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Toxic and genotoxic effects of the imazethapyr-based herbicide formulation Pivot H® on montevideo tree frog *Hypsiboas pulchellus* tadpoles (Anura, Hylidae)

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ABSTRACT

Acute lethal and sublethal toxicity of the imidazolinone imazethapyr (IMZT)-based commercial formulation herbicide Pivot H® (10.59% IMZT) was evaluated on *Hypsiboas pulchellus* tadpoles. Whereas mortality was used as the end point for lethality, frequency of micronuclei (MNs) and other nuclear abnormalities as well as DNA single-strand breaks evaluated by the single cell gel electrophoresis assay were employed to test genotoxicity. Behavioral, growth, developmental, and morphological abnormalities were also employed as sublethal end points. Mortality studies revealed equivalent LC_{50} (96 h) values of 1.49 mg/L (confidence limit, 1.09–1.63) and 1.55 mg/L (confidence limit, 1.51–1.60) IMZT for Gosner stage (GS) 25 and GS36, respectively. Behavioral changes, i.e., irregular swimming and immobility, as well as a decreased frequency of keratodonts were observed. The herbicide increased the frequency of MNs in circulating erythrocytes of tadpoles exposed for 48 h to the highest concentration assayed (1.17 mg/L). However, regardless of the concentration of the herbicide assayed, an enhanced frequency of MNs was observed in tadpoles exposed for 96 h. The herbicide was able to induce other nuclear abnormalities, i.e., blebbed and notched nuclei, only when tadpoles were exposed for 96 h. In addition, we observed that exposure to IMZT within the 0.39–1.17 mg/L range increased the genetic damage index in treatments lasting for both 48 and 96 h. This study represents the first evidence of acute lethal and sublethal effects exerted by IMZT on amphibians. Finally, our findings highlight the properties of this herbicide that jeopardize nontarget living species exposed to IMZT.

1. Introduction

Living species are inevitably exposed to pesticides, which represent both a significant ecological and public health concern due to their jeopardizing effects. Furthermore, pesticides are ubiquitous in the world because anthropogenic activities are continuously introducing extensive amounts of them into the environment, regardless of their physicochemical properties that confer persistence, bioaccumulation, and toxicity (Diamond et al., 2015). Pesticides constitute a heterogeneous category of agrochemicals, specifically designed to control pests, weeds, and plant diseases. Unfortunately, it is difficult to decrease the use of pesticides without reducing crop fields (Larramendy and Soloneski, 2012). In addition to causing environmental damage, pesticides frequently affect wild nontarget species that possess physiological or biochemical similarity to the target organisms (Köler and Triebskorn, 2013).

Imidazolinones represent a group of agrochemicals widely used for broad-spectrum weed control as selective pre- or post-emergence herbicides on a variety of crops, including soybean, alfalfa, wheat, and barley, among others. This class of herbicides currently consists of six commercially available enantiomers and their methyl derivatives, namely, imazapic, imazapyr, imazethapyr (IMZT), imazamox, imazaquin, and imazamethabenz-methyl (Lin et al., 2007). These herbicides are among the most popular agrochemicals employed by farmers in more than 200 countries worldwide because while they have proved to be potent and highly selective for plants, they are considered nontoxic for animals. Imidazolinone herbicides control weeds by inhibiting the

Abbreviations: BL, blebbed nucleus; BN, binucleated erythrocyte; CP, cyclophosphamide; GDI, genetic damage index; GS, Gosner stage; IM, immobility; IMZT, imazethapyr; IS, irregular swimming; LB, lobed nucleus; MN, micronucleus; NT, notched nucleus; SCGE, single-cell gel electrophoresis; STG, development stage according to Gosner's classification

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action of the acetohydroxyacid synthase (EC 2.2.1.6), also called acetolactate synthase (EC 4.1.3.18). In plants, the enzyme catalyzes the key reactions in the biosynthesis pathways of three branched-chain aliphatic amino acids, i.e., valine, isoleucine, and leucine, with its activity regulated by the end products of the pathways (Breccia et al., 2013; Zhou et al., 2010). This inhibition causes a disruption in protein synthesis, which, in turn, leads to interference in DNA synthesis and cell growth, and eventually to weed death. However, since acetohydroxyacid synthase does not occur in animals, which rely on plants for these essential amino acids, imidazolinone herbicides generally have very low toxicity in animals (NYS DEC, 2003).

IMZT [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5-dihydroimidazol-1*H*-2-yl) nicotinic acid] is a member of the imidazolinone herbicides used to control grasses and broadleaved weeds, including barnyardgrass, crabgrass, cocklebur, panicums, pigweeds, nightshade, mustard, smartweed, velvetleaf, jimsonweed, foxtails, seedling johnsongrass, lambsquarters, morning glory, and others in a variety of crops and noncrop situations (MacBean, 2012). The herbicide has a low sorption coefficient and is highly soluble in water (1400 mg/L), and therefore has a high affinity with water (Senseman, 2007). The California Department of Pesticide Regulation has classified this herbicide as a potential groundwater contaminant (www.pesticideinfo.org).

IMZT has been classified as a slightly toxic compound (Class III) by the USEPA (1989). Similarly, the herbicide has been reported as a harmful irritant for the respiratory track, skin, and eyes, as well as classified as a dangerous compound for the environment by the European Union (PPDB, 2014). Overall, very little is known about the toxicology and ecotoxicology of IMZT. When IMZT was administered orally, low or moderate acute toxicity was reported in rats. Using algae and aquatic invertebrates, low levels of toxicity have been reported. When aquatic plants were employed as targets, e.g., *Lemna gibba*, high acute levels of toxicity were observed. Among terrestrial invertebrates, insects such as honeybees and annelids such as earthworms have been reported to have extremely high sensitivity and low sensitivity to IMZT, respectively. So far, the levels of acute toxicity exerted by the herbicide were found not to be acutely toxic for fish, including channel catfish, bluegill, and rainbow trout (PPDB, 2014). Although Moraes et al. (2011) reported disorders in oxidative stress parameters in the common carp (*Cyprinus carpio*) after exposure to IMZT- and imazapic-based commercial herbicide formulations. However, no available information has been reported for other aquatic vertebrates, including amphibians, rather than fish (PPDB, 2014).

