

# Toxicity and Genotoxicity of Imidacloprid in Tadpoles of *Leptodactylus Latrans* and *Physalaemus Cuvieri* (Anura: Leptodactylidae)

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## Research Article

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## Abstract

Imidacloprid is a neonicotinoid insecticide widely used worldwide, but which can cause adverse effects on non-target organisms, especially in aquatic environments. This study aimed to evaluate the chronic toxicity of an insecticide-based imidacloprid in amphibians, using *Leptodactylus latrans* and *Physalaemus cuvieri* tadpoles. The parameters of survival, swimming activity, body size, damage to body structures and genotoxicity for both species were analyzed; and the ecological risk of this insecticide calculated. Chronic short-term assay was carried out for 168 h (7 days) and five concentrations of imidacloprid, between 3 and 300  $\mu\text{g L}^{-1}$ , were tested. The insecticide did not affect the tadpoles survival; however, both species tested showed smaller body size, damage to the mouth and intestine and the induction of micronuclei and other erythrocytes nuclear abnormalities after exposure to imidacloprid-based herbicide. Insecticide exposure affected the swimming activity in *L. latrans*, which contributes to the greater sensitivity of *L. latrans* to imidacloprid when compared to *P. cuvieri*. All parameters analyzed indicated that the insecticide presents an ecological risk for both species at concentrations greater than 3  $\mu\text{g L}^{-1}$ . This demonstrates the genotoxicity of the insecticide imidacloprid, which can contribute to the population decline of *L. latrans* and *P. cuvieri* species in natural systems.

## Introduction

The neonicotinoid insecticides were launched in the 90s and almost immediately became a preference over organophosphates and carbamates in the control of herbivorous insects. Currently neonicotinoids are the most used class of insecticides worldwide (Simon-Delso et al. 2015; Bakker et al. 2020; Borsuah et al. 2020). The widespread use of neonicotinoids has become a global environmental issue since the destination, behavior and effects of its residues are poorly understood and scarce (Pietrzak et al. 2020). In addition, overuse often occurs, without adding benefit to cultivation. (Simon-Delso et al. 2015).

The most widely used neonicotinoid worldwide is imidacloprid (Jeschke et al. 2011; Pietrzak et al. 2019; IBAMA 2021). This insecticide is considered moderately toxic, and is indicated for foliar application in crops such as lettuce, coffee, sugar cane, beans, tobacco, corn, tomatoes, wheat and grapes (ANVISA 2021). In insects, it has a neurotoxic action, chemically interacting to mimic the action of acetylcholine, binding to the nicotinic receptors (nAChRs) of this important neurotransmitter (Kagabu 2011). By acting selectively on insect nAChRs (Liu and Casida 1993; Tomizawa and Casida 2005), this interaction triggers excessive neuron stimulation resulting in the death of these animals (Simon-Delso et al. 2015).

Imidacloprid has low sorption and slow soil degradation, and great potential for leaching into groundwater (Hashimoto et al. 2020; Pietrzak et al. 2020). Although imidacloprid is not suitable for the water to be consumed, it can reach water bodies through spraying, draining or leaching (Wiggins et al. 2018; Chen et al. 2019). This insecticide is persistent in water, with a half-life of 30 days and may not be readily biodegradable (Hladik et al. 2018). Due to these characteristics, it is a pesticide often found in surface water (Sánchez-Bayo and Hynes 2014; Batikian et al. 2019; Jurado et al. 2019; Pietrzak et al. 2019), and one of the most detected insecticides in drinking water in Brazil, in the last 10 years (Montagner et al. 2019).

Some countries have standardized limits for imidacloprid in water. In Canada, the maximum allowed concentration of imidacloprid for the protection of aquatic life is 0.23  $\mu\text{g L}^{-1}$ ; in the USA, the limit established by the Environmental Protection Agency (EPA) is 1.05  $\mu\text{g L}^{-1}$ ; and in Netherlands the environmental risk index is 0.2  $\mu\text{g L}^{-1}$  to acute toxicity and 0.067  $\mu\text{g L}^{-1}$  to chronic toxicity (Hrynyk et al. 2018). The use of imidacloprid is prohibited in the field in the European Union, and its use is allowed only in greenhouses, since 2018 (Jactel et al. 2019). In Brazil, there is a limit of 300  $\mu\text{g L}^{-1}$  in drinking water in Rio Grande do Sul state (Brazil 2014), but this is the only restriction defined in the country.

The high solubility of neonicotinoids in water can cause adverse effects in non-target organisms, such as in vertebrates, such as genotoxicity, cytotoxicity, changes in immune functions, reduced growth or even reproductive failure (Gibbons et al. 2015; Hrynyk et al. 2018). The toxicity of imidacloprid has been verified for several non-target organisms belonging to aquatic communities, such as aquatic insects (Kobashi et al. 2017; Cavallaro et al. 2016), benthic macroinvertebrates (Bartlett et al. 2019), fish (Rajput and Singh 2012; Islam et al. 2019) and amphibians (Pérez-Iglesias et al. 2014; Sievers et al. 2018). Previous works have verified behavioral, physiological and neurological changes; in addition to cell damage and genotoxicity, and decreased survival in anuran amphibians, such as *Boana pulchella* (cited as *Hypsiboas pulchellus*; Pérez-Iglesias et al. 2014) and *Limnodynastes tasmaniensis* (Sievers et al. 2018).

Amphibians, among vertebrates, are considered excellent bioindicators, as they have permeable skin and are sensitive to changes in environmental conditions (Haddad et al. 2008). In addition, amphibians are considered of special interest because they are in decline in population and with the increase in endangered species (Beasley 2019), which has recently been associated with pesticides exposure (Agostini et al. 2020). These organisms become more susceptible to pollutants because they occupy a transitional niche between terrestrial

and aquatic ecosystems (Mason et al. 2013; Jing et al. 2017), mainly during the reproductive stage, which occurs mainly in a humid environment; which coincides with the periods of application of pesticides for large crops, during spring and summer (Tavalieri et al. 2020).

Toxicological studies using native species are important to assess the sensitivity of these species and to understand the impacts of toxic substances. *Leptodactylus latrans* (Fitzinger 1826) and *Physalaemus cuvieri* (Steffen 1815) are two species native to South America, with reproductive strategy in foam nests (Mijares et al. 2010; Sá et al. 2014). Even listed as least concern (LC) by the International Union for Conservation of Nature Red List of Threatened Species (IUCN 2021), both species, *L. latrans* and *P. cuvieri*, showed sensitivity to glyphosate, such as development, behavioral and morphological changes, genotoxic effects and lethality (Bach et al. 2018; Herek et al. 2020); and glyphosate combined with 2,4-D was also toxic to *L. latrans* (Pavan et al. 2021).

*Leptodactylus latrans*, popularly known as butter frog, has a wide geographical distribution in South America, east of the Andes. It is very adaptable, occurring in different types of habitats, both in preserved areas and in disturbed and modified environments. Their spawns are deposited in large foam nests produced on the surface of the water (Heyer et al. 2010). *Physalaemus cuvieri* popularly known as dog-frog, inhabit open and anthropized areas (Kwet and Di Bernardo 1999; Eterovick and Sazima 2004). The organisms are widely distributed in Brazil, Argentina, and Paraguay, preferably reproducing in temporary bodies of water; they reproduce preferentially in temporary bodies of water, where they deposit their spawning also in foam nests, close to the vegetation that borders the lagoons, on the water surface (Mijares et al. 2010).

