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Genotoxicity evaluation of the insecticide imidacloprid on circulating blood cells of Montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae) by comet and micronucleus bioassays

C. Ruiz de Arcaute[a,c,1], J.M. Pérez-Iglesias[a,c,1], N. Nikoloff[a,c,1], G.S. Natale[b,c], S. Soloneski[a,c], M.L. Larramendy[a,c,*]

[a] Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Calle 64 N° 3, B1904AMA La Plata, Argentina
[b] Centro de Investigaciones del Medio Ambiente (CIMA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 y 115, B1900GGA La Plata, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

Acute toxicity and genotoxicity of imidacloprid (IMI) was evaluated on *Hypsiboas pulchellus* (Anura: Hylidae) tadpoles exposed under laboratory conditions. A lethal effect was used as the end point for lethality, whereas the frequency of micronuclei (MNs) and DNA single-strand breaks evaluated by the single cell gel electrophoresis assay were employed as end points for genotoxicity. Experiments were performed on tadpoles at stage 36 (range, 35–37) according to the classification proposed by Gosner. Mortality studies revealed an LC_{50} (96 h) value of 84.91 mg/L IMI (95% confidence limits, 77.20–93.04). While increased frequency of MNs was observed when 15 and 30 mg/L were assayed for 48 h, only 15 mg/L increased the frequency of MNs in tadpoles exposed for 96 h. Furthermore, other nuclear abnormalities, *i.e.*, binucleated cells and blebbed and notched nuclei, were induced in tadpoles exposed for both 48 h when treated with 15 mg/L and 96 h when treated with 15 and 30 mg/L. An increase in the genetic damage index was observed in tadpoles treated with 30 mg/L for 48 and 96 h. This study represents the first evidence of acute lethal and sublethal effects exerted by IMI on tadpoles of an amphibian species native to Argentina. Finally, our findings highlight the hazardous properties of this insecticide for nontarget living species exposed to this agrochemical.

1. Introduction

Amphibians represent important vertebrates in natural and agricultural ecosystems since they are included among the most important natural enemies of several agricultural pests worldwide. They possess certain characteristics rendering them a useful indicator species for measuring the effects of changes of the environment. Several reports agree in demonstrating that these vertebrates can be regarded as bioindicators of aquatic and agricultural ecosystems due not only to their sensitivity to habitat modification, but also to the presence of larvae stage. Amphibian larvae live in the aquatic environment and are sensitive to the pollutants (Brodeur et al., 2012; Pollet and Bendell-Young, 2000). At population level, decline of amphibian abundances have been observed, a phenomenon in most cases attributed to pollution of agricultural areas exerted by emerging pollutants, including agrochemicals (Mann et al., 2009; Relyea, 2009). However, other factors, *e.g.*, overexploitation, diseases, habitat loss and/or modification, introduced species, and climate change, also contribute to reduction of amphibian population (Mann et al., 2009). At the organism level, the growth, development, and susceptibility to disease are affected. Furthermore, at the molecular level, the induction of genetic injury into DNA after exposure to agrochemicals is perhaps the most relevant biological effect. A correlation between the use of agrochemicals and the decline of amphibian populations has been demonstrated (Beebee, 2005). The effects of pesticides, including insecticides and herbicides, are particularly detrimental to amphibian species. Several factors contribute to the high sensitivity of amphibians to pesticides: living in the aquatic environment and therefore exposure to various pollutants, the laying of unprotected eggs, and possessing highly permeable skin (Brühl et al., 2011; Sparling and Fellers, 2009).

* Corresponding author at: Facultad de Ciencias Naturales y Museo, Calle 64 Nro. 3 (esq. 120), B1904AMA La Plata, Argentina. Tel.: +54 221 424 9049.
E-mail address: marcelo.larramendy@gmail.com (M.L. Larramendy).
[1] These authors contributed equally to this work.

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Hypsiboas pulchellus, the Montevideo tree frog, also called the common tree frog, is an arboreal anuran species in the family Hylidae. Its natural habitats are subtropical or tropical dry, seasonally wet or flooded lowland grassland, intermittent freshwater lakes, marshes and pastureland (Kwet et al., 2004). Is a very widespread and abundant species with an extensive distribution in Neotropical America, including Argentina, Brazil, Paraguay, and Uruguay, and species commonly found in the Pampasic region of Argentina (Cei, 1980). Previous studies have stressed that tadpoles of this species can be considered a suitable *in vivo* model for detecting lethal and sublethal effects induced by several emerging pollutants, including agrochemicals. Among them, the oxidizing agent potassium dichromate (Natale et al., 2006); the chemotherapeutic cyclophosphamide (Lajmanovich et al., 2005); the insecticides fenitrothion (Junges et al., 2010), cypermethrin (Agostini et al., 2010), and endosulfan (Lajmanovich et al., 2005); as well as the herbicide glufosinate ammonium (Peltzer et al., 2013) can be included.