Nowadays, there is an increasing concern in monitoring biomarkers to provide measurements as well as estimations of biological exposure to emergent pollutants. To accomplish this target, several sublethal end points for testing both genotoxicity and cytotoxicity have been employed on aquatic organisms, including amphibians. Among them, analysis of micronucleus (MN) frequency and the induction of DNA single-strand breaks by the single cell gel electrophoresis (SCGE) assay are the most frequently used and recommended end points for detecting cytogenetic and primary DNA damage in circulating nucleated cells, respectively (Hartmann et al., 2003; Nikoloff et al., 2014b; Pérez-Iglesias et al., 2014; Vera Candioti et al., 2010).

The aim of the present study is to characterize the acute toxicity of the IMZT-based herbicide formulation Pivot H® (10.59% IMZT) on *Hypsiboas pulchellus* tadpoles exposed under laboratory conditions employing a static acute experimental method. Whereas mortality was used as end point for lethality, the frequency of MNs and other nuclear abnormalities as well as DNA single-strand breaks evaluated by the SCGE assay were employed as sublethal end points for genotoxicity on circulating blood cells. Furthermore, behavioral, growth, developmental, and morphological abnormalities were also employed as sublethal end points.

2. Materials and methods

2.1. Chemicals

Pivot H® (10.59% IMZT, CAS 081335-77-5) was purchased from BASF Argentina S.A. Cyclophosphamide (CP, CAS 6055-19-2) was purchased from Sigma Chemical Co. (St. Louis, MO), and $K_2Cr_2O_7$ [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. Chemical analysis

Concentration levels of IMZT in the test solutions were analyzed according to U.S. Geological Survey Report 01-4134. Imazethapyr levels were analyzed by high performance liquid chromatography. Active ingredient samples from test solutions (0.78, 1.17, and 1.63 mg/L) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit for IMZT was 0.5 μg/L.

2.3. Test organisms

H. pulchellus is an arboreal species in the family Hylidae. This species has an extensive distribution in the Neotropical America and is an abundant species in the Pampasic region of Argentina (Cei, 1980). Its natural habitats are subtropical or tropical dry lowland grasslands, subtropical or tropical seasonally wet or flooded lowland grasslands, intermittent freshwater lakes, intermittent freshwater marshes, and pasturelands (Kwet et al., 2004). This species lays its eggs in masses attached to the submerged stems of aquatic plants, and it is easy to handle and acclimate to laboratory conditions (Lajmanovich et al., 2005; Pérez-Iglesias et al., 2014).

2.4. Experimental design

All tadpoles used for this study were collected from a temporary and unpolluted pond away from agricultural areas, in the vicinity of La Plata City (35°10′S, 57°51′W; Buenos Aires Province, Argentina), at late cleavage stage (GS) 9 according to Gosner's (1960) classification. 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: temperature, 25.0 ± 1 °C; pH 8.0 ± 0.1; dissolved oxygen, 6.3 ± 0.3 mg/L; conductivity, 663 ± 15.0 μS/cm; hardness, 181 ± 35.0 mg/L $CaCO_3$. Commercially available fish food (Tetra-Min®, Tetra Werke, Germany) as a food source was supplied twice a week until the beginning of the experimental procedures, when the individuals reached development stage 25 (GS25) or 36 (GS36) (range, 35–37) according to Gosner (1960). Afterwards, individuals were randomly deposited in test chambers according to the experimental design. Hatches were collected with the permission of the Flora and Fauna Direction from the Buenos Aires Province (Buenos Aires, Argentina) (code 22500-22339/13) and the Ethical Committee from the National University of La Plata (code 11/N619).

Two acute toxicity tests were conducted. One of them employed both GS25 and GS36 tadpoles to estimate lethal and sublethal effects including behavioral, growth, developmental, and morphological effects. The second, employing GS36 tadpoles, was

conducted to evaluate genotoxicity, including induction of MNs, other erythrocytic nuclear abnormalities, and DNA single-strand breaks.

Acute toxicity tests were performed following standardized methods proposed by the USEPA (2002) and ASTM (2007), with minor modifications reported previously (Natale et al., 2006; Vera Candioti et al., 2010). For each experimental point, experiments were performed using five tadpoles, maintained in a 500 ml glass container and exposed to different concentrations of IMZT for 96 h. To determine the concentrations used in the acute toxicity tests, preliminary tests were performed. Whereas GS25 tadpoles were exposed to 0.27, 0.41, 0.54, 0.68, 0.81, 0.87, 0.95, 1.02, 1.09, 1.22, and 2.72 mg/L IMZT, GS36 tadpoles were exposed to 0.41, 0.81, 1.09, 1.22, 1.36, 1.49, 1.63, 2.17, and 2.72 mg/L IMZT. Whereas the negative control group consisted of five organisms kept in dechlorinated tap water, the positive control group consisted of five tadpoles treated with 23 mg/L Cr(VI) as reported previously (Nikoloff et al., 2014b; Vera Candioti et al., 2010).

For genotoxic analyses, experiments were performed using five GS36 tadpoles for each experimental point, maintained in a 500 ml glass container and exposed to three different concentrations of IMZT equivalent to 25%, 50%, and 75% of the corresponding LC_{50} (96 h) value. To achieve these concentrations, tadpoles were exposed to 0.39, 0.78, and 1.17 mg/L IMZT, respectively. Negative (dechlorinated tap water) and positive controls (40 mg/L CP) were conducted and run simultaneously with treatments for IMZT-exposed tadpoles.

All test solutions were prepared immediately before use and replaced every 24 h. IMZT was first dissolved in dechlorinated tap water at a concentration of 1 g/L to obtain a stable stock solution, and then diluted in test water at the concentrations specified previously (USEPA, 1975). Tadpoles were not fed throughout the experiment. Experiments were performed in quadruplicate and run simultaneously for each experimental point.