The aim of this study was to determine the chronic toxicity of environmentally relevant concentrations of imidacloprid-based insecticide in tadpoles of *L. latrans* and *P. cuvieri* by assessing survival, swimming activity, body size, damage to body structures and genotoxicity. We still calculate the ecological risk to understand the effects of this insecticide on amphibians.

## Material And Methods

### Tested species

Spawning of *L. latrans* and *P. cuvieri* were collected with less than 24 hours of oviposition in a pesticide-free place, at Horto Florestal Municipal de Erechim, RS, Brazil (-27°42'43.77"S and - 52°18'42.94"W), and taken to the Ecology and Conservation Laboratory of the Federal University of Fronteira Sul - Campus Erechim, where they were stored in aquariums with 15 liters of dechlorinated water. The eggs were raised under controlled conditions of temperature ( $24 \pm 2^\circ \text{C}$ ) and photoperiod (12/12 h light/dark) until they reached development stage 25 (Gosner 1960). The water was constantly monitored and presented the following parameters: pH =  $7.5 \pm 0.5$ , dissolved oxygen =  $5.8 \pm 0.4 \text{ mg L}^{-1}$ , turbidity =  $< 5$ , conductivity =  $649 \pm 25 \mu\text{S cm}^{-1}$ , hardness =  $3.57 \text{ mg L}^{-1}$ , Na =  $13.012 \text{ mg L}^{-1}$ , and Ni =  $< 0.002 \text{ mg L}^{-1}$ . The tadpoles were fed daily with complete fish feed (Alcon Basic, Alcon®) with at least 45% crude protein and organic lettuce.

### Experimental design and experimental conditions

Ten tadpoles in development stage 25 were transferred to 500 ml glass containers, and each container was considered an experimental unit. The assays were conducted in triplicate, totaling 30 tadpoles per treatment. The tadpoles used in the tests had complete mouth formation, swimming capacity, and similar and normal length and mass. The tadpoles of *L. latrans* had a length of  $13.25 \pm 0.36 \text{ mm}$  and mass body of  $0.035 \pm 0.008 \text{ g}$ , and *P. cuvieri* had, in average,  $16.60 \text{ mm} \pm 0.60 \text{ mm}$  and  $0.070 \text{ g} \pm 0.011 \text{ g}$ .

Chronic short-term exposure was carried out, with a total duration of 168 hours (7 days) according to ASTM STP 1443 (Herkovits and Perez-Coll 2003), as a static test and the tadpoles were fed daily as previously described. Tadpoles were exposed to five water treatments with a nominal concentration of imidacloprid (48% a.i., Nortox SA Brazil): i)  $3 \mu\text{g a.i. L}^{-1}$ , ii)  $30 \mu\text{g a.i. L}^{-1}$ ; iii)  $100 \mu\text{g a.i. L}^{-1}$ ; iv)  $200 \mu\text{g a.i. L}^{-1}$ , v)  $300 \mu\text{g a.i. L}^{-1}$ , and in parallel a control treatment with clean water only. The concentrations were selected based on the imidacloprid value recorded in the water in Brazil ( $3 \mu\text{g L}^{-1}$ , Bortoluzzi et al. 2006; Bortoluzzi et al. 2007), in rice paddies in Vietnam ( $30 \mu\text{g L}^{-1}$ ; La et al. 2015) and allowed in Rio Grande do Sul state, Brazil ( $300 \mu\text{g L}^{-1}$ ; Brazil 2014), and, still, two intermediate concentrations. The water physical-chemical characteristics were the same as described during the development of the tadpoles. In addition, ammonia was measured daily and it was around  $0.283 \pm 0.038 \text{ mg L}^{-1}$ .

### Survival, swimming activity and body size and structures

Tadpole survival was checked every 24 h, when live and dead tadpoles were recorded. Dead tadpoles were removed from the containers. Swimming activity was also recorded every 24 h by qualitative observation. The tadpoles were gently stimulated with a glass rod and the observed behavior was noted. The behavior was classified as: a) swimming activity equal to the control, b) lethargy (reduced swimming

activity in relation to the control), c) hyperactivity (increased swimming activity in relation to the control), d) unresponsive (without the occurrence of movements) and e) spasms (tremors and convulsions).

At the end of the assay period, the tadpoles were euthanized with lidocaine (5%) following the rules of the National Council for Animal Control and Experimentation (CONCEA 2015). Body size measurements of 10 tadpoles from each treatment were measured. The total length (mm) was verified using a digital caliper (150 mm MTX®, Moscow, Russia) from the face to the tail, and the mass (g) using a precision scale (AUX320, Shimadzu Analítica®, Kyoto, Japan). In addition, body structures were evaluated. Changes in the mouth (denticles or morphology) and changes in the intestine (edema or morphology) were determined. Digital images of morphological traits were taken using a digital camera (P510®, Nikon, Tokyo, Japan) and damage in body structures were determined using a stereomicroscope (SZ51®, Olympus, Tokyo, Japan).

## Micronucleus Assay and Other Nuclear Erythrocytic Abnormalities

For genotoxic analysis, a drop of blood was obtained from 10 tadpoles from each insecticide and control treatments. Each blood sample was placed on a slide, fixed and stained with Panotic Rapid® stain (Laborclin Ltda, Brazil), according to the manufacturer's instructions. The slides were analyzed under optical microscopy under a 100x objective lens (CX31®, Olympus, Tokyo, Japan) and 1,000 cells for each individual, totaling 10,000 erythrocytes for each treatment were analyzed. The presence of erythrocyte nuclear abnormalities (ENAs), including micronuclei (MN) was recorded. The MN was analyzed according to Pérez-Iglesias et al. (2015), and other six ENAs were analyzed according to Montalvão et al. (2017): apoptosis (AP); nuclear bubble/bud (NB); karyolysis (KA); binucleated cell (BC); notched nucleus (NN) and lobed nucleus (LN).

## Ecological risk analysis

The chronic risk analysis was based on the Hazard Quotient (HQ) method for aquatic animals defined by the United States Environmental Agency (USEPA 2020). The calculation is made by the equation: EEC/NOEC, where EEC is the estimated environmental concentration and NOEC represents no observed effect concentration. We used EEC as the limit allowed for imidacloprid in the state of Rio Grande do Sul, Brazil, which is  $300 \mu\text{g L}^{-1}$ . The result of the equation was compared with the level of concern (LOC) of the United States Environmental Protection Agency (USEPA). The LOC indicates whether a pesticide has a potential risk of causing adverse effects to non-target organisms (USEPA 2021). The LOC reference value for chronic risk for aquatic animals is 1; therefore, values greater than 1 represent the existence of adverse effects of the contaminant. We also determined the lowest observed effect concentration (LOEC), which represents the lowest concentration able to cause toxic effects. Thus, it was possible to infer the maximum acceptable toxicant concentration (MATC), which corresponds to the average of the LOEC and NOEC values. When it was not possible to statistically calculate the NOEC, the LOEC values were used to determine HQ, and we defined that the MATC was equal to LOEC.

## Statistical analyses

The data obtained were previously analyzed for normality by the Kolmogorov-Smirnov test (K-S) and for homogeneity of variances by the Barlett test. With the assumptions accepted, the analysis of variance (ANOVA) was performed, and the treatment means were compared with the control treatment by the Dunnett test ( $p < 0.05$ ). Statistical analyses and graphs were performed using Statistic 8.0 and GraphPad Prism 7.0 software, respectively. In the ANOVA results we use acronyms to identify the tested species, *Ll* for *Leptodactylus latrans* and *Pc* for *P. curvieri*.

