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RESEARCH ARTICLE

An imazethapyr-based herbicide formulation induces genotoxic, biochemical, and individual organizational effects in *Leptodactylus latinasus* tadpoles (Anura: Leptodactylidae)

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Abstract

Genotoxic, biochemical, and individual organizational effects on *Leptodactylus latinasus* tadpoles were evaluated after exposure to an imazethapyr (IMZT)-based commercial herbicide formulation, Pivot® H (10.59% IMZT). A determination of the value of the lethal concentration (LC50) was determined as a toxicological endpoint. Alterations in animal behavior and morphological abnormalities as well as cholinesterase (ChE), catalase (CAT), and glutathione *S*-transferase (GST) activities were employed as individual sublethal endpoints. Micronuclei frequencies (MNs), binucleated cells (BNs), blebbed nuclei (BLs), lobed nuclei (LBs), notched nuclei (NTs), erythroplastids (EPs), and evaluation of DNA strand breaks were employed as genotoxic endpoints. All biomarkers were evaluated after 48 and 96 h of exposure to concentrations of IMZT within 0.07–4.89 mg/L. $LC50_{96h}$ values of 1.01 and 0.29 mg/L IMZT were obtained for Gosner stages 25 and 36, respectively. Irregular swimming, diamond body shape, and decreased frequency of keratodonts were detected at both sampling times. Results showed that IMZT increased GST activity and MN frequency at 48 and 96 h of exposure. Other nuclear abnormalities were also observed in the circulating erythrocytes of tadpoles, i.e., NT and BL values after 48 h, and LN, BL, and EP values after 96 h. Finally, results showed that IMZT within 0.07–0.22 mg/L increased the genetic damage index in tadpoles exposed for both exposure times (48 and 96 h). This study is the first to report the sublethal biochemical effects of IMZT in anurans and is also the first report using *L. latinasus* tadpoles as a bioindicator for ecotoxicological studies.

Keywords Imazethapyr herbicide formulation · Lethality · Genotoxicity · Oxidative stress · Morphological and behavioral alterations

Introduction

When pesticides are applied to crops, a fraction of the product may be lost to the environment, affecting non-target organisms through contamination processes such as drift, runoff, or percolation. Pesticide contamination has been linked to adverse effects on amphibian health (Egea-Serrano et al. 2012; Smalling et al. 2015) and has been proposed as a potential cause for the amphibian declines observed worldwide (Brühl et al. 2013; Egea-Serrano et al. 2012; Kiesecker et al. 2001; Mann et al. 2009). Amphibians possess a number of special traits that make them especially sensitive to environmental contamination. Among these are permeable skin, the shell-less eggs, exposed embryogenesis, free-living aquatic larvae, and dependence upon both the terrestrial and aquatic environments (Brühl et al. 2013; Egea-Serrano et al. 2012; Kiesecker et al. 2001; Mann et al. 2009; Sparling et al. 2010). The

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combination of these characteristics together with others such as their narrow home range make amphibians effective bioindicators of environmental health (Brühl et al. 2013; Kiesecker et al. 2001; Sparling et al. 2010).

Since biochemical changes are generally detectable before adverse effects are observed at higher levels of biological organization, the biochemical and/or cytogenetic marker approaches are often applied as an early warning or proactive tool in environmental studies (Hagger et al. 2006; Newman 2014). In this sense, the application of a battery of biomarkers is considered a strong and powerful tool to support environmental management decisions before irreversible damage occurs within the environment (Josende et al. 2015; Ossana et al. 2013; Smalling et al. 2015; Sparling et al. 2010; Venturino and Pechen de D'Angelo 2005; Venturino et al. 2003).

The imidazolinones are a class of herbicides used in a variety of situations to control grasses and broad leaf weeds (MacBean 2012). Imazethapyr (IMZT) is one of the best-known active ingredients belonging to this family chemical group. IMZT has a low sorption coefficient and high solubility in water (Senseman 2007) and affects targeted weeds by altering their growth patterns through both an inhibition of synthesis and increased catabolism of branched chain amino acids (Qian et al. 2015). Available data summarized by the Toxicology Data Network (TOXNET 2019) indicate that if IMZT is released into air, it will exist solely in the particulate phase in the atmosphere being removed by wet or dry deposition. In soil, the herbicide is expected to have very high to moderate mobility. Although low amounts of IMZT were observed to evaporate from dry soil, volatilization is not expected. IMZT photodecomposition was 8% for 43 h. Reported aerobic soil biodegradation half-lives for imazethapyr are 23.5 days to 10.6 months. IMZT is expected to adsorb to suspended solids and sediment. Its bioconcentration factor of 3 suggests the low potential for bioconcentration in aquatic organisms and photodegradation half-lives is estimated varying from 4 up to 5.1 h. Low to moderate acute toxicity has been reported in rats administered IMZT orally, and although the herbicide is not acutely toxic to fish (Kegley et al. 2016), sublethal effects such as oxidative stress and inhibition of the enzyme acetylcholinesterase (AChE) have been reported (Moraes et al. 2011; Pasha 2013; Pasha and Singh 2005). We recently provided the first evidence of adverse effects associated to IMZT on amphibians by demonstrating lethality and sublethal cytogenetic effects in tadpoles of the Montevideo tree frog *Boana pulchella* (formerly *Hypsiboas pulchellus*) (Pérez-Iglesias et al. 2015). Furthermore, we also demonstrated that the herbicide induces oxidative DNA damage on *B. pulchella* tadpoles at purines bases but not at pyrimidines using the alkaline restriction enzyme-modified SCGE assay (Pérez-Iglesias et al. 2017).

The aim of this study is to characterize both the acute lethal and sublethal toxic effects of the IMZT-based herbicide formulation, Pivot® H (10.59% IMZT), on tadpoles of another amphibian species, the Neotropical frog *Leptodactylus latinasus* (Leptodactylidae). This species is of particular interest in the Pampa Region of Argentina, since it has been previously proposed as a bioindicator species and valid model for ecotoxicological biomonitoring of crop production areas (Brodeur and Vera Candioti 2017; Pérez-Iglesias et al. 2016).

Materials and methods

Chemicals and quality analytical control

The IMZT-based herbicide formulation Pivot® H (10.59% IMZT, CAS 081335-77-5) was obtained from BASF Company Argentina S.A. (Buenos Aires, Argentina). Cyclophosphamide (CP, CAS 6055-19-2), acetylcholine iodide (CAS 2260-50-6), L-glutathione reduced (GSH, CAS 70-18-8), and other chemicals and solvents of analytical grade were obtained from Sigma Chemical Co. (St. Louis, MO).