Imidacloprid (IMI, $C_9H_{10}ClN_5O_2$), is a nicotine-derived systemic insecticide belonging to the neonicotinoid pesticide group. These insecticides act as insect neurotoxins and belong to a class of chemicals, the chloronicotinyl nitroguanidine chemical family, that affect the central nervous system of insects (Blacquière et al., 2012; Tomizawa and Casida, 2005). IMI works by interfering with the transmission of stimuli in the insect nervous system by blocking the nicotinergic neuronal pathway. This blockage leads to the accumulation of acetylcholine, resulting in the insect's paralysis and eventually death. It is effective on contact and *via* stomach action (http://extoxnet.orst.edu/pips/imidaclo.htm). Because IMI binds much more strongly to insect nicotinic neuron receptors than mammal neuron receptors, this insecticide is selectively more toxic to insects than mammals (NPIC, 2010; Tomizawa and Casida, 2005). IMI has been ranked as a Class II chemical (moderately hazardous) by the World Health Organization (WHO, 2002), whereas the U.S. Environmental Protection Agency (NPIC, 2010) has included the insecticide in Group E, compounds with no evidence of carcinogenicity, based on studies with rats and mice. Furthermore, it has not been included as a carcinogen by the International Agency for Research on Cancer (NPIC, 2010). Neonicotinoid insecticides, including IMI, are successfully applied to control pests in a variety of agricultural crops, affecting not only pest insects but also non-target organisms such as pollinators and aquatic invertebrates, *e.g.*, insect larvae living in water (Blacquière et al., 2012).

Studies of the deleterious effects induced by IMI have revealed that the insecticide should be considered as not acutely toxic for fish and amphibians, slightly toxic for zooplankton, moderately toxic for crustaceans, and highly toxic for annelids, but very highly toxic for insects (www.pesticideinfo.org). Among aquatic invertebrates, arthropods such as chironomids (Langer-Jaesrich et al., 2010; Stoughton et al., 2008) as well as ostracods and amphipods (Sánchez-Bayo and Goka, 2006; Stoughton et al., 2008) are extremely sensitive to imidacloprid exposure, with adverse effects observed on survival, growth, and reproductive success. Similarly, toxic effects have also been reported in aquatic vertebrates, namely, fish (Sánchez-Bayo and Goka, 2005) and amphibians, including two species widely distributed in Southeast Asia, *Pelophylax nigromaculatus* and *Rana limnocharis* (Feng et al., 2004).

There is an increasing interest in biomonitoring markers to provide a measurement as well as an estimation of biological exposure to genotoxic pollutants. To achieve this goal, several end points for testing both genotoxicity and cytotoxicity have been employed on aquatic organisms, including amphibians. However, analysis of the frequency of micronuclei (MNs) and the induction of DNA single-strand breaks by the single cell gel electrophoresis (SCGE) assay are the most frequently employed and recommended end points for detecting cytogenetic and DNA damage in circulating nucleated erythrocytes, respectively (Lajmanovich et al., 2005, 2013; Mouchet et al., 2007; Nikoloff et al., 2014; Vera-Candioti et al., 2010).

The aim of the present study is to characterize the acute toxicity of the insecticide imidacloprid on *H. pulchellus* tadpoles exposed under laboratory conditions using a static acute experimental method. This study was performed employing lethal and several sublethal short-term end points for genotoxicity, namely, the frequency of micronuclei (MNs) and the induction of DNA single-strand breaks.

2. Materials and methods

2.1. Chemicals

IMI [95.1%; CAS 138261-41-3; recommended application field ratio up to 700 g a.i. per hectare (CASAFE, 2011)] was kindly provided by Gleba, Argentina. Cyclophosphamide (CP; CAS 6055-19-2) and dimethyl sulfoxide (DMSO; CAS 67-68-5) were purchased from Sigma Chemical Co. (St. Louis, MO), whereas $K_2Cr_2O_7$ [$Cr_{(VI)}$] (CAS 7778-50-9) was obtained from Merck KGaA (Darmstadt, Germany). All other chemicals and solvents of analytical grade were purchased from Sigma Chemical Co.

2.2. Quality control

Determination of the concentration level of IMI in the stock and the test solutions was performed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to U.S. Geological Survey Report 01-4134. Imidacloprid levels were analyzed by high-performance liquid chromatography using an ultraviolet detector. Active ingredient samples from test solutions (30 and 100 mg/L) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit for IMI was 0.5 mg/L.