## Results

Exposure to imidacloprid-based insecticide did not significantly influence the survival of *L. latrans* and *P. curvieri* after 168h in chronic assay. In *L. latrans* the survival of the exposed tadpoles was on average 84.67%

( $F_{L5,12} = 1.16$ ;  $p = 0.380$ ) and in *P. curvieri* was 100% in all treatments. The data are shown as supplementary material (Online Resource 1).

## Body size

Tadpoles of both species had smaller body sizes than control tadpoles, after exposition to imidacloprid treatments (Fig 1; Online Resource 1). The body length of exposed *L. latrans* tadpoles was on average 12.52% less than the control treatment ( $F_{L5,54} = 16.12$ ;  $p < 0.0001$ ), and 7.11% for *P. curvieri* ( $F_{Pc5,54} = 8.96$ ;  $p < 0.0001$ ). *L. latrans* and *P. curvieri* imidacloprid-treated tadpoles weighed 48.9% and 7.4% less than control, respectively ( $F_{L5,54} = 23.19$ ;  $p < 0.0001$ ;  $F_{Pc5,54} = 12.25$ ;  $p < 0.0001$ ).

## Damage to body structures

Mouth damage was observed in tadpoles of *L. latrans* and *P. cuvieri* ( $F_{LJ5,12} = 19,28; p < 0,0001; F_{Pc5,12} = 24,16; p < 0,0001$ ) at the lowest tested concentration ( $3 \mu\text{g L}^{-1}$ ), reaching up to 90% of the individuals when exposed to  $300 \mu\text{g L}^{-1}$  of imidacloprid for 168 h (Figs 2, 3; Online Resource 2). Intestine damage appeared in *L. latrans* ( $F_{LJ5,12} = 20,21; p < 0,0001$ ) from  $100 \mu\text{g L}^{-1}$  (43.3%) to  $300 \mu\text{g L}^{-1}$  (88.4%); while *P. cuvieri* ( $F_{Pc5,12} = 30,22; p < 0,0001$ ) showed significant intestine damage in all concentrations of imidacloprid (Fig 2C-D). Total damages (mouth and intestine) in the corporal structures ( $F_{LJ5,12} = 15,42; p < 0,0001, F_{Pc5,12} = 34,05; p < 0,0001$ ) were observed between 50 and 90% of the tadpoles exposed to imidacloprid between 3 and  $300 \mu\text{g L}^{-1}$ , for 168 h (Fig 2e-f; Online Resource 2). No eye damage was observed.

## Swimming activity

Only *L. latrans* showed changes in swimming activity when exposed to imidacloprid (Online Resource 3). The most frequent behavior was lethargy (30.67% of the exposed tadpoles), followed by hyperactivity (20.67%), and spasms (18.67%). Unresponsive was observed in 18% of treated tadpoles (Fig 4).

In all imidacloprid treatments, *L. latrans* showed a significant frequency of lethargy in comparison to the control ( $F_{LJ5,12} = 7.33; p = 0.002$ ). Hyperactivity was significant from the concentration of  $30 \mu\text{g L}^{-1}$  ( $F_{LJ5,12} = 31.45; p < 0.0001$ ) and spasms were significant only at the highest concentration ( $300 \mu\text{g L}^{-1}$ ) ( $F_{LJ5,12} = 7,086, p = 0,003$ ) (Fig 4). Tadpoles unresponsive were recorded mainly in 3 and  $300 \mu\text{g L}^{-1}$  treatments ( $F_{LJ5,12} = 6.60; p = 0.004$ ). The number of tadpoles with changes in swimming activity increased with the exposure time, being significant in 144 and 168 hours (time  $\times$  concentration: lethargy,  $F_{L6,35} = 7.80; p < 0.0001$ ; unresponsive,  $F_{L6,35} = 4.79; p = 0.001$ ; spasms,  $F_{L6,35} = 11.00; p < 0.0001$ ). Unresponsive was observed for hyperactivity and time of exposure to the insecticide ( $F_{L6,35} = 0.20, p = 0.97$ ).

## Micronucleus (MN) and other Nuclear Erythrocytes Abnormalities (ENAs)

The frequency of MN was significant in *L. latrans* when tadpoles were exposed to 200 and  $300 \mu\text{g L}^{-1}$  of imidacloprid ( $F_{LJ5,54} = 8.22; p < 0.0001$ ; Fig 5A), and in *P. cuvieri* exposed to the highest concentration ( $300 \mu\text{g L}^{-1}$ ) ( $F_{Pc5,54} = 4.15; p = 0.003$ ; Fig 5B). Total ENAs ( $F_{LJ5,54} = 19,57; p < 0,0001; F_{Pc5,54} = 13,09; p < 0,0001$ ) and MN plus ENAs ( $F_{LJ5,54} = 19,13; p < 0,0001; F_{Pc5,54} = 12,88; p < 0,0001$ ) were significantly higher from the concentration of  $30 \mu\text{g L}^{-1}$  of imidacloprid for both species (Fig 5E-F).

Except for the karyolysis (KA) in *P. cuvieri*, all other ANEs as nuclear bubble/bud (NB), binucleated cell (BC), notched nucleus (NN), and lobed nucleus (LN) were found both in *L. latrans* and *P. cuvieri*, mainly at the concentration of  $300 \mu\text{g L}^{-1}$  (Table 1). The most frequent ANEs were notched nucleus and lobed nucleus, which were significantly higher from the concentration of  $30 \mu\text{g L}^{-1}$  of imidacloprid (Table 1).

**Table 1** Frequency de Erythrocyte Nuclear Abnormalities (ENAs) as apoptosis (AP), nuclear bubble/bud (NB), binucleated cell (BC), notched nucleus (NN), and lobed nucleus (LN) in *Leptodactylus latrans* and *Physalaemus cuvieri* tadpoles exposed to different concentrations of imidacloprid for 168 h.