2.5. Lethal end points

Mortality was evaluated by visual observation every 24 h. Individuals were considered dead when no movement was detected after gentle prodding with a glass rod compared to control organisms. Dead individuals were taken at each observation time, labeled, and fixed in 10% v/v formaldehyde. After 96 h, all (live and dead) individuals were labeled and fixed in 10% v/v formaldehyde for further evaluation of growth and abnormalities.

2.6. Sublethal end points

2.6.1. Behavioral changes

Behavioral changes were registered every 24 h in four independent experiments setup as described in Section 2.4. Changes were registered after gently swirling the water five times with a glass rod and observing for 1 min the swimming activity of each organism. Irregular swimming (IS) and immobility (IM) were categorized according to descriptions made by Brunelli et al. (2009). The prevalence of a type of abnormality was calculated by dividing the number of larvae with the particular abnormality by the number of individuals examined.

2.6.2. Growth and development

Whereas growth was assessed, after 96 h of exposure, by measuring body length according to McDiarmid and Altig (1999), with a digital caliper of 0.01 mm, developmental stage was ranked according to Gosner (1960).

2.6.3. Morphological abnormalities

Abnormalities were assessed at 96 h after herbicide exposure in four independent experiments setup as described in Section 2.4. Morphological characteristics were registered under a Wild Heerbrugg M8 binocular stereoscope microscope and determined according to the categories proposed elsewhere (Bantle et al., 1998), with minor modifications. Briefly, among them were included the presence of edema, axial abnormalities (severe flexure of the tail), decrease in keratodont numbers, and gut abnormalities. The prevalence of a type of abnormality was calculated as indicated in Section 2.6.1.

2.6.4. Micronuclei and other erythrocytic nuclear abnormalities

The MN assay was performed on mature erythrocytes in peripheral circulating blood according to the procedure described previously (Vera Candioti et al., 2010). Tadpoles were killed according to the American Society of Ichthyologists and Herpetologists criteria (ASIH, 2004). The frequency of MNs was determined at 48 and 96 h after initial treatment. Experiments were performed in quadruplicate and run simultaneously for each experimental point, and 15 specimens were randomly selected for further analysis. At the end of each experiment, tadpoles were anesthetized 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 a 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 of that of the main nucleus, nonrefractibility, the same staining intensity as or staining intensity lighter than that of the main nucleus, no connection or link with the main nucleus, no overlapping with the main nucleus, a MN boundary distinguishable from the main nucleus boundary, and no more than four MNs associated with the nucleus.

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 for the species (Pérez-Iglesias et al., 2014; Ruiz de Arcaute et al., 2014). Briefly, cells with two nuclei were considered binucleated cells (BNs), whereas cells with one nucleus presenting a relatively small evagination of the nuclear membrane that contained euchromatin were classified as blebbed nuclei (BLs). Nuclei with evaginations of the nuclear membrane larger than the BL, which could have several lobes, were considered lobed nuclei (LBs). Finally, nuclei with vacuoles and appreciable depth into a nucleus without containing nuclear material were recorded as notched nuclei (NTs).

2.6.5. Single cell gel electrophoresis assay

Specimens employed for the MN assay (see Section 2.6.4) were also used for the SCGE assay. Experiments were performed in quadruplicate and run simultaneously for each experimental point. Negative (dechlorinated tap water, see Section 2.4) and positive (40 mg/L CP) controls were conducted simultaneously with IMZT treatments. The SCGE assay was performed following the alkaline procedure described elsewhere (Vera-Candioti et al., 2013). Blood samples were diluted with 1 ml phosphate-buffered saline, centrifuged (2000 rpm, 9 min), and resuspended in a final volume of 50 μl of phosphate-buffered saline. An aliquot of 30 μl of the diluted samples was mixed with 70 μl of 0.5% low-melting-point agarose and was then layered on a slide precoated with 100 μl of 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 of 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 sarcosinate, 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 20 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 (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) using the formula GDI=[I(I)+2(II)+3(III)+4(IV)/*N*(0–IV)], where 0–IV represents the nucleoid type, and N_0–N_{IV} represent the total number of nucleoids scored.

2.7. Statistical analysis

LC_{50} and EC_{50} values were calculated using the U.S. EPA Probit Analysis statistical software, version 1.5 (http://www.epa.gov/nerleerd/stat2.htm) based on Finney's (1971) method. Concentration–response (*C*–*R*) curves at different times (24, 48, 72, and 96 h) were estimated with their 95% confidence limits. Tests of significance for the regression and correlation coefficients and comparison of linear regression equations were performed following Zar (1999).

The proportion of individuals affected per test chamber ($n=5$) was calculated for lethal and sublethal end points (mortality, behavior, morphological abnormalities, MNs, BNs, BLs, LBs, NTs, and damaged cell frequencies). Each proportion was angular transformed, and a one-way analysis of variance (ANOVA) with Dunnett's test was performed (Zar, 1999), obtaining the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values. ANOVA assumptions were corroborated with Barlett's test for the homogeneity of variances and χ^2 test for normality. In cases that did not meet the assumptions of normality, a Kruskal–Wallis test was performed. A *t*-test of mean difference was performed for comparisons in chemical analyses, and a paired *t*-test was performed for comparisons between both development stages in NOEC and LC_{50} values.

The relationship between mortality, behavioral changes, growth and development averages, morphological abnormalities, MNs and other nuclear abnormalities, and frequencies of damaged cells with herbicide concentrations were evaluated with a correlation matrix using a Pearson product moment correlation coefficient and a principal component analysis using concentration as a groping variable. The significance of regressions and correlations between them was evaluated by simple linear regression and correlation analyses (Zar, 1999). The level of significance chosen was $\alpha=0.05$ for all tests, unless indicated otherwise.