Levels of IMZT in test solutions were analyzed by HPLC in the CIMA Laboratory (National University of La Plata, La Plata, Argentina) following procedures described in Report 01-4134 by the U.S. Geological Survey. Triplicate samples from test nominal solutions equivalent to 0.22 mg/L IMZT were taken and immediately measured after the preparation (0 h) and 24 h thereafter with the detection limit of IMZT being 0.5 μg/L.

Test organisms

Leptodactylus latinasus (Jiménez de la Espada, 1875) is a medium-sized anuran (28–40 mm) which is present throughout the North to central region of Argentina, occupying different habitats such as the Chaco forests, the Pampean grasslands, and different cereal crop agroecosystems (IUCN 2019). Males of *L. latinasus* build small caves in the ground where the mating vocalizations occur. After mating, eggs are layered in a foam nest within the cave. Embryos begin their development and the tadpoles emerge when inundations occur. The species is categorized as non-threatened, with stable populations (IUCN 2019; Vaira et al. 2012) are easily maintained under laboratory conditions as described elsewhere (Pérez-Iglesias et al. 2016).

Tadpoles for bioassays were manually collected in a unpolluted temporary pond located in rural areas of La Plata city, Buenos Aires Province, Argentina (34°59′54″ S, 57°52′28″ W) at the early embryo stage GS 9 according to Gosner (1960). Foam nests containing the embryos were transported to the laboratory and conditioned under controlled parameters as reported elsewhere (Pérez-Iglesias et al. 2015, 2016). Tadpoles were fed commercial fish food (TetraMin®; Tetra Werke, Germany) daily until the bioassays began. Tadpoles

were collected under permissions from the Flora and Fauna Direction of Buenos Aires Province (Argentina) (License Code 22500-22339/13). Experimental manipulations of tadpoles were approved by the Ethical Committee of the National University of La Plata (License Code 11/N847).

Experimental design

Lethal effects

LC50, NOEC, and LOEC values were determined in GS25 and GS36 tadpoles according to Gosner (1960). Bioassays were performed according to standardized methods proposed by international environmental agencies (ASTM 2007; USEPA 1975, 2002) with slight modifications as previously reported (Ruiz de Arcaute et al. 2014). Preliminary bioassays were carried out to determine the range of IMZT concentrations to be used for the evaluation of lethality. GS25 tadpoles were treated with 0.27, 0.41, 0.68, 0.95, 1.36, 2.72, 3.25, and 4.89 mg/L IMZT whereas the tadpoles in GS36 were treated to 0.07, 0.15, 0.22, 0.27, 0.29, 0.81, 1.22, 1.63, 2.72, 3.26, and 4.89 mg/L IMZT. For each replicate, five organisms were kept in dechlorinated tap water acting as a negative control group, as reported elsewhere (USEPA 1982, 2002). For each concentration, five tadpoles were placed in quadruplicate in 500-mL glass jars for 96 h. Test solutions for the bioassays were replaced every 24 h and tadpoles were not fed during the assay.

Mortality was recorded daily before test solution replacement. Tadpoles were considered dead when no motility was present after gentle body stimulation with a glass rod. Dead individuals were removed every 24 h, labeled, and fixed in Carnoy's fixative. All live individuals were fixed in Carnoy's fixative for further morphological abnormalities evaluations.

Sublethal effects

Swimming activity Alterations in swimming activities were registered every 24 h before changing test solutions. Briefly, the swimming activity of each tadpole was observed for 1 min after gently moving the solution with a glass rod as previously described (Pérez-Iglesias et al. 2015). Categories used to evaluate effects on swimming activity were described by Brunelli et al. (2009) and include irregular swimming (IS) and immobility (IM). Frequency of swimming activity was determined by the quotient between number of tadpoles presenting a variation in the tested phenotype and the total number of tadpoles evaluated.

Morphological abnormalities Morphological abnormalities were evaluated at the end of the bioassay (96 h) and were identified following procedures outlined by Bantle et al. (1998). The categories of morphological abnormalities registered were as follows: presence of edema, flexure of the tail or axial abnormalities, decrease in keratodont numbers, and abnormalities in the gut. The frequency of a given type of abnormality was determined by the quotient of the number of tadpoles presenting the abnormality and the total number of tadpoles evaluated. Abnormalities in the morphology of the tadpoles were examined using a stereoscope microscope (Wild Heerbrugg M8 binocular, Switzerland).

Evaluation of biochemical and genotoxic biomarkers Evaluation of biochemical and genotoxicological biomarkers was carried out in GS36 tadpoles after IMZT exposure. Tadpoles were exposed to 0.07, 0.15, or 0.22 mg/L IMZT, which represents 25%, 50%, and 75% of the $LC50_{96h}$ value determined as described above (see "Lethal effects" Section). Each experimental chamber consisted of five tadpoles placed in 500-mL glass jars for 96 h and was performed in quadruplicate. Negative and positive controls (40 mg/L CP) were tested simultaneously with IMZT-treatment groups. Test solutions used in the bioassays were replaced every 24 h and the tadpoles were not fed during the experiments. To evaluate biochemical and genotoxicological biomarkers, 20 tadpoles per treatment were euthanized after 48 and 96 h of exposure following the American Society of Ichthyologists and Herpetologists criteria (ASIH 2004).

Determination of GST, ChE, and CAT activities

Evaluation of biomarkers was performed after 48 and 96 h of exposure. For each tested concentration, activity was determined from homogenates obtained from a whole tadpoles individually processed ("Evaluation of biochemical and genotoxic biomarkers" Section) according to the procedures described by Brodeur et al. (2011, 2017). Briefly, the enzymatic activity was determined by homogenizing an entire tadpole using a Teflon-glass Potter–Elvehjem homogenizer with ice-cold homogenate buffer (Tris 50 mM, 1 mM EDTA, 0.25 M sucrose, 1 N HCl, 1 N NaOH, pH 7.4). Each homogenate was centrifuged at 10,000×*g* (5 min, 4 °C) and the supernatant was collected and maintained at −80 °C for further use.