Species	Imidacloprid ( $\mu\text{g L}^{-1}$ )	ENAs (per 1,000 cells)					
		MN	AP	NB	BC	NN	LN
<i>Leptodactylus latrans</i>	0	0 <sup>a</sup>	0 <sup>a</sup>	0,9±0,10 (0-1) <sup>a</sup>	0,2±0,13 (0-1) <sup>a</sup>	1,8±0,42 (0-4) <sup>a</sup>	1,7±0,15 (1-2) <sup>a</sup>
	3	0.4±0.22 (0-2) <sup>a</sup>	0.8±0.33 (0-3) <sup>.ab</sup>	2.2±0.76 (0-7) <sup>.ab</sup>	1.3±0.45 (0-4) <sup>.ab</sup>	4.8±1.03 (2-13) <sup>.ab</sup>	4.8±1.14 (0-12) <sup>.ab</sup>
	30	0.5±0.17 (0-1) <sup>a</sup>	0.7±0.30 (0-3) <sup>.ab</sup>	2.9±0.53 (0-5) <sup>.ab</sup>	1.3±0.45 (0-4) <sup>.ab</sup>	9.6±1.24 (5-17) <sup>.bc</sup>	9.5±0.76 (6-13) <sup>.bc</sup>
	100	1.3±0.45 (0-4) <sup>.ab</sup>	1.3±0.62 (0-5) <sup>.ab</sup>	3.8±1.06 (0-9) <sup>.ab</sup>	2.7±0.73 (0-6) <sup>.ab</sup>	13.4±1.37 (6-20) <sup>.cd</sup>	12.1±1.04 (8-16) <sup>.cd</sup>
	200	2.3±0.42 (0-5) <sup>b</sup>	0.6±0.22 (0-2) <sup>.ab</sup>	4±0.99 (0-10) <sup>.ab</sup>	2.3±0.67 (0-6) <sup>.ab</sup>	14.8±1.87 (6-21) <sup>.cd</sup>	12.9±1.06 (7-17) <sup>.cd</sup>
	300	2.6±0.62 (0-5) <sup>b</sup>	2.0±0.45 (0-4) <sup>b</sup>	5.7±1.98 (0-16) <sup>b</sup>	3.1±0.98 (0-9) <sup>b</sup>	18.3±1.96 (6-27) <sup>d</sup>	15.5±2.02 (3-22) <sup>d</sup>
<i>Physalaemus cuvieri</i>	0	0 <sup>a</sup>	0 <sup>a</sup>	0.1±0.10 (0-1) <sup>a</sup>	0 <sup>a</sup>	1.3±0.50 (0-5) <sup>a</sup>	0.6±0.34 (0-3) <sup>a</sup>
	3	0 <sup>a</sup>	0.2±0.20 (0-2) <sup>.ab</sup>	1.1±0.28 (0-2) <sup>a</sup>	0.5±0.22 (0-2) <sup>.ab</sup>	4.9±0.82 (1-10) <sup>.ab</sup>	6.7±0.92 (0-10) <sup>.ab</sup>
	30	0.4±0.16 (0-1) <sup>.ab</sup>	0.4±0.16 (0-1) <sup>.ab</sup>	2.5±0.65 (0-5) <sup>.ab</sup>	1.5±0.60 (0-5) <sup>.ab</sup>	8.1±1.08 (3-13) <sup>.bc</sup>	9.3±1.61 (3-17) <sup>.abc</sup>
	100	0.6±0.22 (0-2) <sup>.ab</sup>	0.2±0.13 (0-1) <sup>.ab</sup>	3.6±1.14 (0-10) <sup>.a.abc</sup>	1±0.26 (0-2) <sup>.ab</sup>	9.7±1.14 (6-18) <sup>.cd</sup>	14.4±3.47 (5-36) <sup>.bc</sup>
	200	0.7±0.22 (0-2) <sup>.ab</sup>	0.6±0.22 (0-2) <sup>.ab</sup>	5.8±1.31 (1-15) <sup>.bc</sup>	2.1±0.57 (0-5) <sup>b</sup>	12.6±0.88 (9-19) <sup>d</sup>	12.6±2.11 (6-28) <sup>.bc</sup>
	300	1.0±0.56 (0-6) <sup>b</sup>	0.7±0.15 (0-1) <sup>b</sup>	6.9±1.68 (0-16) <sup>c</sup>	2.1±0.59 (0-6) <sup>b</sup>	13.7±1.59 (4-20) <sup>d</sup>	18±3.93 (3-48) <sup>c</sup>

Data represent mean  $\pm$  SE (minimum-maximum). Different letters indicate significant differences by the Tukey test ( $p < 0.05$ ).

## Ecological risk analysis

All parameters analyzed presented ecological risk for *L. latrans* and *P. cuvieri* (Table 2), considering hazard quotient (*HQ*) values higher than the reference value LOC (level of concern) = 1, as determined by USEPA (2021). The maximum acceptable toxicant concentration (MATC) was between 3.00 and 9.49  $\mu\text{g L}^{-1}$  of imidacloprid for *L. latrans*, and between 3.00 e 9.49  $\mu\text{g L}^{-1}$  for *P. cuvieri* (Table 2).

**Table 2** No observed effect concentration (NOEC), lowest observed effect concentration (LOEC), maximum acceptable concentration of the toxicant (MATC) and hazard quotient of ecological risk (*HQ*), calculated for the species of *Leptodactylus latrans* and *Physalaemus cuvieri* exposed to different concentrations of imidacloprid based insecticide for 168 h.

Species	Parameter	NOEC	LOEC	MATC	HQ
		( $\mu\text{g L}^{-1}$ )	( $\mu\text{g L}^{-1}$ )	( $\mu\text{g L}^{-1}$ )	
<i>L. latrans</i>	Swimming activity	-	3.00	3.00	100
	Body size	-	3.00	3.00	100
	Structures damages	-	3.00	3.00	100
	MN + ENAs	3.00	30.00	9.49	100
<i>P. cuvieri</i>	Body size	3.00	30.00	9.49	100
	Structures damages	-	3.00	3.00	100
	MN + ENAs	3.00	30.00	9.49	100

(-) indicate values not calculated due to the NOEC being the lowest concentration used.

## Discussion

Imidacloprid exposure caused morphological and genotoxic changes in tadpoles of *L. latrans* and *P. cuvieri*, although it did not affect the survival of individuals. Recent studies have also shown that, in controlled assays, survival is not necessarily a sensitive parameter, as reported in the exposure of *Xenopus laevis* to imidacloprid (Hrynyk et al. 2018), and *Lithobates sylvaticus* and *L. pipiens* to other neonicotinoids (Robinson et al. 2019). However, developmental impairment and the presence of nuclear changes in surviving individuals, as seen in *L. latrans* and *P. cuvieri*, indicate the tadpoles' ability to survive in the long term (Robinson et al. 2017).

Changes in the development of tadpoles were verified by the shorter length and body mass observed in both species, with *L. latrans* being more sensitive to imidacloprid than *P. cuvieri*. Low development has already been related to imidacloprid, in concentrations well below those associated with mortality (Gibbons et al. 2015).

In stressful situations, such as in the presence of contaminants, the expenditure of resources to try to tolerate the presence of pesticides can reduce the resources available for growth (DiGiacopo and Hua 2020). The impairment of the development of tadpoles can also contribute to greater predation in natural environments, both due to the smaller size (e.g. Carlson and Langkilde 2017), as well as the lower physical performance. Although body size is a highly variable characteristic, this analysis is generally correlated between the life stages of tadpoles and adult individuals (Phung et al. 2020). It has been found that tadpoles with reduced body size can result in smaller adults and, consequently, with a lower rate of survival and reproduction (Beasley 2019). *L. latrans*, for example, has a body size considered large and important for the defense of eggs and tadpoles, in addition to being important for predation (Toledo et al. 2011). Thus, morphological changes may contribute to the population decline of anurans, since it has been demonstrated that larger females of *Leptodactylus* sp. and *Physalaemus* sp. have greater fertility (Hartmann et al. 2010; Pereira and Manyero 2012; Pupin et al. 2010).

Associated with the smaller body size of the tadpoles it was possible to verify damage in the mouth and intestine of the individuals due to the increase in the concentration of the pesticide in the environment. Damage to the mouth can restrict body mass, as this characteristic is considered a primary indicator of food acquisition (Zhao et al. 2019). Mouthpieces have important lip teeth in the food ingestion process, in addition to playing a fundamental role in supporting the substrate and scraping food (Venesky et al. 2013). Changes in this organ affect the ability to forage by tadpoles; in addition, inefficiency in feeding can impact the growth rate (Bach et al. 2016), and generate energy waste until metamorphosis, increasing susceptibility to predation (Toledo et al. 2014).