3. Results

3.1. Chemicals analysis

Results of chemical analyses showed no significant changes ($P>0.05$) in the concentration of the toxicant in treatments during the 24 h interval renewals of the testing solutions (concentration range, 98 ± 5% recovery).

3.2. Lethal end points

Probit analysis of the mortality showed a concentration-dependent increase following treatment with IMZT at GS25 ($r=0.89$, $P<0.0001$) and at GS36 ($r=0.75$, $P<0.01$). The data allowed determination of the LC_{50} values of IMZT after 24, 48, 72, and 96 h of exposure. The variation of LC_{50} values along the exposure time does not present significant differences ($P>0.05$). When GS25 tadpoles were assayed, results revealed the same mean values of LC_{50} for all times, LC_{50} (24, 48, 72, 96 h)=1.48 mg/L IMZT (confidence limits, 95%, 1.08–1.62). The NOEC and LOEC reached values of 1.22 and 2.72 mg/L IMZT, respectively. In GS36, the values also remained constant at all times, 1.55 mg/L IMZT (confidence limits, 95%, 1.51–1.60). For this development stage, the NOEC and LOEC values were 1.36 and 1.49 mg/L IMZT, respectively.

LC_{50} values were not affected by either the exposure time ($P>0.05$) or the development stage of the tadpoles ($P>0.05$). Similarly, no differences were observed between LOECs values obtained for GS25 and GS36 tadpoles ($P>0.05$).

3.3. Behavior

Results revealed the induction of IS and IM in GS25 IMZT-exposed tadpoles compared to the control group for treatments lasting 24 h ($P<0.001$), but not in treatments lasting 48 h ($P>0.05$). However, when GS36 tadpoles were exposed to the herbicide, IS was observed both 24 h ($P<0.05$) and 48 h after exposure ($P<0.01$). NOEC and LOEC values obtained for GS25 after 24 h of exposure were 0.95 and 1.02 mg/L IMZT and 1.02 and 1.09 mg/L IMZT for IS and IM, respectively. When exposed GS36 tadpoles were analyzed, the NOEC and LOEC for IS reached values, regardless of the exposure time, of 1.09 and 1.17 mg/L IMZT, respectively. Overall, a regression analysis demonstrated that IS in GS25 IMZT-exposed tadpoles varied in a concentration-dependent manner only when treatments lasted 24 h ($r=0.98$, $P<0.001$). We estimated an EC_{50} (24 h) value of 1.15 mg/L IMZT (confidence limits, 95%, 1.04–1.27). On the other hand, in GS36 treated tadpoles, IS was not affected by the concentration of IMZT ($P>0.05$).

3.4. Growth and development

No alteration on growth and development were observed in those GS25 and GS36 IMZT-exposed tadpoles in relation to control specimens ($P>0.05$).

3.5. Morphological abnormalities

Among all morphological abnormalities analyzed, ANOVA demonstrated a decreased frequency of keratodonts only in GS36 IMZT-exposed tadpoles, in which the rows of keratodonts were partially lost compared to control values ($P<0.01$) (Fig. 1). For this morphological abnormality, NOEC and LOEC values were 0.00 and 0.41 mg/L IMZT, respectively. Overall, a regression analysis demonstrated that the loss of keratodonts varied as a positive function of the herbicide concentration assayed ($r=0.33$, $P<0.05$).

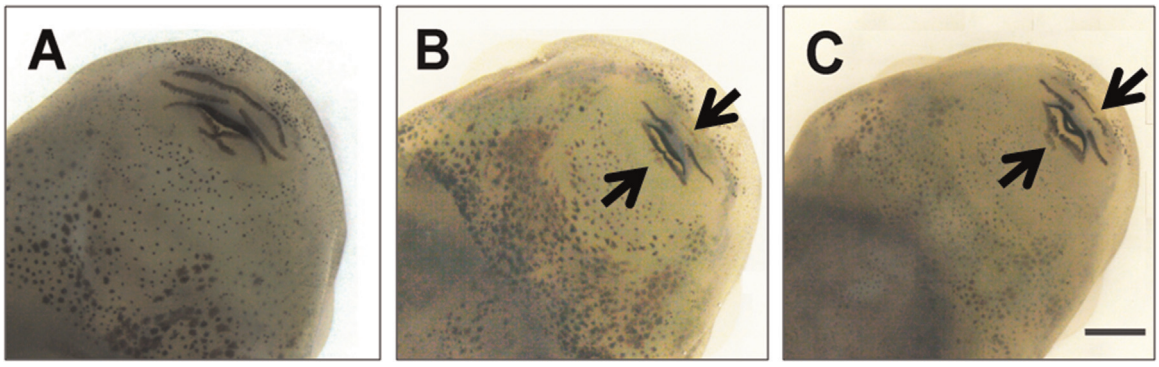

Fig. 1. Abnormalities in keratodonts observed in *Hypsiboas pulchellus* (Anura, Hylidae) tadpoles exposed for 96 h to the imazethapyr-based commercial formulation herbicide Pivot H®. (A) A negative control individual depicting normal rows of keratodonts; (B) a 0.41 mg/L imazethapyr-treated individual showing complete loss of the upper first row and the lower last row; (C) a 0.81 mg/L imazethapyr-treated individual showing partial loss of the upper first row and complete loss of the lower last row. Arrows indicate missing complete (B, C) or partial (C) rows of keratodonts. Bar represents 1 mm.

3.6. MN and other erythrocytic nuclear abnormality frequencies

Table 1 shows the results of the analysis of IMZT-induced MNs in peripheral blood erythrocytes of *H. pulchellus* tadpoles. An increased frequency of MNs was observed in CP-exposed tadpoles (positive control) at 96 h ($P<0.001$), but not in those treatments lasting 48 h ($P>0.05$). In tadpoles exposed for 48 h, a significant increase in the frequency of MNs was found in 1.17 mg/L IMZT-treated individuals compared to negative controls ($P<0.05$). On the other hand, no increased MN frequency was observed in tadpoles exposed to 0.39 and 0.78 mg/L IMZT ($P>0.05$).