2.3. Test organisms

Egg masses from *H. pulchellus* were collected from a temporary and unpolluted pond free from pluvial runoff from agricultural areas, in the vicinity of La Plata City (35°10′ S, 57° 51′ W; Buenos Aires Province, Argentina), at the late cleavage stage, stage 9 according to Gosner's classification (Gosner, 1960). Hatches were transported to the laboratory and then acclimatized to 16/8 h light/dark cycles in aquaria at 25 °C with dechlorinated tap water with artificial aeration. The physical and chemical parameters of the water were as follows (mean ± SE): temperature, 25.0 ± 1 °C; pH 7.5 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; conductivity, 994 ± 8.5 μS/cm; hardness, 143 ± 23.5 mg/L $CaCO_3$. Boiled lettuce was supplied as a food source twice per week until the beginning of the experimental procedures.

2.4. Determination of LC_{50}

Experiments for toxicity assessment were performed on tadpoles at Gosner stage 36 (range, 35–37) (Gosner, 1960) following standardized methods proposed by the U.S. EPA (1975, 2002) and ASTM (2007) with minor modifications reported previously for native species (Nikoloff et al., 2014; Vera-Candioti et al., 2010). To determine IMI concentrations used in the acute toxicity tests, preliminary assays were performed. Experiments were performed in quadruplicate and run simultaneously for each experimental point employing five tadpoles maintained in a 500 ml glass container per replicate ($N = 20$), and exposed to six different concentrations of IMI (50, 75, 100, 150, 200, and 250 mg/L) for 96 h. Prior to use,

IMI was first dissolved in DMSO and then diluted in dechlorinated tap water (pH 7.55 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; ammonium (NH_4^+) < 0.2 mg/L; hardness, 143 ± 23.5 mg/L $CaCO_3$). The final solvent concentration was lower than 0.5% for all treatments. The negative control group consisted of five organisms kept in dechlorinated tap water, and the positive control group consisted of five tadpoles treated with 23 mg $Cr_{(VI)}$/L as reported previously (Nikoloff et al., 2014; Vera-Candioti et al., 2010). In addition, a solvent control exposed to 0.5% (v/v) DMSO was included. Negative, solvent, and positive controls were conducted simultaneously with IMI treatments. All test solutions were prepared immediately before use and replaced every 24 h. Tadpoles were not fed throughout the experiments.

2.5. Sublethal end points

2.5.1. Micronuclei and other erythrocytic nuclear abnormalities

The MN assay was performed on peripheral circulating blood erythrocytes according to the procedure described previously (Vera-Candioti et al., 2010). Experiments were performed in quadruplicate and run simultaneously for each experimental point employing five tadpoles maintained in a 500 ml glass container per replicate ($N = 20$ per time of sampling). Tadpoles were exposed to three different concentrations of IMI equivalent to 25%, 50%, and 75% of the corresponding LC_{50} (96 h) value. To achieve these concentrations, tadpoles were exposed to 15, 30, and 45 mg/L IMI, respectively (see Section 2.4). Negative (dechlorinated tap water, see Section 2.4), positive (40 mg/L CP), and solvent (0.5%, v/v DMSO) controls were conducted simultaneously with IMI treatments. All test solutions were prepared immediately before each experiment. The frequency of MNs was determined in peripheral mature erythrocytes at 48 and 96 h after initial treatment. Fifteen specimens were randomly selected for analysis at each experimental time. Tadpoles were euthanized according to American Society of Ichthyologists and Herpetologists (ASIH, 2004) criteria. At the end of each experiment, tadpole anesthesia was induced by immersion in ice water, and blood samples were obtained by sectioning behind the operculum. Peripheral blood smears were performed for each animal onto clean slides, air dried, fixed with 100% (v/v) cold methanol (4 °C) for 20 min, and then stained with 5% Giemsa solution for 12 min. Slides were coded and blind-scored by one researcher at 1000× magnification. Data are expressed as the total number of MNs per 1000 cells, as suggested previously (Vera-Candioti et al., 2010). MN frequency was determined following the examination criteria reported previously (Vera-Candioti et al., 2010). Briefly, the criteria employed in identifying MNs were as follows: a diameter smaller than 1/3 that of the main nuclei, nonrefractability, the same staining intensity as or staining intensity lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, an MN boundary distinguishable from the main nuclei boundary, and no more than four MNs associated with the nuclei.

Other erythrocytic nuclear abnormalities were blind-scored from 1000 erythrocytes per experimental point from each experiment at 1000× magnification. Examination criteria followed those established previously (Cavaş and Ergene-Gözükara, 2003; Strunjak-Perovic et al., 2009). Briefly, cells with two nuclei were considered binucleated, whereas cells with one nucleus presenting a relatively small evagination of the nuclear membrane which contains euchromatin were classified as having blebbed nuclei. Nuclei with evaginations of the nuclear membrane larger than those of the blebbed nuclei, which could have several lobes, were considered as lobed nuclei. Finally, nuclei with vacuoles and appreciable depth into a nucleus without containing nuclear material were recorded as notched nuclei.