The structural integrity of the intestine guarantees functional fitness for digestion, a fundamental process for nutrient absorption (Sun et al. 2018). This process requires the presence of the intestinal microbiota, which is related to the stress response and can be affected even by pesticides (Gao et al. 2018). Previous studies have shown that imidacloprid is capable of inducing microbiota dysbiosis in crabs (Hong et al. 2020) and mice (Yang et al. 2020). According to Yang et al (2020), the reduction of the intestinal barrier weakens the organ to toxic substances. Thus, over time, the tadpoles would have difficulties in feeding.

In natural populations of amphibians, morphologically malformed individuals generally constitute a small fraction of less than 2% (Ouellet 2000); however, the high number of damage (> 50% of individuals) by imidacloprid observed in this study demonstrates the high toxicity of this compound to the studied species. This neonicotinoid also caused damage to body structures in at least two other groups of vertebrates, birds (Hussein and Singh 2016) and fish (Islam et al. 2019), showing the importance of evaluating different groups of animals.

Despite changes in the morphology of both tadpoles, only *L. latrans* showed changes in swimming activity when exposed to imidacloprid. In addition to dietary changes due to damage to the mouth and intestine, energy expenditure in hyperactivity or even less food intake due to lethargy behavior may have contributed to the lower growth and greater sensitivity by *L. latrans* to imidacloprid. The change in activity swimming by exposure to imidacloprid has also been found in other amphibians (eg, Lee-Jenkins and Robinson 2018; Sievers et al. 2018) and fish (Crosby et al. 2015), as well as a decrease in spontaneous locomotor activity in rats (Lonare et al. 2014), possibly due to the neurobehavioral effects of this pesticide (Crosby et al. 2015). Imidacloprid is a neurotoxin that acts to excite nicotinic acetylcholine receptors (nAChRs) in mammals (Kimura-Kuroda et al. 2012) and decrease in acetylcholinesterase activity (Lonare et al. 2014) in amphibians, which may be related to the impairment of swimming activity in *L. latrans*.

Imidacloprid in concentrations higher than those used in this study demonstrated genotoxic effects in amphibians *Rana* sp. (Feng et al. 2004) and *Boana pulchella* (Arcaute et al. 2014), and fishes *Australoheros facetus* (Iturburu et al. 2017) and *Prochilodus lineatus* (Vieira et al. 2018). The presence of micronuclei and some ENAs, such as AP, NB and BC were significant in both *L. latrans* and *P. cuvieri* species, mainly in the highest concentrations evaluated. The presence of micronuclei usually occurs due to failures in mitotic division (Amaral et al. 2019), and can be triggered by the presence of nuclei with bubbles or cell binucleation (Crott and Fenech 2001); whereas nuclei with apoptosis are characterized by nuclear disintegration without alteration of the cytoplasm (Fenech 2000) and often associated with neurological disorders (Podratz et al. 2011).

Total ENAs and NN and LN were highly responsive from the concentration of  $30 \mu\text{g L}^{-1}$  of imidacloprid, which demonstrates the highly genotoxic potential of this pesticide. Although the mechanisms of action of pesticides in nuclear abnormalities have not yet been fully described, these changes are already known to induce the presence of micronuclei and are recently considered biomarkers of the toxic action of pesticides on amphibians (Rutkoski et al. 2020; Herek et al. 2021; Pavan et al. 2021). Among ANEs, notched nucleus was the most sensitive in *Boana pulchella* tadpoles exposed to pirimicarb-based formulation (Natale et al. 2018), as observed in the present study for both species. Any external factor that affects cell proliferation, differentiation or apoptosis can produce embryotoxic or teratogenic effects, and can result in permanent congenital malformations, functional abnormalities or even the death of individuals (Gilbert 2006).

Based on the morphological traits, swimming activity and genotoxicity, the ecological risk analysis indicated that the maximum acceptable concentration of imidacloprid for *L. latrans* and *P. cuvieri* is  $3 \mu\text{g L}^{-1}$ . Above this concentration, both species may show damage to body structures, and *L. latrans*, smaller body size and changes in swimming activity. Above  $9.49 \mu\text{g L}^{-1}$  *P. cuvieri* has smaller body size, in addition to genotoxic cell damage in both species. The fact that chronic short-term assays is long enough to cause cytotoxic damage to cells in tadpoles shows how toxic pesticides used in environmentally relevant concentrations and allowed in Brazil are toxic to amphibians. Considering the response of non-target species that live in aquatic environments, such as amphibians, the allowed concentration for imidacloprid in water should be  $3 \mu\text{g L}^{-1}$ , which is 100 times less than the concentration allowed in the state of Rio Grande do Sul, Brazil.  $300 \mu\text{g L}^{-1}$  (Brazil 2014). This demonstrates the need for further studies focusing on limit concentrations at different trophic levels to subsidize legislation and protect aquatic life.

Although *L. latrans* and *P. cuvieri* belong to the same family (Leptodactylidae), and have a wide distribution in South America and adaptability to different habitats (Heyer et al. 2010; Mijares et al. 2010), *L. latrans* tadpoles showed sensitivity greater when exposed to imidacloprid, and this is the first study that characterizes this differentiation. This is important to determine the choice of environmental bioindicator species that can be, at the same time, easily found, and are sensitive to contaminants.

In the tadpole phase, amphibians are unable to escape exposure to environments contaminated with neocotinoids, as they use these habitats for larval development until they reach the appropriate stage to survive in the terrestrial environment (Robinson et al. 2017). Neonicotinoids such as imidacloprid have high persistence and toxicity, and impact on trophic levels in aquatic environments, as they are able to decrease the biomass of organisms, harming the dynamics of the food chain, especially of higher-level consumers (Yamamuro et al. 2019). For this reason, conservation measures regulated in legislation for national applicability are needed, which currently does not exist in the country where the study was carried out. Still, showing the toxicity of this insecticide for anuran amphibians and the existence of other neonicotinoids, further studies are needed to detail the risks of these pesticides in populations of anurans.

## Conclusion

We found that environmentally relevant concentrations of the neonicotinoid imidacloprid induced changes in the development of *L. latrans* and *P. cuvieri*. Morphological and genotoxic changes were observed in both species, however *L. latrans* was more sensitive to the insecticide than *P. cuvieri*. Imidacloprid presents a high ecological risk for the two species studied, where the maximum acceptable concentration of this insecticide is  $3 \mu\text{g L}^{-1}$ , 100 times below what is allowed by law in Brazil. Thus, we emphasize the importance of conservation actions associated with the review or creation of specific legislation that correlate the impacts of pesticides on the extinction of anuran amphibians.

# Declarations

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## Data availability

Data are available in Supplementary Information and on request from the corresponding author.

## Author contribution

Caroline G. Samojeden: Conceptualization, Methodology, Writing - original draft, Visualization. Felipe A. Pavan, Camila F. Rutkoski Alexandre Folador and Sílvia P. da Fré: Conceptualization, Methodology, Writing. Caroline Müller, Paulo A. Hartmann and Marília T. Hartmann: Conceptualization, Methodology, Writing - review & editing, Supervision.

## Ethics declarations

## Conflict of interest

The authors declare no competing interests.

