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Toxicity of *Bacillus thuringiensis var. israelensis* in aqueous suspension on the South American common frog *Leptodactylus latrans* (Anura: Leptodactylidae) tadpoles ☆

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ABSTRACT

The effects of commercial formulations of *Bacillus thuringiensis var. israelensis* (*Bti*) on non-target organisms are still a matter of debate; in amphibians, the risks of *Bti* are little known. To evaluate the toxicity of a commercial liquid (aqueous suspension, AS) formulation of *Bti* (Introban®) on *Leptodactylus latrans* tadpoles, including median lethal concentration (LC_{50}) and no-and lowest–observed-effect concentrations (NOEC and LOEC, respectively), as well as the possible effects of *Bti* on oxidative responses, erythrocytes genotoxicity, and histology of the intestines. In the laboratory, tadpoles were exposed to nominal concentrations of 0 (control), 2.5, 5, 10, 20 and 40 mg/L of formulated *Bti*-AS. Glutathione S-transferase (GST) and catalase (CAT) activities, as well as formation of erythrocyte nuclear abnormalities (ENAs), and histological effect were measured in tadpoles displaying survival rates $>85\%$. *L. latrans* tadpoles were sensitive to exposure to *Bti*-AS, reaching 100% mortality after 48 h of exposure at the highest concentration. *Bti*-AS induced GST and CAT enzymes and genotoxicity (erythrocyte's nuclear abnormalities), and caused intestine's histopathology. Our results demonstrate that toxicity of *Bti*-AS is dose-dependent for *L. latrans* tadpoles and that sublethal exposure alters enzymes of oxidative stress, induces genotoxicity, and causes intestine damage. Further research is needed to evaluate the ecotoxicological risk of the massive use of *Bti* formulations on amphibian populations that commonly used suburban wastewater or urban waterbodies to reproduce and where this biopesticide is frequently applied.

1. Introduction

The worldwide use of pesticides amounts to about two million tons per year (Arnab et al., 2014). Half of the pesticide trade involves biopesticides, with more than 60% of this trade being related to commercial *Bacillus thuringiensis* (*Bt*) products (Marzban, 2012). *Bt* is a rod-shaped Gram-positive entomopathogenic bacterium abundant in soil and plants. During the sporulation phase (resting stages), it produces intracellular crystal proteins (Cry proteins), which are effective only when ingested by susceptible insects (Soberón et al., 2009). These crystals are mostly composed of one or more proteins (Cry and cytolytic toxins), also called δ-endotoxins. These toxins are responsible for the selective *Bt* insecticide activity, since they activate upon ingestion, and then bind to specific receptors in the midgut cells of insects, leading to the loss of homeostasis and septicemia, and, ultimately, producing the death (Bravo et al., 2007, 2011).

In the past years, *Bt* genes were used for production of insect-resistant genetically modified (GM) crops (Key et al., 2008). For instance, *Bt* maize with insecticidal properties is one of the most widely planted GM crops in the world (James, 2011). Although *Bt* is a viable alternative for the control of herbivores in agriculture (Pardo-López et al., 2013), it is also very important in the control vectors of human diseases (Fernandez–Luna et al., 2010). *Bt* var. *israelensis* (*Bti*)-based products are used worldwide mostly due to

☆Animals used in this research have been treated according to ASIH (2004) criteria and with approval from the animal ethics committee of the Faculty of Biochemistry and Biological Sciences. http://www.fbcb.unl.edu.ar/pages/investigacion/comite-de-etica.php.

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their efficacy to control mosquitoes, and it is one of the most recommended methods to control the dengue vector (*Stegomyia aegypti*) [World Health Organization (WHO), 2009], with a global use of 70–300 metric tons of formulated product per year (van den Berg et al., 2012).

Favorable conditions of temporary ponds provide breeding sites for many aquatic organisms, such as amphibians and mosquitoes (Blaustein and Margalit, 1996). Because tadpoles and mosquito larvae coexist naturally in these ponds and compete for resources (Mokany and Shine, 2003) tadpoles might be exposed to mosquitoes larvicides. According to many investigations conducted in several parts of the world, *Bti* is harmless for non-target organisms due to its specific and target-oriented mode of action [Environmental Protection Agency (EPA), 2008]. The effects of *Bti* on amphibians have received little attention, but it has been suggested that *Bti* endotoxins are not a direct toxic concern for amphibians (Mathavan and Velpandi, 1984; Paulov, 1985). Accordingly, Dos Santos et al. (2013) discuss that the tests used to justify approval for use and release of *Bt* products into the market, as well as the protocols used to assess its toxicity, are extremely superficial.