2.5.2. Single cell gel electrophoresis assay

Specimens employed for the MN assay were also used for the SCGE assay (see Section 2.5.1). Negative (dechlorinated tap water, see Section 2.4), positive (40 mg/L CP), and solvent (0.5%, v/v DMSO) controls were conducted simultaneously with IMI treatments. The SCGE assay was performed following the alkaline procedure described by Singh (1996) with minor modifications reported elsewhere (Nikoloff et al., 2014; Vera-Candioti et al., 2013c). Briefly, after severing the spinal column behind the opercula, two drops of peripheral blood from each specimen were collected. Blood samples were diluted with 1 ml phosphate-buffered saline, centrifuged (2000 rpm, 8 min), and resuspended in a final volume of 50 μl of phosphate-buffered saline. An aliquot of 30 μl the diluted samples was mixed with 70 μl 0.5% low-melting-point agarose and was then layered on a slide precoated with 100 μl 0.5% normal-melting-point agarose. The slide was covered with a coverslip and placed at 4 °C for 10 min. After solidification, the coverslip was removed, and the slide was covered with a third layer of 50 μl 0.5% low-melting-point agarose. After solidification, the coverslip was removed, and slides were immersed in ice-cold freshly prepared lysis solution (1% sodium sarcocinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, 10% DMSO) and then lysed in darkness for a 1 h period at 4 °C. Then, slides were placed in an electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH) for 25 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 30 min at 25 V and 250 mA. Finally, the slides were neutralized with a solution comprising 0.4 m Tris–HCl, pH 7.5, stained with 4′,6-diamino-2-phenylindole (DAPI; Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Slides were examined under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and nonoverlapping cells. DNA damage was classified in four classes (0-I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Cavaş and Könen, 2007). Data are expressed as the mean number of damaged cells (sum of Classes II, III, and IV) and the mean comet score for each treatment group. The genetic damage index (GDI) was calculated for each test compound following Pitarque et al. (1999).

2.6. Statistical analysis

A *t*-test was performed for comparisons in chemical analyses. Mortality data were analyzed using the U.S. EPA Probit Analysis statistical software, version 1.5 (http://www.epa.gov/nerleerd/stat2.htm.), based on Finney's method (Finney, 1971). The proportion of individuals affected per test chamber ($n = 5$) was calculated for lethal and sublethal end points (mortality, MNs, binucleated cells, blebbed nuclei, lobed nuclei, notched nuclei, damaged cells, and GDI). Each proportion was angular transformed, and a one-way ANOVA with Dunnett's test was performed (Zar, 1999), whereas a one-way ANOVA with Tukey's test was performed for comparison between negative and solvent control data. ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and χ^2 test for normality. The relationships between concentration and GDI and MN data were evaluated by simple linear regression and correlation analyses. Concentration–response (C–R) curves at 96 h were estimated with their 95% confidence limits. Regression and correlation coefficients were calculated for each C–R curve. Tests of significance of the regressions and correlation coefficients were performed (Zar, 1999). The level of significance chosen was 0.05 unless indicated otherwise.

Table 1
Imidacloprid-induced mortality on *Hypsiboas pulchellus* exposed tadpoles.

Time (h)	LC50[a]	95% CI[a,b]
24	131.161	63.468–271.213
48	92.584	74.439–107.032
72	92.584	74.439–107.032
96	84.909	77.201–93.044

[a] mg/L.
[b] 95% confidence interval.

3. Results

Results obtained from the *t*-test between chemical analyses showed no significant changes ($P > 0.05$) in the concentration of the toxicant (30 and 100 mg/L IMI) during the 24 h interval renewals of the testing solutions (concentration range, 98 ± 5% recovery).

3.1. Mortality

Probit analysis of the mortality data allowed determination of the LC_{50} values of IMI after 24, 48, 72, and 96 h of exposure. Results are summarized in Table 1. As revealed by regression analysis, LC_{50} values were not affected by the exposure time ($r = -0.85$, $P > 0.05$).

3.2. Micronuclei and other erythrocytic nuclear abnormalities

Table 2 shows the results of the analysis of IMI-induced MNs in peripheral blood erythrocytes of *H. pulchellus* tadpoles. Since no differences in MN frequencies were observed between negative and solvent control values ($P > 0.05$), pooled data were used for further statistical analyses. An increased frequency of MNs was observed in tadpoles exposed to CP (positive control) for 96 h ($P < 0.05$), but not in those treated for 48 h ($P > 0.05$). In tadpoles exposed for 48 h, a significant increase in the frequency of MNs was found in 15 mg/L ($P < 0.001$) and 30 mg/L ($P < 0.05$) IMI-treated individuals with respect to control values. On the other hand, no increased MN frequency was observed in tadpoles exposed to 45 mg/L ($P > 0.05$). In addition, only tadpoles exposed to 15 mg/L of IMI for 96 h showed a significant increase in MN frequency in regard to controls ($P > 0.01$). When the other nuclear abnormalities were analyzed, an increase in the frequency of notched nuclei was observed only in tadpoles exposed to 15 mg/L for 48 h ($P < 0.01$) and 96 h ($P < 0.001$). In addition, only the treatment of 15 mg/L for 48 h showed an increased frequency of binucleated cells ($P < 0.001$). Moreover, an increase in the frequency of blebbed nuclei was observed in tadpoles exposed to 15 mg/L for 48 h ($P < 0.01$) and 96 h ($P < 0.05$), and individuals exposed to 30 mg/L IMI for 96 h ($P < 0.001$). IMI treatments, regardless of both concentration and exposure time, did not modify the frequencies of lobed nuclei in regard to control values ($P > 0.05$) (Table 2).

3.3. Single cell gel electrophoresis assay

The results of the SCGE assay obtained in peripheral blood erythrocytes of *H. pulchellus* tadpoles exposed for 48 and 96 h to IMI are presented in Table 3, and mean frequencies of cells from each damage grade are depicted in Fig. 1. CP treatment (positive control) induced an enhancement of the GDI as well as in the frequency of damaged cells compared to negative controls in specimens exposed for both 48 and 96 h ($P < 0.001$) (Table 3, Fig. 1). In IMI-exposed tadpoles, a significant increase of the GDI was observed only in 30 mg/L treatments lasting for 48 and 96 h ($P < 0.001$) (Table 3). In tadpoles exposed for 48 h, such alteration was due to an enhanced frequency of type II and III nucleoids ($P < 0.01$) and a concomitant decrease of type 0-I nucleoids ($P < 0.01$) (Fig. 1A). In addition, an increased frequency of type IV nucleoids in tadpoles treated with 45 mg/L IMI for 48 h was observed ($P < 0.05$), although this did not affect the final GDI (Fig. 1A). The results demonstrate that the alteration in the GDI for tadpoles treated for 96 h with 30 mg/L was due to an increase in the frequency of type III ($P < 0.001$) and a concomitant decrease of type 0-I nucleoids ($P < 0.01$) (Fig. 1B). In addition, enhanced frequencies of type II ($P < 0.01$) and type IV nucleoids ($P < 0.05$) were also observed in tadpoles treated with 15 and 45 mg/L of IMI for 96 h, but without affecting the GDI value (Fig. 1B). Overall, a regression analysis demonstrated that alteration of the GDI was not dose dependent in tadpoles treated for either 48 h ($r = -0.0026$, $P > 0.05$) or 96 h ($r = -0.128$, $P > 0.05$).

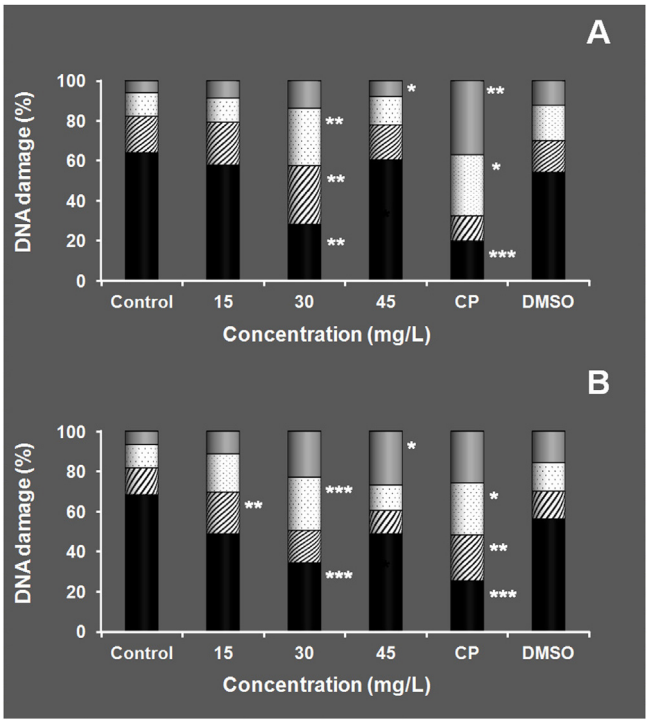

Fig. 1. Imidacloprid-induced DNA damage measured by comet assay in circulating blood cells from *Hypsiboas pulchellus* (Anura, Hylidae) tadpoles exposed for 48 h (A) and 96 h (B). The frequencies of undamaged (type 0-I nucleoids; black column sections), type II (stripped column sections), type III (dotted column sections), and type IV (gray column sections) were determined by analyzing 100 nucleoids from each tadpole. Results are presented as percentages of pooled data from three independent experiments. Negative (untreated tadpoles), positive (CP, 40 mg cyclophosphamide/L-treated tadpoles), and solvent (DMSO, 0.5% (v/v) dimethyl sulphoxide/L-treated tadpoles) controls were conducted and run simultaneously with imidacloprid-exposed larvae. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; significant differences with respect to control values.

4. Discussion

In the current study, the acute lethal toxicity and sublethal effects of the insecticide IMI were evaluated on *H. pulchellus* (Anura, Hylidae) tadpoles exposed under laboratory conditions. Regarding the acute lethal effects of the studied insecticide on the species, the chemical could be ranked, according the scoring proposed by the Office of Pollution Prevention and Toxics of the U.S. EPA (Smrchek et al., 1993; Wagner et al., 1995), as a compound with moderate ecotoxicity concern for larvae at premetamorphic stage. However, it can be classified as harmful compound for aquatic organisms (category III) following either the European Union directives (Mazzatorta et al., 2002) or the classification criteria proposed by the United Nations (2011). The results demonstrate that IMI can be considered a damaging agent with genotoxic effects at both chromosomal and DNA levels. IMI increased the frequency of MNs in peripheral erythrocytes of *H. pulchellus* tadpoles exposed within

Table 2
Frequencies of micronuclei (MN) and other nuclear abnormalities in peripheral blood erythrocytes of *Hypsiboas pulchellus* tadpoles exposed to the insecticide imidacloprid.[a]

Exposure time (h)	Concentration (mg/L)	No. of animals analyzed	No. of cells analyzed	MN	Other nuclear abnormalities			
					Notched nuclei	Lobed nuclei	Binucleated nuclei	Blebbed nuclei
48	Control	15	14,357	4.87 ± 1.05	4.60 ± 1.24	0.50 ± 0.43	0.20 ± 0.11	6.93 ± 1.92
	Positive control[b]	15	15,139	4.23 ± 0.87	3.69 ± 0.98	0.26 ± 0.12	0.59 ± 0.16	5.61 ± 0.95
	15	15	15,222	17.35 ± 2.44***	7.45 ± 1.43**	0.53 ± 0.33	1.84 ± 0.50***	11.13 ± 1.65**
	30	15	15,342	6.71 ± 1.89*	6.04 ± 1.48	0.26 ± 0.20	0.72 ± 0.28	5.39 ± 1.59
	45	15	15,194	4.14 ± 0.58	4.41 ± 0.69	0.26 ± 0.15	0.46 ± 0.23	4.28 ± 1.01
	Solvent control[c]	15	13,483	2.02 ± 0.57	1.87 ± 0.35	0.26 ± 0.15	0.86 ± 0.53	2.88 ± 0.96
96	Control	15	15,288	5.54 ± 0.98	2.51 ± 0.56	0.45 ± 0.19	1.11 ± 0.27	6.15 ± 1.32
	Positive control[b]	15	15,108	9.95 ± 2.01*	6.42 ± 2.11	0.66 ± 0.16	0.93 ± 0.18	8.39 ± 1.55
	15	15	15,209	10.14 ± 2.01**	9.59 ± 2.30***	0.39 ± 0.18	0.72 ± 0.26	9.29 ± 1.54*
	30	15	15,440	7.78 ± 1.53	4.51 ± 1.16	0.53 ± 0.35	0.31 ± 0.15	12.52 ± 1.57***
	45	14	13,922	4.23 ± 1.59	5.27 ± 1.70	0.92 ± 0.37	0.77 ± 0.39	4.07 ± 1.35
	Solvent control[c]	15	15,160	3.17 ± 0.84	2.18 ± 0.50	0.46 ± 0.19	0.46 ± 0.16	3.49 ± 0.74

* $P < 0.05$; significant differences with respect to control values.
** $P < 0.01$; significant differences with respect to control values.
*** $P < 0.001$; significant differences with respect to control values.
[a] Results are expressed as mean number of abnormalities/1000 cells ± SE.
[b] Cyclophosphamide (CP, 40 mg/L) was used as positive control.
[c] Dimethyl sulphoxide (DMSO, 0.5%, v/v) was used as solvent control.

Table 3
Analysis of DNA damage measured by comet assay in *Hypsiboas pulchellus* tadpoles cells exposed to the insecticide Imidacloprid.

Chemicals	Concentration (mg/L)	Exposure time (h)	No. of animals analyzed	No. of cells analyzed	% of damaged cells (II + III + IV)	GDI ± SE[a]
Control		48	15	1577	36.63	1.31 ± 0.23
		96	15	1590	31.72	1.26 ± 0.17
Imidacloprid	15	48	15	1603	40.85	1.51 ± 0.16
		96	15	1590	50.40	1.77 ± 0.14
	30	48	15	1673	71.29**	2.25 ± 0.15***
		96	15	1515	65.02***	2.29 ± 0.11***
	45	48	15	1520	38.69	1.52 ± 0.16
		96	15	1446	51.3	1.99 ± 0.22
Positive control[b]	40	48	14	1418	76.47***	2.84 ± 0.22***
		96	15	1528	74.82***	2.42 ± 0.24**
Solvent control[c]		48	15	1493	40.97	1.52 ± 0.20
		96	15	1537	41.42	1.65 ± 0.14

** $P < 0.01$; significant differences with respect to control values.
*** $P < 0.001$; significant differences with respect to control values.
[a] GDI, genetic damage index.
[b] Cyclophosphamide (CP, 40 mg/L) was used as positive control.
[c] Dimethyl sulphoxide (DMSO, 0.5%, v/v) was used as solvent control.

the 15–30 mg/L concentration range for 48 h or to 15 mg/L for treatments lasting 96 h. Furthermore, the insecticide was able to induce other nuclear abnormalities, *i.e.*, binucleated cells and blebbed and notched nuclei, in tadpoles exposed for 48 h, when treated with 15 mg/L, and 96 h, when treated with 15 and 30 mg/L. Besides, we observed that acute exposure to all IMI concentrations increased the frequency of primary DNA lesions estimated by alkaline SCGE. Additionally, our data revealed that the SCGE assay was more sensitive than the MN test in detecting early DNA damage when the same IMI concentrations were employed for tadpole exposure.

The variability in pesticide-induced toxicity in different amphibian species is a phenomenon known worldwide (Relyea, 2009; Vera-Candioti et al., 2010). To the best of our knowledge, acute lethality data of IMI have been reported previously for two amphibian species. Years ago, Feng et al. (2004) reported 96 h LC_{50} values of 82.0 and 129.0 mg/L for *R. limnocharis* and *P. nigromaculatus* premetamorphic tadpoles exposed to the active ingredient under laboratory conditions, respectively. However, no indication of the developmental growth state of the tadpoles was given (Feng et al., 2004). Accordingly, the species *H. pulchellus* could be considered the anuran species most sensitive to IMI reported so far.

Our results reveal a concentration of 84.91 mg/L IMI (confidence limit, 77.20–93.04) as the 96 h LC_{50} value for *H. pulchellus* tadpoles at Gosner stage 36. Previous studies have demonstrated that the tested hylid frog tadpoles can be considered adequate reference organisms in the risk assessment of toxicity induced by different xenobiotics, including pesticides. Maximum 96 h LC_{50} values of 29.60 mg/L for Cr(VI) (Natale et al., 2006), 0.47 mg/L for the insecticide cypermethrin or 0.175 mg/L for the cypermethrin-based commercial formulation Cypermethrin Sherpa® (Agostini et al., 2010), and 0.00013 mg/L for an endosulfan-based insecticide formulation (Agostini et al., 2009) have been reported. Furthermore, a 48 h LC_{50} value of 21.47 mg/L for the herbicide glufosinate ammonium (Peltzer et al., 2013) has been reported, whereas our results reveal a 48 h LC_{50} value of 92.58 mg/L (confidence limit, 74.44–107.03) for IMI. Accordingly, it seems evident this anuran species is nearly 653,154, 485, 181, 4, and 3, times less sensitive to IMI than to endosulfan, Cypermethrin Sherpa®, cypermethrin,

glufosinate ammonium, and Cr(VI), respectively. In other words, IMI is the least toxic emerging pollutant reported so far for *H. pulchellus* tadpoles.

The MN analysis is used worldwide as a bioassay of genotoxic to detect small chromosomal fragments, *i.e.*, acentric fragments and chromatid fragments, induced by clastogens or vagrant chromosomes produced by aneugens (OECD, 2010). Previous reports demonstrated the induction of MNs in circulating erythrocytes from amphibian tadpoles as a consequence of pesticide treatments (Bouhafs et al., 2009; Li et al., 2010; Nikoloff et al., 2014; Vera-Candioti et al., 2010). Furthermore, *H. pulchellus* has been used previously as an experimental model in genotoxicity studies. Lajmanovich et al. (2005) demonstrated the induction of MNs after exposure to the insecticide endosulfan. In the current study, the lowest concentration of IMI used (15 mg/L) was able to induce DNA damage leading to MN formation when tadpoles were exposed for 96 h. Furthermore, an enhanced MN frequency was noticed when 15 and 30 mg/L IMI were assayed after 48 h of treatment. However, the highest IMI concentration (45 mg/L) was unable to modify the basal level of MNs, regardless of the exposure time. So far, we do not have any explanation for this particular observation. However, a plausible possibility could be related to the induction of a selective cell loss by insecticide-induced cell death of the most damaged cells after treatment, leaving only a reduced proportion of cells capable of reaching the M1 status after nearly 96 h of exposure. Whether the latter is true or not, the present results highlight and verify the genotoxic capability of the test compound under study, although previous studies on vertebrates, including amphibians, exposed to IMI are scarce (Feng et al., 2005, 2004; Stivaktakis et al., 2012; Vlastos et al., 2010). Finally, nuclear abnormalities other than MNs were also induced after IMI exposure in those tadpoles exposed within the 15–30 mg/L concentration range, regardless of the exposure time. Previous reports have demonstrated that these morphological nuclear abnormalities, *e.g.*, binucleated erythrocytes as well as notched and blebbed nuclei, can be considered genetic damage indicators (Cavaş and Ergene-Gözükara, 2005; Gökalp Muranli and Güner, 2011). Our results agree well with these observations.