ChE activity was determined by following Ellman's method (1961). The reaction was evaluated in 200 μL PBS (100 mM, pH 8), 10 μL acetylthiocholine iodide (1 mM), 10 μL 5,5′-dithiobis-(2-nitrobenzoic acid) (0.5 mM), and 100 μL diluted sample. The kinetic of absorbance was recorded at 412 nm (37 °C) after 1 min. Enzymatic activity of ChE was determined using a molar extinction coefficient of 14.150/Mcm. The reaction of CAT consisted of 300 μL of PBS (100 mM, pH 7), 10 μL H_2O_2 (1:10 *v*/*v*), and 10 μL diluted sample. The kinetic of absorbance post hydrogen peroxide (H_2O_2) consumption was recorded at 240 nm (37 °C) after 2 min. Activity of CAT was determined using a molar extinction coefficient of 43.6/Mcm. Finally, the reaction to determine GST activity consisted of 300 μL of GSH (3 mg of GSH diluted in 10 mL of PBS 100 mM, pH 7.0), 10 μL 1-chloro-

2,4-dinitrobenzene (0.1 M), and 10 μL diluted sample. The kinetic of absorbance was recorded at 340 nm (2 min, 37 °C). Enzymatic activity of GST was determined using a molar extinction coefficient of 9.6/mMcm. Protein concentrations were determined by Lowry's method (1951) using bovine serum albumin as a standard. All enzymatic reactions and protein determinations were performed in microplates and measured using a microplate reader, SPECTROstar Nano (BMG Labtech, Ortenberg, Germany).

Micronuclei (MN) and other erythrocytic nuclear abnormalities

The MN assay was carried out on mature erythrocytes obtained from the peripheral circulating blood of tadpoles as reported in detail elsewhere (Pérez-Iglesias et al. 2015). MN frequencies were determined after 48 and 96 h of exposure. For each treatment, blood samples were obtained from 15 randomly selected tadpoles. Mature erythrocyte samples were acquired by sectioning exposed tadpoles behind the gill system, following procedures proposed elsewhere (ASIH 2004). The analysis of the MNs and other nuclear abnormalities were performed following those criteria established and previously (Çavaş and Ergene-Gözükara 2003; Fenech 2007; Vera Candioti et al. 2010) and included the determination of micronucleated and binucleated cells (BNs), blebbed nuclei (BLs), lobed nuclei (LBs), and notched nuclei (NTs). Finally, circulating red blood cells with anucleated forms were considered as erythroplastids (EPs) according to Barni et al. (2007). Slides were analyzed by one researcher in an optical microscope at ×1000 magnification. MNs and other erythrocytic nuclear abnormality frequencies were determined as the ratio between total number of MNs or erythrocytic nuclear abnormalities per 1000 cells as previously described (Çavaş and Ergene-Gözükara 2003; Fenech 2007; Vera Candioti et al. 2010).

Single-cell gel electrophoresis (SCGE) assay

A 30-μL blood sample collected from specimens taken for evaluation of MNs and other erythrocytic nuclear abnormalities ("Evaluation of biochemical and genotoxic biomarkers" Section) were used for the SCGE assay. The SCGE assay was performed following the alkaline procedure described by Singh (1996). Quantification of DNA damage was determined in each blood sample by the length of DNA migration visually analyzed in 100 randomly selected and non-overlapping cells. The categories of DNA damage were classified as 0–I, undamaged; II, minimum damage; III, medium damage; and IV, maximum damage, as described by Çavaş and Könen (2007). Results are expressed as the sum of classes II, III, and IV nucleoids (mean number of damaged cells), and the mean comet score obtained for each treatment group. The genetic damage index (GDI) was calculated for each treatment group according to Pitarque et al. (1999).

Statistical analysis

U.S. Environmental Protection Agency Probit Analysis statistical software version 1.5 (http://www.epa.gov/nerleerd/stat2.htm) was used for obtaining LC50 values. Concentration–response (C-R) curves and ecotoxicological parameters (slope, intercept, and correlation coefficient) with their 95% confidence limits were estimated for all exposure times (24, 48, 72, and 96 h) with the package "ecotoxicology" within R software v.2.11.1 (R Core Team 2010, October 14, 2015). The regression and correlation coefficients were obtained from tests of significance according to Zar (2010). The proportion of individuals affected for each lethal and sublethal endpoints were calculated. Each affected proportion of individuals was angular transformed. One-way ANOVA and Dunnett's post hoc test was performed to explore the differences between treated groups with respect to the negative control group. One-way ANOVA also was used to evaluate NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) values. ANOVA assumptions such as homogeneity of variances and normality were corroborated by using Barlett's test and χ^2 test, respectively. When these assumptions could not be met, a non-parametric Kruskal–Wallis test was performed. For comparisons between the analytical IMZT concentrations at the begging of the bioassay and after 24 h, a *t* test was applied, whereas when comparing NOEC and LC50 values between the two developmental stages, a paired *t* test was performed.

The relationship between response variables (mortality, behavioral alterations, morphological abnormalities, CAT, GST, ChE, MNs, other nuclear abnormalities, and GDI) and IMZT concentrations (grouping variable) were evaluated with a principal component analysis (PCA) that provides a correlation matrix, eigenvectors, and eigenvalues (Jackson 1993). The significance of the regressions and the corresponding correlations were evaluated by simple linear regression (Zar 2010). The analyses were performed with R software v.2.15. Significance level for all tests was set at $\alpha = 0.05$, unless otherwise indicated.

Results

Chemical determinations

Chemical determinations demonstrated that concentrations of IMZT were stable over the 24-h period between daily renewals of the test solution (concentration range 98 ± 5% recovery). Specifically, HPLC analytic determinations revealed that nominal concentrations of IMZT were 0.220 mg/L, a value equivalent to the real concentration of 0.243 ± 0.02 and 0.222 ± 0.01 mg/L for 0 and 24 h, respectively ($P < 0.05$).

LC50, LOEC, and NOEC values

A concentration-dependent increase in mortality was observed after IMZT treatments with both GS25 ($r = 0.97$; $P < 0.05$) and GS36 ($r = 0.96$; $P < 0.01$) tadpoles. LC50 values calculated after 24, 48, 72, and 96 h of exposure for GS25 tadpoles were stable at 1.01 mg/L IMZT (confidence limits, 0.27–3.20). The NOEC and LOEC values for GS25 tadpoles were also stable at 0.95 and 1.36 mg/L IMZT at all exposure times. At GS36, the LC50 value remained constant at 1.63 mg/L IMZT (confidence limits, 1.42–1.99) between 24 and 48 h of exposure, while it significantly decreased to 0.29 mg/L IMZT (confidence limits, 0.08–0.46) after 72 and 96 h of exposure ($P < 0.05$). The NOEC and LOEC values for GS36 tadpoles were 1.63 and 2.72 mg/L IMZT at 24 and 48 h of exposure, respectively, and decreased to 0.81 and 1.22 mg/L IMZT after 72 and 96 h of exposure, respectively. The $LC50_{96h}$ value was significantly lower in tadpoles at GS36 than GS25 ($P < 0.05$).

Swimming activity

IS was observed in GS25 tadpoles after 48 h of IMZT exposure ($P < 0.01$), but this effect disappeared after 72 h of exposure ($P > 0.05$). $NOEC_{48h}$ and $LOEC_{48h}$ values for IS in GS25 tadpoles were 0.40 and 0.81 mg/L IMZT, respectively. Analysis of regression showed that the occurrence of IS at 48 h was concentration dependent in GS25 tadpoles ($r = 0.87$; $P < 0.01$).

In GS36 IMZT-exposed tadpoles, regression analysis demonstrated the presence of a concentration-dependent inhibition of IS at both 24 h ($r = 0.91$; $P < 0.001$) and 48 h ($r = 0.76$; $P < 0.01$) of exposure. NOEC and LOEC values obtained for IS were 0.40 and 1.63 mg/L IMZT and 1.63 and 3.26 mg/L IMZT exposures lasting 24 and 48 h, respectively. For GS25, this altered swimming capacity was also transient at GS36 ($P < 0.01$) and disappeared at 72 and 96 h of exposure ($P > 0.05$).

Morphological abnormalities

Morphological abnormalities detected include body deformations, including the appearance of a diamond body shape and mouth abnormalities. Mouth anomalies involve loss or absence of keratodonts. Both types of abnormalities were detected only in GS36 IMZT-exposed tadpoles. Mouth anomalies and the diamond body shape reached values equivalent to 8.0% and 3.3%, respectively, over controls regardless of exposure time.

In IMZT-exposed tadpoles, loss of keratodonts was detected after 96 h ($P < 0.05$) in more than 43.8% of the individuals, but no evidence of this anomaly was present after 48 h of exposure ($P > 0.05$). The $NOEC_{48h}$ and $LOEC_{48h}$ values for this abnormality were 0.67 and 0.81 mg/L IMZT, respectively. Similarly, a diamond body shape was present in at least 24.0% of the IMZT-exposed tadpoles, regardless of exposure time, with NOEC and LOEC values equivalent to 0.14 and 0.21 mg/L IMZT, respectively ($P_{48h} < 0.01$ and $P_{96h} < 0.001$). A regression analysis showed that both the absence of keratodonts ($r = 0.38$, $P < 0.05$) and the incidence of a diamond body shape ($r = 0.67$, $P < 0.01$) augmented as a positive correlation with the IMZT concentration.

Enzyme activities

Figure 1 depicts the response of GST activity in *L. latinasus* tadpoles after exposure to IMZT. Tadpoles exposed to 0.15 mg/L IMZT, exhibited a GST-activity increase after both exposure times ($P < 0.05$, Fig. 1). IMZT exposure did not significantly influence ChE nor CAT activities at either 48 or 96 h post exposure ($P > 0.05$).

MNs and other nuclear abnormalities

Results showed that IMZT induces an increase in MNs and other nuclear abnormality frequencies in mature erythrocytes from treated tadpoles (Table 1). In addition, tadpoles treated with CP exhibited an increase in MN frequency after both exposure time points ($P_{48h} < 0.05$; $P_{96h} < 0.001$). Results also demonstrated an increase in MNs frequency in tadpoles exposed to only 0.22 mg/L IMZT both at 48 and 96 h post treatment ($P < 0.05$), when comparing to the negative control group (Table 1).

The results of other nuclear abnormality analysis showed a significant increase in frequency when tadpoles were exposed to CP after both 48 and 96 h of exposure. An increase in NTs frequency was only observed in tadpoles exposed to CP for

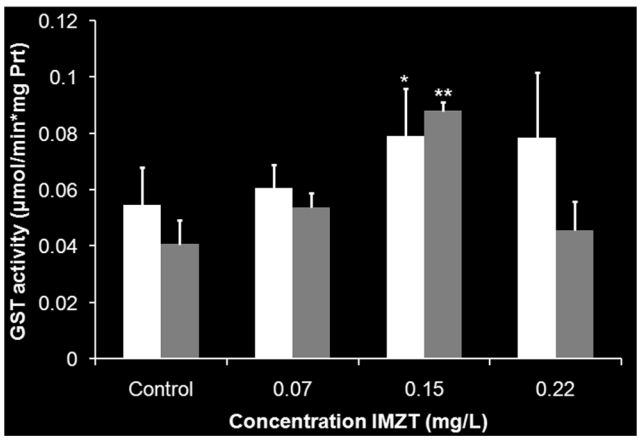

Fig. 1. GST activity in *Leptodactylus latinasus* tadpoles after exposure to the imazethapyr-based herbicide formulation, Pivot® H. The black and white bars represent GST activity at 48 and 96 h, respectively. Significant difference comparisons between treated and control values are denoted as follows by a single asterisk (*) representing $P < 0.05$, or a double asterisk (**) representing $P < 0.01$

Table 1 Frequencies (‰) of MNs and other nuclear abnormalities in peripheral blood erythrocytes from *Leptodactylus latinasus* exposed to the imazethapyr-based herbicide formulation Pivot® H

Chemicals	Concentration (mg/L)	Exposure time (days)	Number of animals analyzed	Number of cells analyzed	MNs[a]	Other nuclear abnormalities[a]				
						NTs	LNs	BNs	BLs	EPs
Control		48	14	14653	1.70 ± 0.25	0.07 ± 0.07	1.03 ± 0.28	0.34 ± 0.16	3.00 ± 0.40	1.23 ± 0.38
		96	14	14572	1.43 ± 0.27	0.41 ± 0.17	0.62 ± 0.37	0.41 ± 0.21	2.36 ± 0.68	0.49 ± 0.24
Pivot® H	0.07	48	15	15662	1.80 ± 0.24	0.07 ± 0.07	0.31 ± 0.12	0.51 ± 0.22	3.77 ± 0.59	0.81 ± 0.29
		96	13	13138	1.63 ± 0.32	0.65 ± 0.37	0.60 ± 0.21	1.43 ± 0.62	2.65 ± 0.63	3.61 ± 1.63*
	0.15	48	15	15540	2.57 ± 0.51	0.13 ± 0.09	0.70 ± 0.20	0.26 ± 0.11	3.93 ± 0.77	1.17 ± 0.29
		96	15	15351	1.83 ± 0.37	0.19 ± 0.10	0.85 ± 0.25	0.45 ± 0.16	2.29 ± 0.69	2.67 ± 0.52**
	0.22	48	15	15325	3.12 ± 0.53*	0.58 ± 0.24*	1.73 ± 0.41	0.74 ± 0.18	6.36 ± 1.29**	2.22 ± 0.96
		96	15	15533	2.40 ± 0.32*	0.52 ± 0.16	2.52 ± 0.51***	0.84 ± 0.23	8.55 ± 0.95***	0.58 ± 0.23
CP [b]	40.00		15	15606	3.13 ± 0.41*	0.82 ± 0.29*	1.47 ± 0.48	0.51 ± 0.25	6.07 ± 1.32*	1.68 ± 0.34
			13	13055	5.52 ± 1.56***	0.52 ± 0.32	2.31 ± 0.58**	0.60 ± 0.28	6.81 ± 1.05***	1.57 ± 0.73