On the other hand, a significant increase in the frequency of MNs was observed at 96 h of treatment for all tested concentrations of IMZT compared to negative controls ($P<0.05$). Overall, a regression analysis demonstrated that the frequency of MNs varied as an independent function of the IMZT concentration in tadpoles treated for 96 h ($r=0.06$, $P>0.05$). When the other nuclear abnormalities were analyzed, an increase in the frequency of BLs was observed after 48 h only in tadpoles exposed to 1.17 mg/L IMZT ($P<0.05$) and after 96 h in tadpoles exposed to 0.39 ($P<0.01$), 0.78 ($P<0.05$), and 1.17 mg/L IMZT ($P<0.01$). In addition, only the treatment of 0.39 mg/L IMZT for 96 h showed an increased frequency of NTs ($P<0.05$). IMZT treatments, regardless of both concentration and exposure time, did not modify the frequencies of LBs and BNs in regard to control values ($P>0.05$) (Table 1).

3.7. DNA damage

The results of the SCGE assay obtained in peripheral blood erythrocytes of *H. pulchellus* tadpoles exposed for 48 and 96 h to IMZT are presented in Table 2, and the mean frequencies of cells from each damage grade are depicted in Fig. 2. CP treatment (positive control) induced an enhancement of the GDI as well as the frequency of damaged cells compared to negative controls in specimens exposed for either 48 or 96 h ($P<0.01$) (Table 2, Fig. 2). In tadpoles exposed to IMZT, a significant increase of the GDI was observed at 48 h of treatment for all tested concentrations ($P<0.05$) (Table 2). In those tadpoles exposed for 48 h, an increased frequency of type III nucleoids within the 0.39–1.17 mg/L IMZT concentration range was observed ($P<0.05$ to $P<0.01$), and a concomitant decrease of type 0–I nucleoids ($P<0.05$) for all tested concentrations (Fig. 2A). When the analysis was performed in tadpoles exposed for 96 h, an increase in the GDI was observed for all tested concentrations ($P<0.01$) (Table 2). Furthermore, such increase was due to an increase in the frequency of type II nucleoids ($P<0.05$) in all concentrations, an increased frequency of type IV nucleoids in tadpoles exposed to 1.17 mg/L IMZT ($P<0.01$), and a concomitant decrease of type 0–I nucleoids ($P<0.01$) (Fig. 2B). Overall, a regression analysis demonstrated that the GDI varied as a dependent function of the IMZT concentration in tadpoles treated for 96 h ($r=0.35$, $P<0.05$), but not those exposed for

Table 1
Frequencies (‰) of MNs and other nuclear abnormalities in peripheral blood erythrocytes of *Hypsiboas pulchellus* (Anura, Hylidae) tadpoles exposed to the imazethapyr-based herbicide formulation Pivot H®.

Exposure time (h)	Concentration (mg/L)	No. of animals analyzed	No. of cells analyzed	MNs[a]	Other nuclear abnormalities[a]			
					NTs	LNs	BNs	BLs
48	Control	15	15,192	4.67 ± 0.84	1.24 ± 0.45	0.52 ± 0.18	1.30 ± 0.62	3.96 ± 0.69
	CP[b]	15	15,527	4.75 ± 1.02	3.08 ± 0.93	0.45 ± 0.16	0.77 ± 0.28	4.47 ± 0.80
	0.39	15	15,641	4.36 ± 0.75	3.04 ± 1.26	0.26 ± 0.14	0.57 ± 0.22	3.65 ± 0.52
	0.78	15	15,468	5.34 ± 1.20	1.97 ± 0.58	0.57 ± 0.15	1.03 ± 0.30	3.10 ± 0.83
	1.17	15	15,445	12.78 ± 3.81*	3.48 ± 0.80	0.84 ± 0.24	2.12 ± 0.47	9.30 ± 1.89*
96	Control	15	16,170	4.38 ± 0.83	1.58 ± 0.47	0.64 ± 0.26	1.28 ± 0.40	4.49 ± 0.51
	CP[b]	15	15,259	10.54 ± 1.80***	5.08 ± 2.53	0.74 ± 0.50	0.99 ± 0.28	11.45 ± 2.28**
	0.39	15	13,442	9.22 ± 1.89**	4.51 ± 0.89*	0.78 ± 0.19	1.12 ± 0.34	11.95 ± 2.08**
	0.78	15	15,944	7.02 ± 0.98*	3.27 ± 1.38	0.97 ± 0.46	1.06 ± 0.28	8.96 ± 0.62*
	1.17	15	16,371	7.97 ± 1.53*	1.36 ± 0.46	0.46 ± 0.13	1.54 ± 0.89	10.08 ± 1.77**

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

[a] MNs, micronuclei; NTs, notched nuclei; LNs, lobed nuclei; BNs, binucleated nuclei; and BLs, blebbed nuclei.

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

* $P<0.05$; significant differences with respect to control.

** $P<0.01$; significant differences with respect to control.

*** $P<0.001$; significant differences with respect to control.

Table 2
Analysis of DNA damage measured by comet assay in *Hypsiboas pulchellus* (Anura, Hylidae) tadpoles blood cells exposed to the imazethapyr-based herbicide formulation Pivot H®.

Chemicals	Concentration (mg/L)	Exposure time (h)	No. of animals analyzed	No. of cells analyzed	% of damaged cells (II+III+IV)	GDI ± SE[a]
Negative control		48	17	1801	30.72	1.04 ± 0.12
		96	14	1355	27.60	1.09 ± 0.13
Pivot H®	0.39	48	15	1589	52.75**	1.61 ± 0.11**
		96	15	1742	51.55**	1.49 ± 0.11*
	0.78	48	15	1537	47.03*	1.46 ± 0.15*
		96	15	1595	60.41***	1.61 ± 0.07**
	1.17	48	16	1582	49.18*	1.50 ± 0.15*
		96	15	1487	59.93***	1.81 ± 0.20***
CP[b]	40	48	15	1555	68.41***	2.14 ± 0.22***
		96	16	1681	59.14**	1.85 ± 0.21**

[a] GDI, genetic damage index.
[b] Cyclophosphamide (CP, 40 mg/L) was used as positive control.
* $P < 0.05$; significant differences with respect to control.
** $P < 0.01$; significant differences with respect to control.
*** $P < 0.001$; significant differences with respect to control.