## Ethics approval

This study was approved by the Ethics Committee for the Use of Animals (CEUA) of the Federal University of Fronteira Sul under protocol n° 8822130919 and n° 8742250320 and authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) under n° 72719-

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## Figures

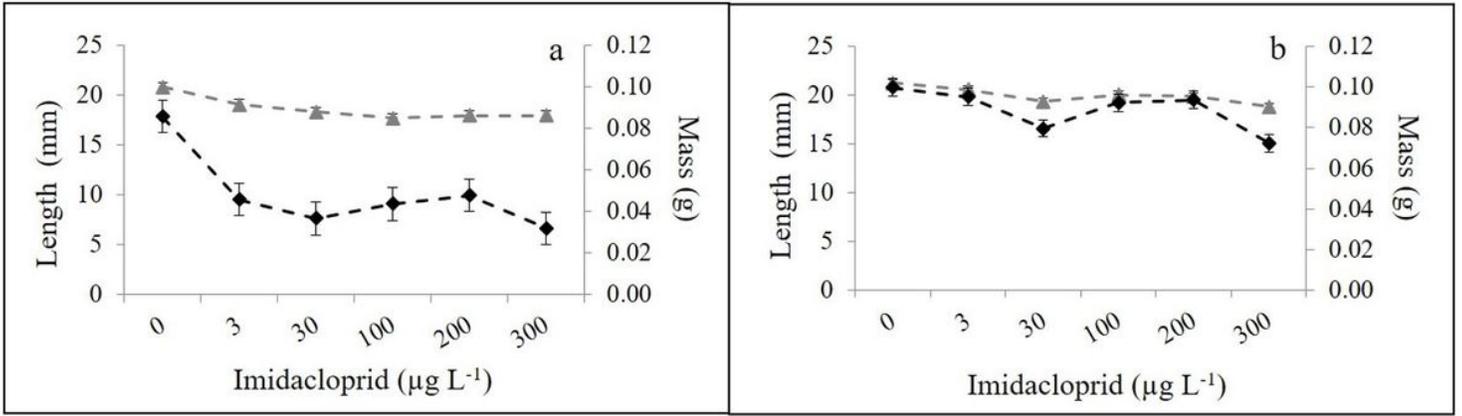


Figure 1

Total length (mm,  $\blacktriangle$ ) and body mass (g,  $\bullet$ ) of *Leptodactylus latrans* (a) and *Physalaemus cuvieri* (b) tadpoles in the control (0) and exposed for 168 h to different concentrations of herbicide-based imidacloprid

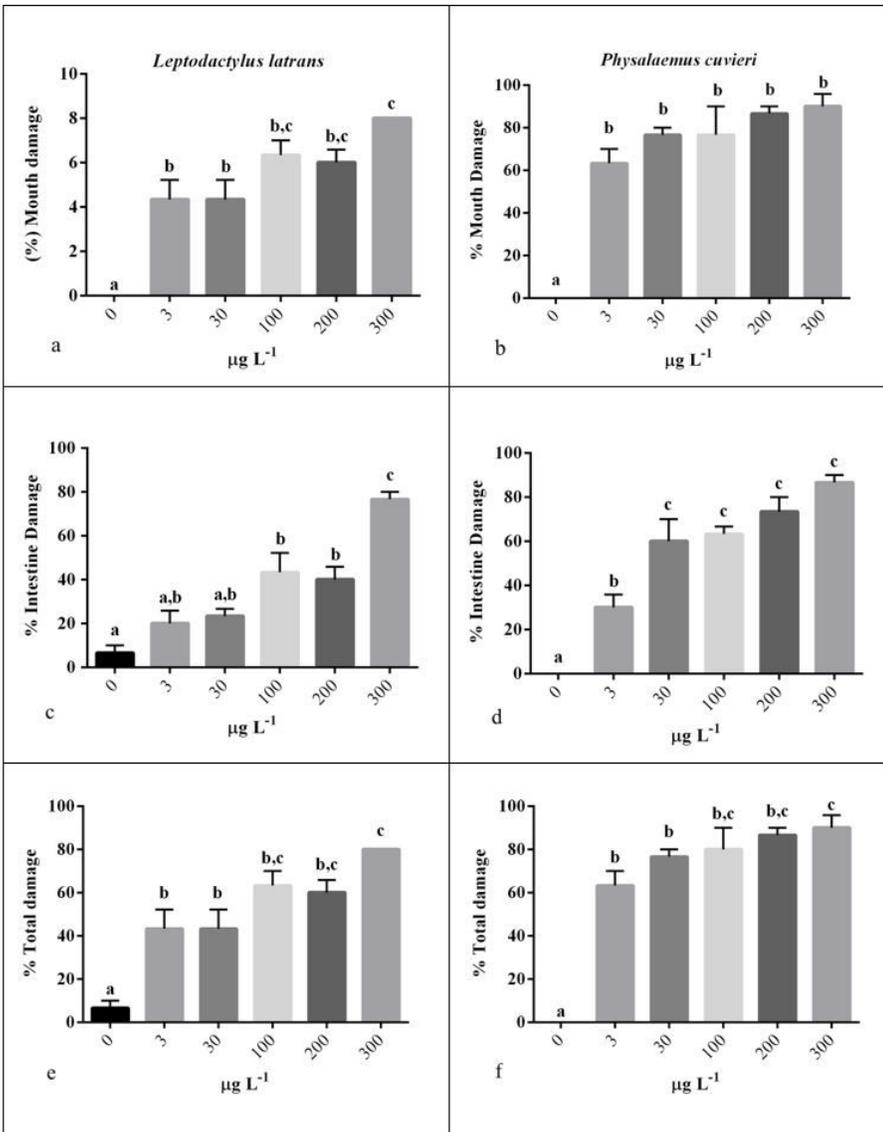
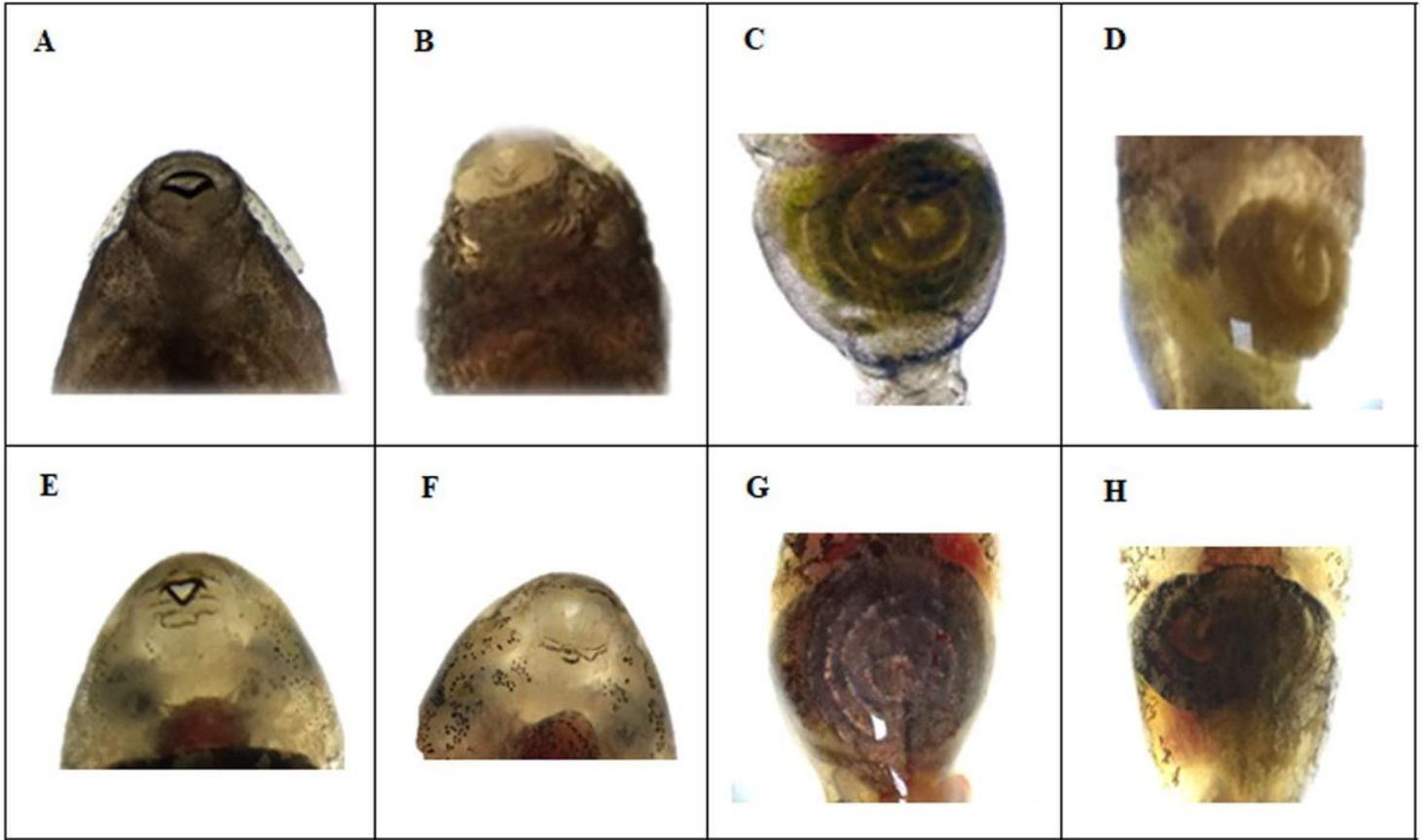