Results are expressed as mean number of abnormalities/1000 cells ± SE

[a] MNs, micronuclei; NTs, notched nuclei; LNs, lobed nuclei; BNs, binucleated cells; BLs, blebbed nuclei; EPs, erythroplastids

[b] Cyclophosphamide (CP, 40 mg/L) was used as positive control

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; significant differences with respect to control values

48 h ($P < 0.05$) while an increase in LN frequency was observed when treatment lasted 96 h ($P < 0.01$). Finally, in tadpoles exposed to CP, an increase in BL frequency was observed after both 48 ($P < 0.05$) and 96 h ($P < 0.001$) of exposure (Table 1).

Increased NT, LN, and BL frequencies were observed in tadpoles exposed to 0.22 mg/L IMZT (Table 1). Increased BLs frequency was found in tadpoles exposed for both durations of exposure ($P_{48h} < 0.01$ and $P_{96h} < 0.001$). An increase in the frequency of NTs was detected after 48 ($P < 0.05$) but not after 96 h of exposure ($P > 0.05$). An enhancement of LNs frequency was observed in IMZT-treated tadpoles only after 96 h ($P < 0.001$). EPs values only increased in tadpoles treated with 0.07 ($P < 0.05$) and 0.15 mg/L IMZT ($P < 0.01$) after 96 h (Table 1). Finally, none of the IMZT treatments induced alterations in the frequencies of BNs ($P > 0.05$) (Table 1).

DNA damage

The SCGE assay results showed that IMZT induced DNA damage in the circulating red blood cells of *L. latinasus* tadpoles (Table 2 and Fig. 2). Treatment with CP induced an increase in GDI and damaged cell frequencies when comparing to negative control values in tadpoles treated at both exposure times ($P_{48h} < 0.001$ and $P_{96h} < 0.05$) (Table 2).

GDI showed an increase in tadpoles treated with 0.07 ($P < 0.05$), 0.15 ($P < 0.05$), and 0.22 mg/L IMZT ($P < 0.01$) for 48 h (Table 2). This alteration was achieved by enhancing the frequency of type II nucleoids at 0.07 mg/L IMZT ($P < 0.05$), of type III nucleoids at 0.15 and 0.22 mg/L IMZT ($P < 0.05$), and type IV nucleoids at 0.22 mg/L IMZT ($P < 0.05$) (Fig. 2a). A concomitant decrease of non-damaged nucleoids (type 0–I) was detected for all assayed concentrations ($P < 0.01$) (Fig. 2a).

In tadpoles treated with IZMT for96 h, GDI increases were observed in specimens treated with 0.07 and 0.15 mg/L IMZT ($P < 0.05$) but not with 0.22 mg/L IMZT ($P > 0.05$) (Table 2). The GDI increase was due to an enhancement of the damaged cell frequency of types II and III nucleoids at 0.07 mg/L IMZT ($P < 0.05$), and types II, III, and IV nucleoids in 0.15 mg/L IMZT-exposed tadpoles ($P < 0.05$). In addition, a concomitant reduction in the frequency of 0–I nucleoid types occurred in samples treated with 0.07 and 0.15 mg/L IMZT ($P < 0.05$) (Fig. 2b).

Correlations between biomarkers

Biomarkers found to exhibit a significant difference between IMZT-exposed tadpoles and controls were included in further analyses. When analyzing correlations by PCA, the responses obtained in 48-h-exposed tadpoles for the seven biomarkers studied (loss of keratodonts, diamond body shape, GST activity, and cytogenetic parameters (MNs, NTs, BLs, and GDI)) were used. Variables were divided into three major factors or principal components (PC) that explained the 78.4% of the variability (PC1 = 53.1%, PC2 = 13.5%, and PC3 = 11.8%). In addition, at this sampling time, the reduction of the dimensionalities through the PCA demonstrated a concentration gradient separating the lowest concentrations of IMZT (0.07 and 0.15 mg/L) from the higher concentration (0.22 mg/L). GST activity was negatively correlated with the other biomarkers, exhibiting a Pearson's correlation coefficient of $r = -0.42$ and $r = -0.33$ with morphological and cytogenetic biomarkers, respectively. On the other hand, morphological and

Table 2 Analysis of DNA damage measured by comet assay in *Leptodactylus latinasus* tadpoles cells exposed to the imazethapyr-based herbicide formulation Pivot® H

Chemicals	Concentration (mg/L)	Exposure time (h)	Number of animals analyzed	Number of cells analyzed	% of damaged cells (II + III + IV)	GDI ± SE[a]
Control		48	14	1447	29.04	1.12 ± 0.09
		96	13	1557	28.53	1.17 ± 0.05
Pivot® H	0.07	48	14	1436	50.79**	1.46 ± 0.11*
		96	15	1817	49.54*	1.53 ± 0.11*
	0.15	48	13	1356	46.06*	1.47 ± 0.12*
		96	15	1861	49.24*	1.52 ± 0.14*
	0.22	48	14	1466	47.39*	1.54 ± 0.10**
		96	13	1363	24.93	1.09 ± 0.08
CP [b]	40.0	48	15	1694	57.75***	1.76 ± 0.12***
		96	13	1431	42.94*	1.43 ± 0.10*

[a] GDI, Genetic Damage Index

[b] Cyclophosphamide (CP, 40 mg/L) was used as positive control

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significant differences with respect to control values

cytogenetic biomarkers were highly correlated with Pearson's correlation coefficients ranging from $r = 0.68$ to $r = 0.81$.