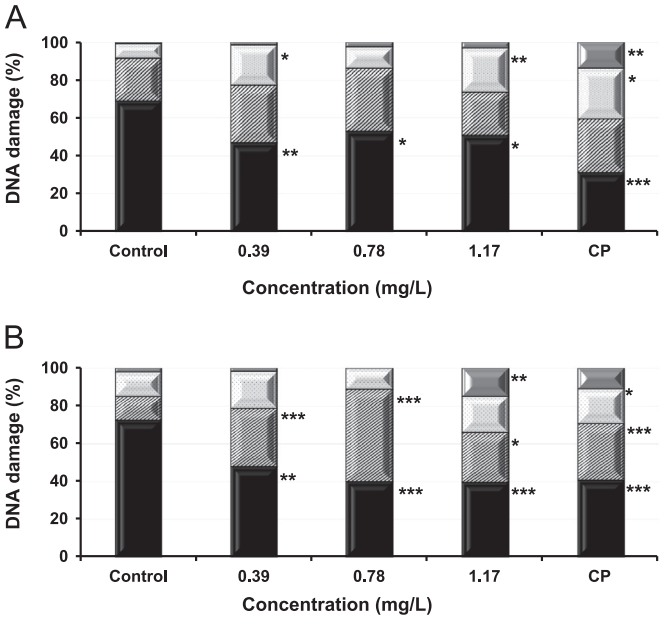

Fig. 2. Imazethapyr-based commercial formulation herbicide Pivot H®-induced DNA damage measured by comet assay in circulating blood cells from *Hypsiboas pulchellus* (Anura, Hylidae) tadpoles exposed for 48 h (A) and 96 h (B). The frequencies of undamaged (type 0–I nucleoids; black bar sections), type II (stripped bar sections), type III (dotted bar sections), and type IV nucleoids (gray bar 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) and positive controls (CP, 40 mg/L cyclophosphamide-treated tadpoles) were conducted and run simultaneously with treatments for Pivot H®-exposed tadpoles. *$P < 0.05$; **$P < 0.01$;***$P < 0.001$ (significant differences with respect to control values).

48 h ($r = 0.03$, $P > 0.05$).

3.8. Relationships between lethal and sublethal end points

Statistical analyses demonstrated no significant differences in mortality, behavioral changes, and morphological abnormalities between GS25 and GS36 tadpoles after IMZT exposure ($P > 0.05$). Similarly, no relationship between behavioral changes, morphological abnormalities, and MNs and DNA single-strand breaks induction and IMZT exposure was observed ($P > 0.05$).

4. Discussion

H. pulchellus, called the common tree frog and also the Montevideo tree frog, is an arboreal anuran species in the family Hylidae. The species was recently reported as threatened by agricultural water pollution (specifically pesticide runoff) in the central inner part of Argentina (Kwet et al., 2004). Previous studies have stressed that the tested hylid frog tadpoles can be considered suitable reference organisms in the risk assessment of 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 (Agostini et al., 2013; Lajmanovich et al., 2005), as well as the herbicide glufosinate ammonium (Peltzer et al., 2013) can be included. Recently, we used *H. pulchellus* as a laboratory target species to evaluate the lethal and sublethal toxicity of other agrochemicals, the insecticide imidacloprid (Ruiz de Arcaute et al., 2014) and the imidacloprid-based insecticide formulation Glacoxan Imida® (Pérez-Iglesias et al., 2014). Whereas LC_{50} estimation was employed as biomarker for toxicity, induction of MNs and primary DNA lesions evaluated by alkaline SCGE assay were employed as biomarkers of genotoxicity.

Regarding the acute lethal effects, the studied IMZT-based herbicide formulation Pivot H® could be ranked, according to the scoring proposed by the Office of Pollution Prevention of the U.S. EPA (Smrchek et al., 1993; Wagner et al., 1995), as a compound of moderate ecotoxicological concern for *H. pulchellus* tadpoles, regardless of the premetamorphic development stage of the larvae. It can be classified as a toxic compound for aquatic organisms (Category II) following either the classification criteria proposed by the UN (2011) or the European Union directives (Mazzatorta et al., 2002). In addition, the acute lethality data of LC_{50} values reported here for the herbicide contribute new information for this type of effect not only for *H. pulchellus* in particular, but for amphibians in general. Behavioral changes, i.e., IS and IM, with a concentration of 1.02 mg/L as well as loss of keratodonts after 48 h of exposure to a concentration of 0.41 mg/L were noticed. The herbicide increased the frequency of MNs in peripheral erythrocytes of tadpoles exposed for 48 h to the highest concentration assayed (1.17 mg/L). However, regardless of the concentration assayed, enhanced frequency of MNs was observed in tadpoles exposed for 96 h. Furthermore, the herbicide was able to induce other nuclear abnormalities, i.e., BL and NT nuclei, in tadpoles only when exposed for 96 h. In addition, we observed that acute exposure to all IMZT concentrations within the 0.39–1.17 mg/L range increased the frequency of primary DNA lesions estimated by the alkaline SCGE assay in larvae exposed both for 48 and 96 h. Additionally, our data

showed that the SCGE assay was more sensitive than the MN test in detecting early DNA damage when the same herbicide concentrations were employed for tadpole exposure.