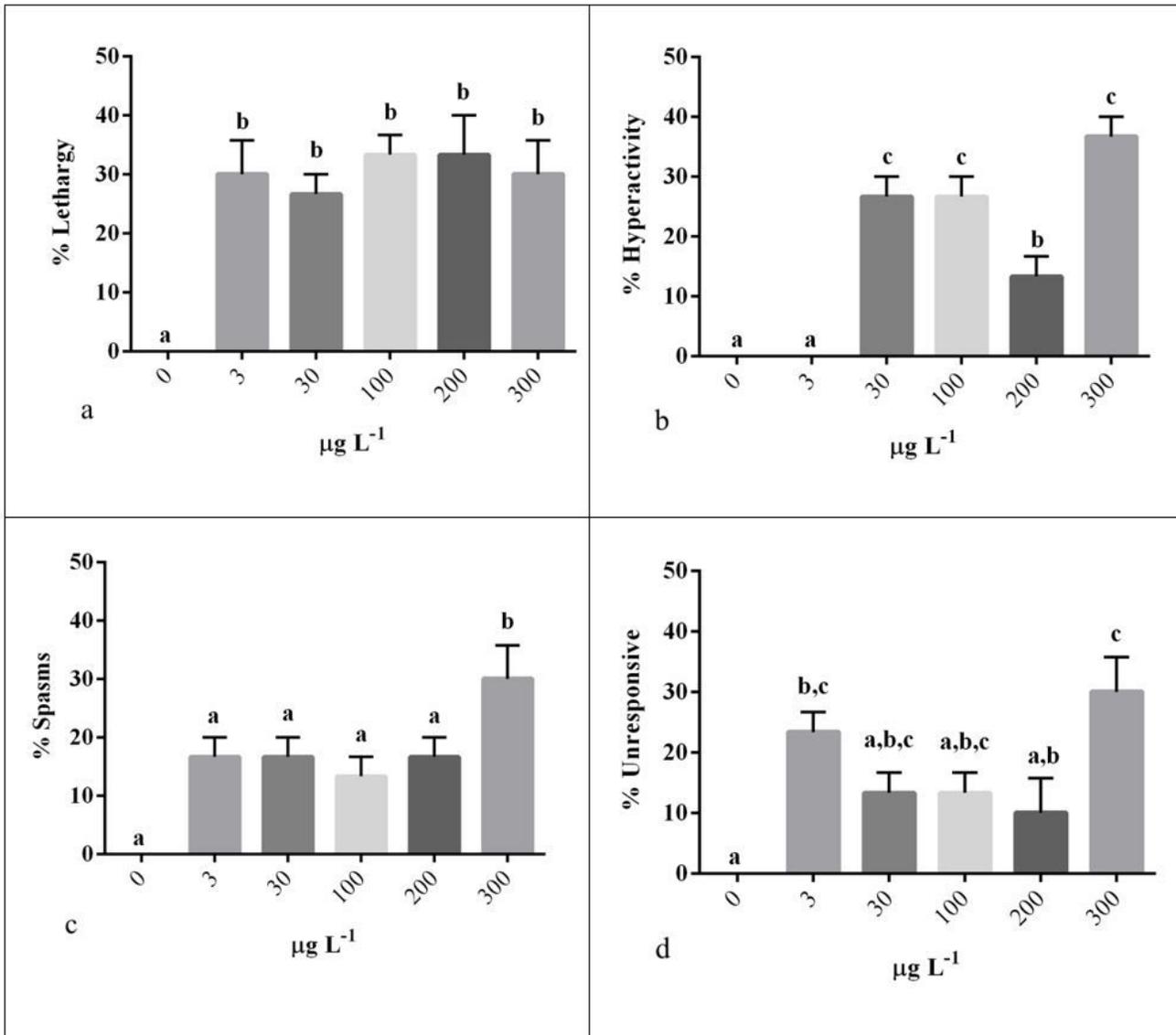
Figure 2

Percentage of occurrence of damage in the mouth, intestine and total damage (mouth + intestine) in tadpoles of *Leptodactylus latrans* (a, c, e) and *Physalaemus cuvieri* (b, d, f) in the control and exposed to different concentrations of imidacloprid based-herbicide for 168 h. Bars represent mean  $\pm$  SE. Different letters indicate significant differences by the Tukey test ( $p < 0.05$ )