When biomarker relationships were analyzed at the 96 h of IMZT exposure timepoint, the PCA analysis showed the existence of a concentration gradient in the intensity of the biomarker responses, as depicted in Fig. 3. In this case, PC1 and PC2 account for the 77.6% of observed variability (PC1 = 45.6% and PC2 = 32.0%). Results showed that at lowest sublethal concentration, namely 0.07 mg/L IMZT, that such scenario is committed to the GDI. In the intermediate sublethal concentration (i.e., 0.15 mg/L IMZT), the difference between the other concentrations is due to the negatively correlated responses of the GST and EP biomarkers ($r = -0.73$). Finally, at the highest concentration tested (i.e., 0.21 mg/L IMZT), cytogenetic biomarkers (i.e., MNs, LNs, and BLs), and morphological abnormalities are positively correlated ($0.57 < r > 0.68$). Overall, a progression of adverse effects was evidenced by a PCA analysis by the response of biomarkers of lower levels of organization toward a more complex levels (genetic < biochemical < cytological–individual response) as the herbicide exposure increases for the duration of the 96-h assay (Fig. 3).

Discussion

In this work, we examined changes in biomarkers in response to treatment with an IMZT-based herbicide formulation, Pivot® H, after a semi-static acute exposure of GS25 and GS36 oven frog *L. latinasus* tadpoles under laboratory conditions. According to researchers, the species is capable of responding to environmental disturbances, which is one reason for which it has been proposed for use in ecotoxicological evaluations (Agostini et al. 2016; Brodeur et al. 2011; Brodeur and Vera Candioti 2017; Guerra and Aráoz 2016; Medina et al. 2016; Suárez et al. 2016). Our actual results are in accordance with this concept. To the best of our knowledge, tadpoles of this species have not yet been used as laboratory model for ecotoxicological studies. Thus, the present study constitutes the first ecotoxicological report of lethal and sublethal effects exerted by the herbicide IMZT on tadpoles of this Neotropical species.

In our study, $LC50_{96h}$ values of 1.01 and 0.29 mg/L IMZT were obtained for GS25 and GS36 tadpoles *of L. latinasus*, respectively. IMZT should, therefore, be considered as moderately toxic and highly toxic agrochemical for GS25 and GS36 larvae, respectively, according to the ecotoxicological classification proposed for aquatic organisms by the U.S. EPA (1982). IMZT can also be categorized as a very toxic xenobiotic to aquatic organisms (Category I) based on both the United Nations criteria (UN 2011) and European Union directives (Mazzatorta et al. 2002). Information regarding the acute toxicity of IMZT among aquatic vertebrates is scarce. Among amphibians, lethal effects of IMZT have only been reported in our previous study examining developed *Boana pulchella* tadpoles. In this species, $LC50_{96h}$ values of 1.49 and 1.55 mg/L IMZT were reported for GS25 and GS36 tadpoles, respectively (Pérez-Iglesias et al. 2015). It therefore emerges that *L. latinasus* GS25 and GS36 tadpoles are 1.47 and 5.34 times more sensitive to this herbicide than *B. pulchella* tadpoles, respectively.

Furthermore, according to our observations, it seems worth mentioning that *L. latinasus* at the pre-metamorphic GS36 stage is nearly 3.5 times more sensitive to IMZT than tadpoles at an earlier developmental stage such as GS25. Although difficult to interpret, similar observations have been

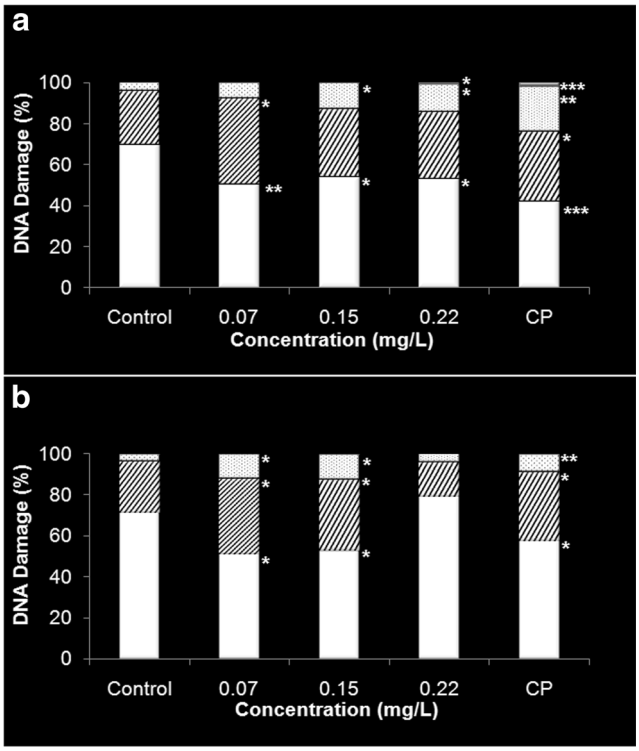

Fig. 2. DNA damage measured by comet assay in circulating blood cells from *Leptodactylus latinasus* tadpoles treated with imazethapyr-based commercial formulation herbicide Pivot® H after 48 (**a**) and 96 h (**b**). Observed frequencies of non-damaged (white column sections; type 0–I nucleoids), type II nucleoids (stripped column sections), type III nucleoids (dotted column sections), and type IV nucleoids (gray column sections) are given. The results are shown as percentages of pooled data. Negative (non-treated tadpoles) and tadpoles treated in positive controls (CP, 40 mg/L of cyclophosphamide). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (significant differences with respect to control values)

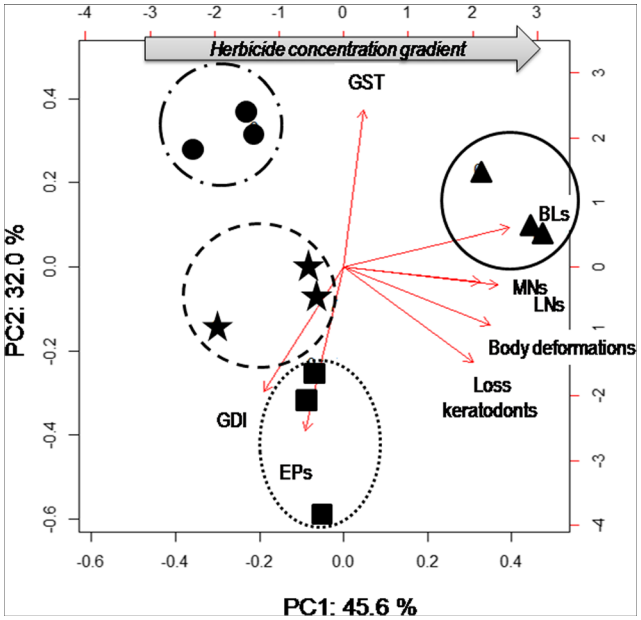

Fig. 3. Biplot representing the responses of each biomarker evaluated in *Leptodactylus latinasus* tadpoles exposed to sublethal concentrations of the imazethapyr-based commercial herbicide Pivot® H for 96 h. The wide gray arrow shows the increasing concentration gradient for each sublethal IMZT concentration such as 0.07 (stars), 0.15 (squares), and 0.22 (triangles) mg/L with respect to the negative control (circles) obtained by the correlation between the biomarkers. Circles showed groupings of the negative control (complete and dotted circles) with respect to the different sublethal concentrations, 0.07 (circle dashed lines), 0.15 (dotted circle), and 0.22 (complete circle) mg/L on an IMZT gradient. The length of the black arrow indicates the magnitude of the response of the different biomarkers. GST, glutathione *S*-transferase activity; GDI, genetic damage index; MNs, micronuclei; BLs, blebbed nuclei; LNs, lobed nuclei