The variability in pesticide-induced toxicity in different amphibian species is a phenomenon known worldwide (Brühl et al., 2013; Relyea, 2009). So far, acute lethality data of IMZT have not been reported previously for any amphibian species. To the best of our knowledge, among aquatic vertebrates, IMZT acute lethality data have been reported only for three fish species, *Ictalurus punctatus* (channel catfish), *Lepomis macrochirus* (bluegill), and *Oncorhynchus mykiss* (rainbow trout). However, acute lethality studies have also been reported for *L. macrochirus*, *O. mykiss*, and *Cyprinodon variegatus* (sheepshead minnow) exposed to IMZT in combination with urea (USEPA, 2013). Our results reveal a concentration of 1.55 mg/L IMZT (confidence limits, 95%, 1.51–1.60) as the LC_{50} (24–96 h) value for GS36 *H. pulchellus* tadpoles. Previously reported maximum LC_{50} (96 h) values for exposed GS37 tadpoles of the species include 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). Recently, we determined 84.91 and 52.62 mg/L as the LC_{50} (96 h) values for the insecticide imidacloprid and the imidacloprid-based formulation Glacoxan Imida®, respectively (Pérez-Iglesias et al., 2014; Ruiz de Arcaute et al., 2014). Furthermore, an LC_{50} (48 h) value of 21.47 mg/L of the herbicide glufosinate ammonium has been reported (Peltzer et al., 2013). Accordingly, it seems evident this anuran species is nearly 11,923, 8.9, and 3.3 times less sensitive to IMZT than to endosulfan, Cypermethrin Sherpa®, and cypermethrin, respectively. On the other hand, *H. pulchellus* appears nearly 0.07, 0.05, 0.03, and 0.02 times more sensitive to IMZT than to glufosinate ammonium, Cr(VI), Glacoxan Imida®, and imidacloprid, respectively. Then, among herbicides, IMZT is the most toxic herbicide reported so far for *H. pulchellus* tadpoles. Thus, it could be valid to suggest that the species seems to be more sensitive to pesticides belonging to the organochlorine and pyrethroid chemical groups than to imidazolinone pesticides, at least to IMZT.

In our study, an IMZT-based herbicide containing only 10.59% of the active ingredient within the formulation, Pivot H®, was assayed. Years ago, the USEPA (1982) claimed that the acute toxicity of a technical active ingredient can differ significantly from that of the end-use formulation containing that active ingredient. Several investigations have demonstrated that the additive compounds present in pesticide commercial formulations have the ability to induce toxicity and cellular damage by themselves, separate from the active ingredient (Belden et al., 2010; Brühl et al., 2013; Grisolia et al., 2004; Mann and Bidwell, 1999; Nikoloff et al., 2014a; Pérez-Iglesias et al., 2014; Ruiz de Arcaute et al., 2014; Soloneski et al., 2007; Soloneski and Larramendy, 2010). Unfortunately, the identities of the additive compounds present in the commercial formulation Pivot H® were not made available to us by the manufacturers. It should be mentioned that according to our Argentinean administration, the excipients present in any agrochemical are not required to be listed on the agrochemical data sheet and can be kept as a "trade secret". Further studies are required to reveal whether the high toxicity exerted by Pivot H® on the *H. pulchellus* tadpoles we observed is attributable to IMZT or results from the presence of xenobiotic(s) within the formulated technical formulation assayed in our study.

Genotoxic studies of IMZT are scarce and contradictory. Whereas IMZT did not induce chromosomal aberrations in rat bone marrow cells, both negative and positive results have been reported for CHO cells with and without metabolic activation, respectively (USEPA, 1989). To the best of our knowledge, the current results we obtained with *H. pulchellus* tadpoles represent, then, the first evidence of the ability of the herbicide to induce acute genotoxic damage, i.e., induction of MNs and primary DNA lesions, on circulating blood cells from vertebrate specimens exposed in vivo under laboratory conditions.

The MN analysis is used worldwide as a bioassay of genotoxicity to detect small chromosomal fragments induced by clastogens or vagrant chromosomes produced by aneugens (OECD, 2007). The induction of MNs in circulating erythrocytes from different amphibian tadpole species as a consequence of pesticide treatments is well documented (Bouhafs et al., 2009; Nikoloff et al., 2014b; Vera Candioti et al., 2010). Among them, *H. pulchellus* has been used previously as an experimental model in genotoxicity studies. Lajmanovich et al. (2005) reported the induction of MNs after exposure to the insecticide endosulfan. Similarly, we recently demonstrated MN induction in circulating erythrocytes from tadpoles exposed to both the insecticide imidacloprid (Ruiz de Arcaute et al., 2014) and the imidacloprid-based formulation Glacoxan Imida® (Pérez-Iglesias et al., 2014). In the current study, the results demonstrated that IMZT increased MN frequency in erythrocytes after 48 h of exposure to a concentration of 1.17 mg/L, the highest concentration assayed, whereas with a concentration range of 0.39–1.17 mg/L it was able to exert to a lesser extent the same genotoxic effect at 96 h of treatment. Accordingly, the results could indicate that after 48 h of exposure, IMZT concentrations lower than 1.17 mg/L are not able to inflict genotoxic damage leading to MN induction. However, a longer exposure time is required to reveal genotoxic damage leading to MN formation in IMZT-exposed tadpoles regardless of the concentration and to allow circulating erythrocytes to reach the first mitosis nearly after 96. The results also highlight that the frequency of MNs in those tadpoles exposed for 48 h to a concentration of 1.17 mg/L IMZT is higher than that found in those exposed for 96 h. So far, we do not have any explanation for this particular observation. A plausible possibility could be related to IMZT-induced erythrocyte cytotoxicity by the induction of a selective cell loss by herbicide-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 the genotoxic capability of the test compound under study on in vivo herbicide-exposed amphibian cells. Finally, nuclear abnormalities other than MNs were also induced after IMZT exposure in those tadpoles exposed within the 0.39–1.17 mg/L concentration range. Previous reports have demonstrated that these morphological nuclear abnormalities, e.g., binucleated erythrocytes as well as notched nuclei, can be considered genetic damage indicators (Cavaş and Ergene-Gözükara, 2005; Ruiz de Arcaute et al., 2014). Our results agree well with these observations.