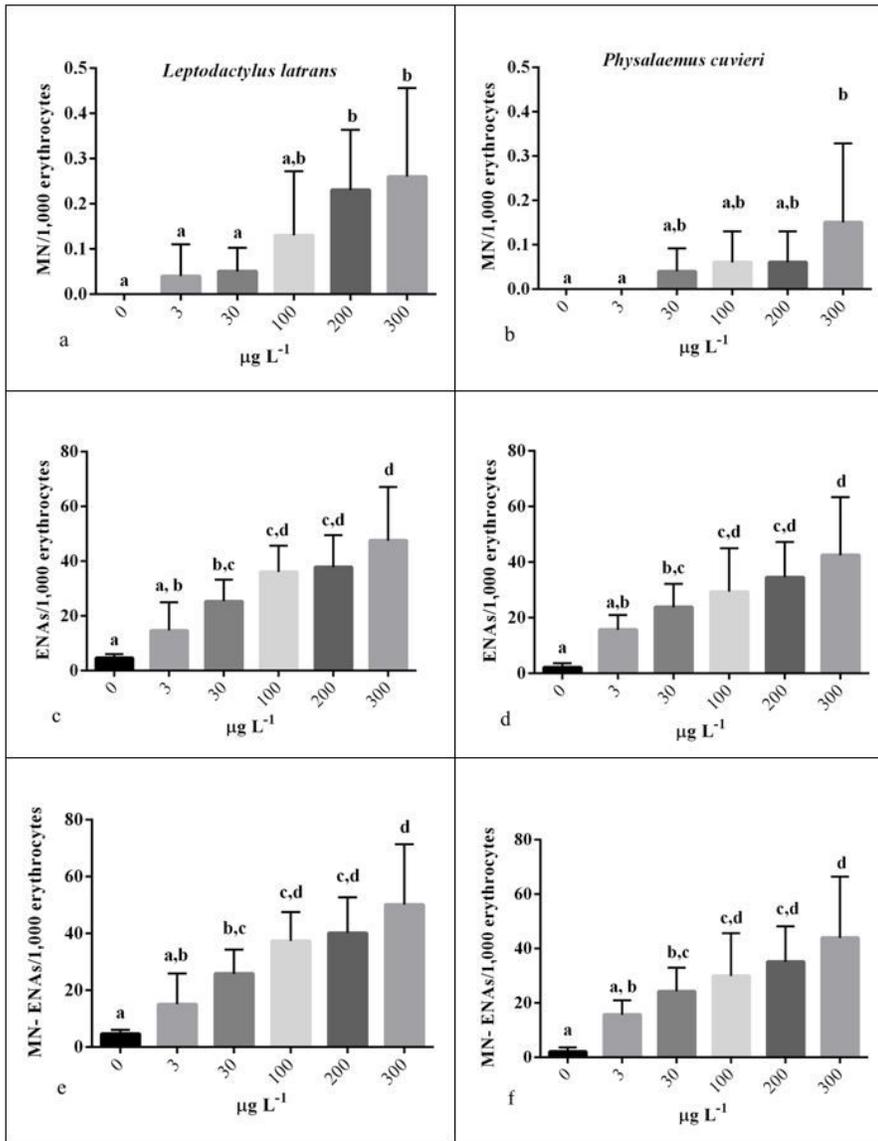
**Figure 3**

Tadpoles of *Leptodactylus latrans* (A-D) and *Physalaemus cuvieri* (E-H). Tadpoles of the control group (A, C and E, G); and exposed to different concentrations of imidacloprid-based herbicide for 168 h: (B) 200  $\mu\text{g L}^{-1}$ , mouth damage; (D) 200  $\mu\text{g L}^{-1}$ , intestine damage; (F) 300  $\mu\text{g L}^{-1}$ , mouth damage; (H) 300  $\mu\text{g L}^{-1}$  intestine damage. (For the color version of this figure, the reader is referred to the web version of this article)



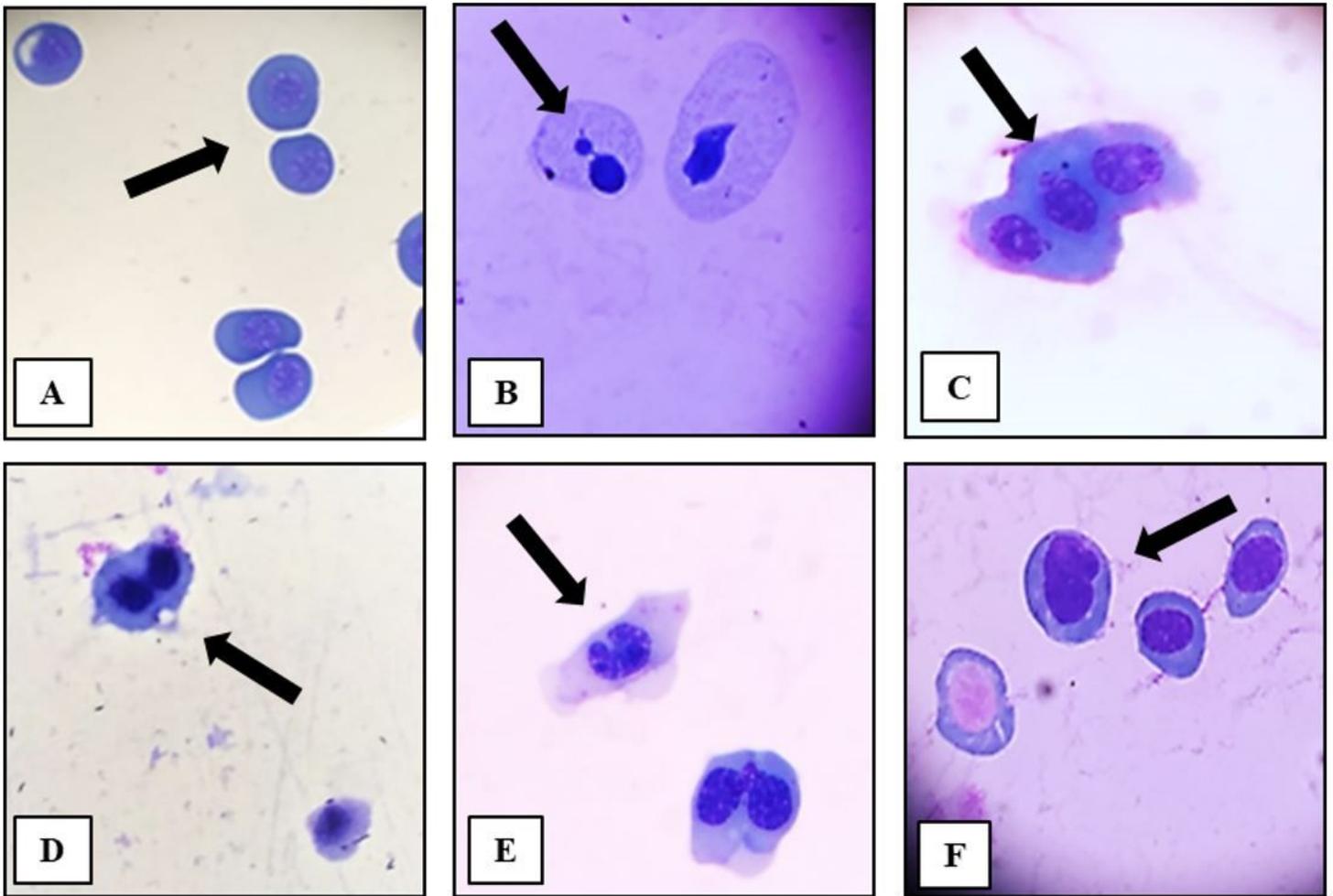
**Figure 4**

Frequency of lethargy (a), hyperactivity (b), spasm (c) and unresponsive (d), during the swimming activity of *Leptodactylus latrans* tadpoles exposed to imidacloprid-based herbicide for 168 h. Bars represent mean  $\pm$  SE. Different letters indicate significant differences by the Tukey test ( $p < 0.05$ ).



**Figure 5**

Micronucleus (MN) frequency and other Nuclear Erythrocytes Abnormalities (ENAs) per thousand erythrocytes (‰) in *Leptodactylus latrans* and *Physalaemus cuvieri* tadpoles in control and exposed to different concentrations of herbicide-based imidacloprid for 168 hours. (a, c, e) *L. latrans*; (b, d, f) *P. cuvieri*. Bars represent mean  $\pm$  SE. Different letters indicate significant differences by the Tukey test ( $p < 0.05$ ).



**Figure 6**

Erythrocyte Nuclear Abnormalities found in tadpoles of *Leptodactylus latrans* and *Physalaemus cuvieri* exposed to different concentrations of imidacloprid for 168 h. (A) Normal cells, (B) Micronucleus (MN); (C) Nuclear bubble/bud (NB); (D) Binucleated cell (BC); (E) Notched nucleus (NN); (F) Lobed nucleus (LN). (For the color version of this figure, the reader is referred to the web version of this article).

## Supplementary Files

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