To monitor contaminated areas (*in situ* assay) (Burlibasa and Gavrila, 2011; Maselli et al., 2010) as well as for screening xenobiotics after direct or indirect exposure (*in vivo* assay) (Binelli et al., 2009; Mouchet et al., 2007; Nikoloff et al., 2014), the SCGE test has become extensively valuable as a biomarker in amphibians. We observed that, regardless of the length of treatment, a concentration of 15 mg/L IMI was unable to increase the frequency of damaged nucleoids in tadpoles treaded for 48 or 96 h. However, acute exposure with concentrations higher than 30.0 mg/L IMI, regardless of the exposure period, increased the frequency of primary DNA lesions estimated by the alkaline SCGE when the frequency of different nucleoids were analyze or by increasing the GDI, an observation opposite that of the MN test. One possible explanation for this observation could be related to the different cellular status of the target cells that are included for analysis in each end point. Whereas DNA damage is estimated by SCGE in resting cells, MNs are determined on proliferating cells with lesions that have lasted for at least one mitotic cell cycle and that probably retain their repair property, as suggested elsewhere (He et al., 2000). Accordingly, the existence of a selective loss of the most damaged cells when increasing IMI concentration could also explain our observation. However, the possibility that circulating blood cells are able to repair IMI-induced DNA damage cannot be ruled out. Whether the latter hypothesis is accepted, the higher the DNA repair capacity, the lower the frequency of the most damaged nucleoids and the higher the frequency of the less damaged nucleoids that should be present among the cells included within the SCGE analysis. Our results agree well with this assumption. Further studies involving IMI-induced DNA damage and repair could bring clear insights to this situation.

Reports in which the frequency of agrochemical-induced DNA single-strand breaks has been used as a bioassay for evaluating genetic damage induced in amphibians from the Noetropical Region, especially for native Argentinean species, are scarce. However, they represent a well-documented issue worldwide (Mouchet et al., 2006, 2007). To the best of our knowledge, only the primary DNA damage induced by the flurochloridone-based commercial formulations Twin Pack Gold® and Rainbow® on tadpoles of *Rhinella arenarum* as the target has been previously reported (Nikoloff et al., 2014). Accordingly, our current results represent the first evidence of the acute genotoxic effects exerted by the active principle IMI on Neotropical tadpoles exposed under laboratory conditions. Furthermore, no other SCGE study has been reported previously employing *H. pulchellus* as the test organism. Finally, our findings support the view that the SCGE assay is a highly sensitive method for the detection of DNA damage induced by environmental pollutants.

Most of the agrochemicals in aquatic environments exert their effects through genotoxic and metabolically toxic mechanisms causing, simultaneously, genotoxic damage, disease, and carcinogenesis (Könen and Cavaş, 2008; Vera-Candioti et al., 2010, 2013a,b). Therefore, IMI, whether inefficiently diluted or degraded after application in the field, might reach levels that pose genotoxic effects, at least on frogs. Conversely, since frogs represent important natural enemies of several agricultural pests, they might be exposed to the residual pesticides in pests through the food chain. Several reports agree, demonstrating that IMI residues can be found in vegetables, crops, and fruits, as well as in pests (Fernandez-Alba et al., 1996; Garrido Frenich et al., 2000; Ko et al., 2014). Our observations revealed 84.91 mg/L (confidence limit, 77.20–93.04) as the 96 h LC_{50} value of IMI for *H. pulchellus* tadpoles. Although the *in vivo* IMI treatments in this study cover a wide range of concentrations, the concentration range represents a relatively high end of the threshold value of 0.1 μg/L IMI found in ground, surface, and drinking water (RIVM, 2008), even considering that the recommended application rates to foliage or soil range from approximately 50–320 g a.i. per hectare (PMRA, 2007), or as high as 700 g a.i. per hectare reported for Argentina (CASAFE, 2011). Thus, the concentrations of IMI employed in this investigation would be expected to be almost improbable in the environment, perhaps observed only when specific events occur (*e.g.*, direct application, drainage ditches or by accidental discharge). Although, we cannot rule out that amphibian populations, and also human workers, could be exposed accidentally to these agrochemicals at this range of concentration. Accordingly, the current awareness of the real/potential hazards of IMI cannot be neglected considering its genotoxicity. Finally, our findings highlight the importance of minimizing the impacts on nontarget living species, such as *H. pulchellus*, exposed to agrochemicals.

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