Amphibians are important indicators of ecosystem health (Waddle, 2006). In this context, ecotoxicological studies on amphibian recognized enzyme and blood biomarkers as important early signals to detect adverse pesticide effects (Attademo et al., 2014; Ossana et al., 2013). In these sense, two antioxidant enzymes, glutathione S-transferase (GST; EC 2.5.1.18) and catalase (CAT; EC 181.11.1.6), are essential to prevent oxidative stress damage. In addition, the formation of erythrocyte nuclear abnormalities (ENAs) such as the presence of micronuclei in erythrocytes, reveal a highly significant genotoxic effect by xenobiotics (Lajmanovich et al., 2013, 2014; Rocha et al., 2012). Thus, ENA scoring is useful to reveal the presence of clastogenic and aneugenic compounds in the aquatic environment (Guilherme et al., 2008; Hoshina et al., 2008). Amphibian tadpoles, similarly to mosquito larvae, are primarily herbivorous (Altig et al., 2007; Bern and Dahl, 2000). The stomach of tadpoles is poorly differentiated, has neutral pH, and serves primarily as a storage site for food, with limited digestive activity (McDiarmid and Altig, 1999), whereas the intestine, as in mosquito's midgut, lacks acid pH (Reeder, 1964). In a comparative way, it is known that in mosquitoes the Cry-proteins bind to specific sites (i.e., receptors) in the midgut cells, whereas in amphibian tadpoles, these receptors remain unknown. Moreover, according to oral mammalian toxicology and in vitro digestibility studies, δ-endotoxins have not shown toxicity to this vertebrate and they are rapidly degraded in simulated gastric fluid (EPA, 1998). However, Fares and El-Sayed (1998) revealed changes in the ileum of a group of mice fed on transgenic potatoes, which carry the CryI gene of *Bt* var. *kurstaki* (strain HD1).

Considering the *Bt* insecticides application on suburban and urban areas to control mosquitoes as principal vector of human disease, highlight the need of ecotoxicological risks assessment on non-target organisms. The present study evaluates the risk of *Bti* commercial formulation on *Leptodactylus latrans* (South American common frog) tadpoles; based on exposition and effects evidences (ecotoxicity assays-acute toxicity; oxidative responses-GST and CAT activities; erythrocyte genotoxicity-ENAs, and histology of intestines).

2. Materials and methods

2.1. Experimental design

Tadpoles of the South American common frog *L. latrans* (Anura: Leptodactylidae) were selected as model test organisms. This common anuran has an extensive neotropical distribution [International Union for Conservation of Nature (IUCN), 2013] and is frequently found resting on the margin of ponds, rivers or small lakes in forests, wetlands, agricultural lands and urban and suburban waterbodies (Peltzer et al., 2006). Larvae are gregarious; they occur in high densities and have an herbivorous diet mainly consisting of diatoms and green algae, detritus and plant residues (Lajmanovich, 1994). Premetamorphic larvae were collected with dip net from temporary ponds in natural floodplains (31°40′29″S-60°20′13″W, Natural Reserve Parque San Martín, La Picada, Entre Ríos province, Argentina) in December 2013; these sites had not been treated with chemical or biological pesticides, as determined by the laws for wildlife protection. Average tadpole size (snout-tail tip) was 18 ± 0.5 mm and weight was 0.60 ± 0.05 g; Gosner stages: 26–30 (Gosner, 1960). Tadpoles were acclimated on glass recipients that contained dechlorinated tap water (DTW; pH 7.40 ± 0.05, conductivity: 165 ± 10.5 μmhos/cm, dissolved oxygen concentration: 6.0 ± 1.5 mg/L and hardness: 54.8 mg/L of $CaCO_3$), under lab conditions (12/12-h light/dark cycle and at 22 ± 2 °C) for 48 h. All tadpoles were fed on boiled lettuce ad libitum throughout the experiments.

Short-term static toxicity tests (48 h) were conducted using Introban®, a commercial liquid (aqueous suspension, AS) formulation of *Bti* produced by Valet BioSciences Corporation (USA) and imported by Chemotecnica S.A. (Argentina). The biopotency of this larvicide is 1200 International Toxic Units (ITU)/mg product plus inert ingredients (q.s. to 100 mL). The safety data sheet provided by the manufacturer describes the physical and chemical characteristics of the product (appearance: light brown suspension, odor: slightly acid, pH: 4.2–4.5, boiling point: 100 °C, and viscosity < 1200 cps). Inert compounds are not declared. In this study, the biological insecticide was tested as a complex commercial mixture (*Bti*-AS) because this is the mode of application and released to the environment. Moreover, some studies demonstrated that other inert ingredients contained in pesticide formulations may contribute to amphibian pesticide toxicity (e.g., Lajmanovich et al., 2014; Relyea and Jones, 2009).