previously reported for other amphibians exposed to different xenobiotics. Harris et al. 2000 found that the developmental stages of Northern leopard frog, *Rana pipiens* (Ranidae), and American toad, *Bufo americanus* (Bufonidae) tadpoles nearest to the metamorphosis were more sensitive to various pesticide formulations than early larval stages. In addition, Sanders (1970) reported a similar pattern for another Bufonidae, the American woodhouse's toad, *Anaxyrus woodhousii*. Tadpoles of this species were more sensitive to DDT at development stages nearest to climax than at earlier stages. In this sense, all aforementioned observations suggest that survival rates may vary substantially depending not only on species employed as biotic matrices but also with developmental stage. This phenomenon is most probably due to internal physiological conditions associated with the metamorphosis period, as suggested elsewhere (Harris et al. 2000; Linder et al. 1990; Sanders 1970). Together with our previous study on *B. pulchella* (Pérez-Iglesias et al. 2015), our current results suggests that the commercial formulation of IMZT, namely Pivot® H, is capable of producing adverse effects by promoting biochemical, genotoxic, and morphological changes in late stage anuran tadpoles, at least from *L. latinasus* and *B. pulchella* species.

Based on biochemical assays, our results showed that 0.15 mg/L IMZT promotes an increase in GST activity regardless of the time tadpoles were exposed to the herbicide. Briefly, an increase in GST activity is considered beneficial and enhances an organisms' ability to handle chemical stress. It is involved in phase II of xenobiotic biotransformation jointly with CYP450-dependent monooxygenases, thus facilitating pesticide elimination and preventing oxidative damage in tadpoles and other vertebrates (Attademo et al. 2014; Ferrari et al. 2008, 2011). However, our observations are opposite to results reported by Moraes et al. (2011) who found a decrease in GST activity in the common carp *Cyprinus carpio* (Cyprinidae) from aquatic IMZT-contaminated environments. Regardless of the real significance of these discrepancies, our present observations represent the first report demonstrating the ability of IMZT to produce biochemical abnormalities in anuran tadpoles, at least of *L. latinasus*.

Results obtained demonstrate that, depending upon length of exposure time, IMZT induces behavioral changes such as

irregular and/or and inhibition of swimming. In addition, exposure to IMZT enhanced MN frequency in mature erythrocytes of those tadpoles treated to the highest concentration assayed regardless of exposure time. Although originally developed for mammalian genotoxicity research, the MN assay has now became profusely employed for ecotoxicological biomonitoring studies employing aquatic species as biotic matrices (Hartmann et al. 2003; Larramendy 2017; Mouchet et al. 2011). It is well known that MNs are originated by condensation of chromosomal fragments and/or whole chromosomes that have not been incorporated into the main nucleus during anaphase, as a consequence of clastogenic (DNA fragmentation) or aneugenic origin (an alteration of the mitotic apparatus) (Balmus et al. 2015; Barni et al. 2007; Hayashi 2016; Heddle et al. 1991). Regardless of a plausible clastogenic or aneugenic origin, the present study demonstrated that the herbicide IMZT is capable of producing genetic damage, as observed through the induction of both MNs as well as other nuclear abnormalities such as NTs, BLs, LNs, and EPs. So far, the mechanisms involved in the induction of these other nuclear abnormalities are still unknown. Previous studies have demonstrated that these abnormalities should be considered genetic biomarkers, highlighting the existence of basic failures in mitosis (Barni et al. 2007; Çavaş and Ergene-Gözükara 2003, 2005). These types of markers include cellular degradation (Ateeq et al. 2002), aneugenicity (Fernandes et al. 2007), and/or gene amplification (Shimizu et al. 1998). However, regardless of their unclear formation, several studies reported that nuclear abnormalities could potentially complement the MN assay as a prospective biomarker for cellular damage in amphibians exposed to a variety of different xenobiotics, including pesticides (Barni et al. 2007; Natale et al. 2018; Nikoloff et al. 2014; Pérez-Iglesias et al. 2015, 2016; Ruiz de Arcaute et al. 2014; Vera Candioti et al. 2010). The presence of enucleated forms of circulating peripheral erythrocytes, i.e., EPs, is a normal situation observable in the blood of several amphibians, especially tadpole forms in which this cellular alteration can attain levels as high as 90–95% of the total circulating red cell population as described in certain members of the tribe Bolitoglossini of salamanders (Plethodontidae) (Glomski et al. 1997). Furthermore, it has been suggested that an increased frequency of EPs could represent a peculiar mechanism for increasing the cell surface/volume ratio to improve the efficiency in oxygen transport, in particular for water pollution conditions (e.g., presence of environmental stressors) (Barni et al. 2007). In accordance with previous observations, EPs were also observed in larval stages of the edible frog *Rana esculenta* (Ranidae) inhabiting heavily polluted sites (Barni et al. 2007) as well as in *H. cordobae* (Hylidae) inhabiting aquatic ecosystems associated to fluorite mine (Pollo et al. 2016), among others.

These results reveal that IMZT induced MNs in *L. latinasus* tadpoles after exposure to only the highest tested concentration (i.e., 0.22 mg/L) of the herbicide. Furthermore, our results corroborate the observation that 0.07–0.22 mg/L and 0.07–0.15 mg/L concentration ranges promote an increase in primary DNA lesion frequency using the comet assay when *L. latinasus* tadpoles were exposed for 48 and 96 h. On the other hand, a decrease in the frequency of damaged nucleoids, and a decreased GDI was observed in those tadpoles exposed to 0.22 mg/L IMZT for 96 h. This finding could be attributable to a cytotoxic potential exerted on the exposed tadpoles by the highest concentration of the herbicide, concomitant to an inhibitory effect induced by the assayed concentration because of alterations in the kinetics of the blood cell population and/or erythrocyte replacement. In agreement with this observation, the authors have previously demonstrated a decrease in primary damage when increasing the exposure time of aquatic organisms including amphibians (Yin et al. 2009), fish (Vera-Candioti et al. 2013), and also invertebrates (Siu et al. 2004) to several environmental stressors.

Overall, it is evident that the SCGE bioassay represents a more sensitive methodology than the MN assay to detect early DNA damage when both parameters are evaluated as consequence of exposure with an equivalent xenobiotic concentration. Previously, our studies report a similar trend, as observed in *B. pulchella* tadpoles when exposed to the same commercial formulation of IMZT (Pérez-Iglesias et al. 2015). In agreement with our previously hypothesized explanation for this scenario (He et al. 2000; Pérez-Iglesias et al. 2015), the differences we observed between both analytical methods could be due to differences in the stages of the peripheral circulating blood cells that are considered in the genotoxicity biomarker analysis. In this context, DNA damage estimated by the SCGE is performed over several circulating cell populations, while assaying for the presence of MNs is an indirect method for making a determination of the presence of lesions on daughter cells that have undergone at least one mitotic cell cycle and probably retain their properties to repair damaged DNA (He et al. 2000). Regardless, current results not only constitute the first in vivo confirmation of the ability of the herbicide IMZT to jeopardize the DNA of the Neotropical oven frog *L. latinasus* but also extend the concept that the SCGE assay represents a highly sensitive methodology for detecting and scoring environmental stress induced damage at the cellular level. Despite this, as previously suggested by other authors, an increase in genomic disorder has been proposed to play important function in the decreased fitness of the vertebrate aquatic population. This is because when primary DNA damage occurs, the level of MNs as well as an alteration in the nuclear morphology of erythrocytes can be observed. This situation can lead to cell death and several pathophysiological conditions that increase overall stress level (Barni et al. 2007; Çavaş and Ergene-Gözükara 2003, 2005; Çavaş and Könen 2007; Jha 2008). On the other hand, another scenario occurs if damaged circulating red blood cells survive. In this case, mis-

or unrepaired cells will have adverse effects on physiological aptitude and will ultimately have an adverse impact on the long-term population level (Barni et al. 2007; Jha 2008).

Regarding effects at the individual level, our results clearly demonstrate that sublethal concentrations of IMZT induced not only alterations in behavior but also morphological abnormalities in IMZT-exposed *L. latinasus* tadpoles. Tadpoles possessing, as proposed elsewhere, morphological abnormalities and alterations in swimming activity could be less competitive not only because they possess a reduced capacity to feed but also because they have greater predisposition to predation (Bach et al. 2016; Junges et al. 2010; Peltzer et al. 2013). In this context, and as suggested by these authors, it seems important not to rule out that these types of alterations including morphological and in the behavior of the exposed specimens can occur simultaneously. Thus, such ultimate instance can inflict negative consequences leading to high mortality at the larval stage of the anurans not only at the population level but also at a community scale. Further studies at the population and community levels of amphibian species exposed to a battery of pesticides are needed to elucidate the effect of environmental stressors on predator–prey relationships as well as on intra- and interspecific competition.

The use of a battery of different biomarkers in amphibians and other vertebrates allows researchers to relate xenobiotic effects with biological responses, and thus provides evidence that pesticides have entered into the organisms in question, have distributed among different tissues, and/or caused adverse effects on non-target cells (Hagger et al. 2006; Newman 2014). In this sense, the application of a battery of biomarkers is considered a strong and powerful tool to support management decisions before irreversible damage occurs (Josende et al. 2015; Ossana et al. 2013; Sparling et al. 2010; Venturino and Pechen de D'Angelo 2005; Venturino et al. 2003). The integrative response observed in the PCA supports this concept demonstrating not only the usefulness of integrated biomarker evaluation but also corroborates the theoretical model of biomarkers proposed by ecotoxicologists, which demonstrates that when the prevalence of environmental stressors increase, the organism concomitantly moves from homeostasis toward a stress status (Amiard-Triquet et al. 2013; Walker 2009). Briefly, the model proposes the importance of using several biomarkers at different levels considering a dose–effect relationship in which the lowest doses do not produce adverse effects, and biological impairments accumulate with increasing dose and are progressively enhanced concomitantly with the dose increment. According to the model, the organism first reaches a state of reversible adverse effects, then reaches a state of irreversible diseases, and finally results in the death of the organism. These states can be monitored by the use of different biomarkers (Amiard-Triquet et al. 2013, Walker 2009). Our observations agree well with this scenario. Results demonstrate that biomarkers show changes at relatively low concentrations when employed at low biological organization levels (e.g., at biochemical and cytogenetic levels), whereas irreversible damages are observed at higher biological organization levels with the highest concentrations assayed, which are also strongly correlated with mortality (e.g., individual level). Furthermore, results also reveal a progression of effects at different levels of biological organization, from the cellular–biochemical to individual level until reaching the mortality of exposed specimens. In agreement with our results, Brunelli et al. (2009) observed the same gradient response when applying PCA analysis to study the effects of the insecticide endosulfan on tadpoles of the European toad, *B. bufo* (Bufonidae).

The IMZT treatment assayed in this study includes a concentration range of 0.07–4.89 mg/L, which represents a relatively high end of the threshold value of 14 μg/L IMZT found in the surface water of the Azul River basin (Buenos Aires, Argentina) reported by Peluso et al. (2008). It should be mentioned that the IMZT concentrations found in Argentinean crop production areas is nearly 7.6 times higher than the highest concentration reported for surface water in the USA (Mattice et al. 2011) or even 51.8 and 40.0 times higher than the highest concentration reported for Brazilian drinking and surface waters, respectively (Souza Caldas et al. 2011). Thus, the concentration of IMZT employed in this investigation would be expected to be almost improbable in the environment. However, considering the recommended application field ratios of 100–150 g a.i./ha reported for Argentina (Bindraban et al. 2009; CASAFE 2017–2019), it cannot be ruled out the possibility that *L. latinasus* populations could be exposed accidentally to IMZT at this range of concentrations when specific events occurred (e.g., direct application, drainage into ditches, or accidental discharge).

In light of the results obtained in the present study, it seems important to highlight that evaluation of this set of different parameters (e.g., cytogenetic, hematological, and antioxidant effects) and biomarkers in amphibian larvae could provide the information necessary to understand the general state of the health of individuals, or at least obtain clues regarding the sanitary status or fitness of the amphibian population. In this sense, results provided by these methods could serve as early warning signs to be used in situations where a comparison between polluted and unpolluted conditions is required.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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