The SCGE test has become extensively valuable as a biomarker in amphibians to monitor contaminated areas (in situ assay) (Burlibasa and Gavrila, 2011; Maselli et al., 2010), as well as for the screening of xenobiotics after direct or indirect exposure (in vivo assay) (Mouchet et al., 2007; Nikoloff et al., 2014b; Pérez-Iglesias et al., 2014; Ruiz de Arcaute et al., 2014). We observed that, regardless of the length of treatment, IMZT acute exposure within the 0.39–1.17 mg/L concentration range increased the frequency of primary DNA lesions estimated by alkaline SCGE, a result opposite that of the MN test. One possible explanation for this observation could be related to the different cellular statuses of the target cells that are included for analysis for each end point. Whereas DNA damage is estimated by SCGE in a heterogeneous circulating cell population, MNs are determined on proliferating cells with lesions that have lasted for at least one mitotic cell cycle and that probably retain their repair properties, as suggested elsewhere (He et al., 2000). Our current results represent the first in vivo evidence of the induction of primary DNA damage exerted by IMZT using the

SCGE assay on Neotropical amphibian tadpoles. 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.

We found IMZT concentrations of 1.48 mg/L (range, 1.08–1.62) and 1.55 mg/L (range, 1.51–1.60) as the LC_{50} (24–96 h) values for GS25 and GS36 *H. pulchellus* tadpoles, respectively. Although the in vivo IMZT treatments in this study do not covered a wide range of concentrations, the concentration range represents a relatively high end of the threshold value of 14 μg IMZT/L found in the surface water of the Azul river basin (Buenos Aires, Argentina) reported by Peluso et al. (2008), even considering the recommended application field ratios of 100–150 g a.i/ha reported for Argentina (Bindraban et al., 2009; CASAFE, 2011). It should be mentioned that the IMZT concentrations found in Argentinean crop production areas is nearly 7.6 times higher than the higher concentration reported for surface water in USA (Mattice et al., 2012). Thus, the concentrations of IMZT employed in this investigation would be expected to be almost improbable in the environment, perhaps only observed when specific events occurred (e.g., direct application, drainage ditches or by accidental discharge). Although, we cannot rule out that amphibian populations and also occupationally exposed human workers could be exposed accidentally to these agrochemicals to this range of concentrations.

Amphibians represent important vertebrates in aquatic and agricultural ecosystems since they are included among the most important natural enemies of several agricultural pests worldwide. 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 changes in their habitats, but also to their larvae living in aquatic or wetland environments (Brodeur et al., 2012; Pollet and Bendell-Young, 2000; Schuytema and Nebeker, 1999). In recent decades, amphibian populations have been reported to suffer significant decline worldwide, a phenomenon in most cases attributed to the pollution of agricultural areas exerted by emerging pollutants, including agrochemicals (Mann et al., 2009; Relyea, 2009). The effects of environmental pollutants on the aquatic communities are a matter of increasing concern due to their potential influence on the prey–predator relationships and the consequences for community structure (Hanlon and Relyea, 2013; Relyea, 2009). It is well known that pesticide effects on predator–prey interactions are often complex and have the potential to alter aquatic community composition (Hanlon and Relyea, 2013). It has been claimed that pesticides may have positive, negative, or no impact on the outcome of predator–prey interactions, depending upon the sensitivity of the species to contaminant exposure (Boone and Semlitsch, 2003; Bridges, 1999; Groner et al., 2013; Junges et al., 2010). We observed that sublethal levels of IMZT induced ethological disturbances, i.e., IS and IM, as well as morphological alterations, i.e., decreased frequency of keratodonts, in exposed tadpoles. Then, it could be assumed that these tadpoles could be less active and with a most probably reduced fitness of nutrition, leading to higher mortality during the larval period, and therefore less conspicuous to predators. Accordingly, a negative impact on the predator–prey interaction could be suggested between IMZT exposure and the outcome on *H. pulchellus* tadpoles. On the other hand, the possibility that the morphological and ethological changes we observed after IMZT exposure could render an increase in predation rate, and therefore indicate a plausible positive impact on the predator–prey interaction, cannot be ruled out. A similar positive impact has been reported previously for *H. pulchellus* tadpoles exposed to the organophosphate insecticide fenitrothion (Junges et al., 2010). Further ecotoxicological studies should be designed and conducted employing *H. pulchellus* as target species to investigate the nature of the mechanisms responsible for the adverse or negative effects of the herbicide IMZT on the predator–prey interaction.

Although environmental pollution might interfere with normal amphibian growth, development, and susceptibility to disease, the induction of genetic injury into DNA after chronic 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 reported (Beebee, 2005; Jones et al., 2009). Increase in genomic instability has been suggested to play important role in decreased fitness of exposed vertebrate aquatic populations not only in laboratory studies but also in field conditions (Clarke, 1993; Hose et al., 1987; Jha, 2008; Parsons, 1992). It has been suggested that particularly, primary DNA damage, MNs as well as alteration in erythrocyte nuclear morphology can lead to cell death, and thus conducting to several pathophysiological conditions which have been reported to be increased upon several stress situations, e.g., diet alterations, pathology, metabolic damage, among others (Barni et al., 2007; Cavaş and Ergene-Gözükara, 2003; Fijan, 2002; Jha, 2008; Lajmanovich et al., 2014). On the other hand, whether other damaged cells rather than circulating red blood can survive, another scenario can be achieved. Clearly, the induction of DNA strands breaks or presence of MNs in both somatic and germ cells of natural biota are of paramount importance. If unrepaired or misrepaired, the damage will have effects on the immediate fitness as well as on reproductive success of the exposed organisms. This will ultimately lead to adverse effects on long-term population survival and, hence, deterioration of the ecosystem quality (Barni et al., 2007; Jha, 2008). In this sense, both cytogenetic and hematological indicators in polluted samples were found to be decidedly different from reference conditions and they are diagnostic indicators of environmental stress in amphibians highlighting that cellular alterations of amphibians represent a significant fragility in these wetland ecosystems (Barni et al., 2007; Josende et al., 2015).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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