Glass aquariums (12.5 cm in diameter and 13.5 cm in height) containing 1 L of test solution were used in the acute experiments. Laboratory toxicity tests were conducted at 22 ± 2 °C with at 12/12-h light/dark cycle. Due to the lack of information in the literature about the effects of *Bti* exposure on native amphibians, the first step was to elucidate the direct toxicity of the biological insecticide on *L. latrans* tadpoles. Range-finding toxicity tests consisted of exposing larvae to *Bti*-AS to estimate the median lethal concentration (LC_{50}), the lowest-observed-effect concentration (LOEC), and the no observed-effect concentration (NOEC). The nominal concentrations used to test toxicity were: 2.5, 5, 10, 20, and 40 mg *Bti*-AS/L, plus a negative control with DTW. Treatments were randomly assigned to the recipients and to the sampling order. Both control and test suspensions were carried out in triplicate with seven tadpoles per aquarium ($n = 126$). A relationship between those nominal concentration of *Bti*-AS and spore plus crystal concentration was also determined. Then, samples (0.5 mL) were taken from each concentration as well as the negative control employed in the toxicity test. Spore plus crystal concentration was determined using a hemocytometer (Neubauer chamber). The relative concentration was determined by submitting the sample to a simple staining method (Crystal Violet, 1 min) using an immersion objective. Spores and crystals were counted using CellNote software (http://cellnote.up.pt/).

Larval mortality was recorded every 24 h, and the cumulative mortality in each treatment was calculated at 48 h of exposure. Dead animals were removed at each observation. To test the effects of *Bti*-AS on oxidative response, genotoxicity, and histological changes to intestines, control and treated animals at all

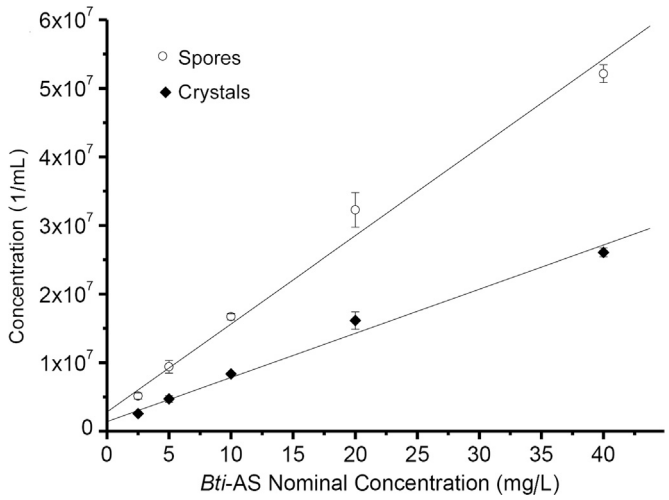

Fig. 1. Relationship between spore and crystal concentration and nominal concentration of *Bti*-AS. Error bars represent SE ($n=2$).

concentrations ($n=10$, respectively) that had a survival rate $>85\%$ at 48 h were euthanized by decapitation, according to ASIH (2004) criteria and with approval from the animal ethics committee of the Faculty of Biochemistry and Biological Sciences.

Fig. 1 shows the relationship between *Bti*-AS/L suspensions as calculated from dilutions obtained from the nominal concentration of Introban®, and spore plus crystal concentration determined microscopically. Spore concentration was twice as high as crystals. On the other hand, a linear relationship was observed between concentrations ($R^2=0{,}990$; $n=5$). According to this result, 1 mg/L of *Bti*-AS corresponded to 4.1×10^6 and 2.1×10^6 spores and crystals per mL of suspension, respectively.

2.2. Oxidative response

Tadpoles (without intestines) were homogenized (on ice) in 20% (w/v) buffer containing 0.1% t-octylphenoxypolyethoxy ethanol (Triton X-100) in 25 mM Tris (hydroxymethyl) aminomethane hydrochloride (pH 8.0) using a homogenizer. The homogenates were centrifuged at 10,000 rpm at 4 °C for 15 min, and supernatants were collected. