULTRA MAX® (RU®), an herbicide based on Glyphosate, and a LC50_{48h} value of 22.45 (19.59-25.73) mg/L for the biopesticide *Bacillus thuringiensis var israelensis* (Introbans), on tadpoles of *L. luctator* (Bach et al., 2016; Lajmanovich et al., 2015). The LC50_{24h} value obtained for DG® in this study is similar to that reported for RU®, which would allow us to affirm that *L. luctator* tadpoles at GS25 stage (the stage evaluated in both studies) present a similar sensitivity range to the commercial formulations of both herbicides. Mean lethal concentration values obtained in laboratory bioassays are useful for comparing the effects of agrochemicals on non-target aquatic organisms (Lajmanovich et al., 2015). Considering this aspect, and that SM is one of the most widely used herbicides, further studies are necessary to evaluate the acute toxicity of different commercial formulations of SM, in order to compare the sensitivity ranges among amphibian species.

Our results demonstrate that the SM-based herbicide DG® induces the formation of MN and other ENA in the peripheral blood of *L. luctator* tadpoles. The frequencies of MN and ENA in tadpoles exposed to different test concentrations and CP were significantly higher than those in the negative control group, both at 48 and 96 h of exposure, except for the lowest concentration of SM tested, for which significant differences were observed only at 96 h. Therefore, we suggest that the genotoxic effects of this herbicide manifest within the first 48 h of larval exposure. Similar results have been previously observed for *Rhinella arenarum* tadpoles (GS 29-31), where genetic alterations caused by sublethal concentrations of the herbicide Liberty® (which contains ammonium glufosinate) were observed within the first

48 h of exposure (Lajmanovich et al., 2015). In contrast, in *Boana pulchella* (Duméril and Bibron, 1841) tadpoles (GS 36), Pérez-Iglesias et al. (2015) found that the herbicide Pivot Hs (which contains imazethapyr) increased the frequency of MN only at the highest sublethal concentration tested at 48 h, while, at 96 h, the herbicide was able to induce other ENA and a higher frequency of MN at all the concentrations tested. In the present study, the lack of significant differences in the frequency of MN between the same treatments at 48 and 96 h would indicate that, at the test concentrations, the maximum genotoxic potential is reached within the first 48 h of exposure to the herbicide. However, we found significant differences in the frequency of ENA between the lowest test concentration (1 mg/L of SM) at 48 and 96 h of exposure, which would indicate that the time of exposure increases the cyto-genotoxicity at these sublethal concentration levels. This result is significant because we consider the concentration of 1 mg/L of SM to be the most relevant among the concentrations tested, as it closely approximates the concentrations of SM reported in agricultural environments (15 µg/L of SM) (Van Opstal et al., 2023).

We identified nine different types of ENA in tadpoles exposed to the different test concentrations, with a predominance of the following abnormalities: notched nuclei (NN), erythroplastids (EP), kidney-shaped nuclei (K), binucleated erythrocytes (BE), and lobed nuclei (LN). The mechanism responsible for generating all known types of ENA has not yet been fully explained; however, several studies have indicated that these aberrations are induced in response to the exposure to genotoxic agents (Tolbert et al., 1992; Serrano-García & Montero-Montoya, 2001; Cavas & Ergene-Gözükara, 2005; Bolognesi et al., 2006; Pérez-Iglesias et al., 2015). The lack of uniform criteria in the literature score ENA makes comparison between results difficult (Bolognesi et al., 2006; Guilherme et al., 2008). Serrano-García & Montero-Montoya (2001), for example, suggested that the origin of budding or nuclear cell sprouts and BE is similar to that of MN and that both are genotoxic events. Bolognesi et al. (2006), on the other hand, found a positive association between the frequency of nuclear buds

and MN and suggested that nuclear invaginations (e.g., kidney-shaped nuclei, notched nuclei) are abnormalities primarily associated with cytotoxicity, whereas Cavas & Ergene-Gözükara (2005) suggested that blebbed and lobulated nuclei are also indicators of genotoxic damage. Pyknotic nuclei are associated with apoptosis, and their prevalence allows inferring the occurrence of cell death (Tolbert *et al.*, 1992), since their frequency increases in the presence of cell damage (Ray *et al.*, 2005). Tolbert *et al.* (1992) mentioned that a level of apoptosis above normal would indicate genotoxic aggression. In the present study, the high frequency of apoptotic cells observed in tadpoles exposed to the concentration of 6.2 mg/L of SM at 48 h would be related to the high larval mortality recorded for this treatment at 96 h of exposure. Finally, anucleated red blood cells or erythroplastids have been proposed to be more frequent in the blood of larvae exposed to contaminated water and to arise to make oxygen transport more efficient by improving the cell surface-to-volume ratio (Barni *et al.*, 2007).

Nikoloff *et al.* (2013) also showed the genotoxic and cytotoxic effects of SM-based herbicides, such as Twin Pack Gold® (96 % active ingredient), in human hepatoma cells (HepG2). These authors showed that the commercial formulation was capable of inducing the formation of MN, as well as a significant reduction in mitochondrial activity, while pure SM did not have these effects. Similar results have been reported for amphibians by Lajmanovich *et al.* (2014) for the herbicide ammonium glufosinate and its commercial formulation Liberty®, in *Rhinella arenarum* larvae. The differences found would indicate that the inert ingredients of the commercial formulation play a key role in the induction of genotoxicity. Therefore, we emphasize the importance of evaluating commercial formulations, as has been done in the present study, in order not to underestimate effects. On the other hand, Paunescu *et al.* (2018) demonstrated the toxic effects of sublethal doses of DG® (the same commercial formulation used in the present study) on adult individuals of *Pelophylax ridibundus*, highlighting physiological, biochemical, and histological alterations in exposed frogs. Our results complement those obtained by these authors and allow us to infer that

the SM-based herbicide DG® has potential cytotoxic and genotoxic effects on *L. luctator* tadpoles and that it thus poses a risk to the amphibians that inhabit agroecosystems.

In conclusion, *Leptodactylus luctator* tadpoles have demonstrated sensitivity to the SM-based herbicide DG®, as well as to other herbicides (e.g., Lajmanovich *et al.*, 2015; Bach *et al.*, 2016) and insecticides (Samojeden *et al.*, 2022). Therefore, this species could be considered a good biological model to assess the potential risk of pesticides. Additionally, we highlight the importance of blood biomarkers as early warning signals to detect adverse effects caused by agrochemicals (Ossana *et al.*, 2013; Attademo *et al.*, 2014; Lajmanovich *et al.*, 2021). In the Pampa Biome, agricultural activity plays a central role, and this study would be the first to evaluate the effects of acute toxicity (LC50) and cytogenotoxicity of an SM-based herbicide on amphibians in the region.

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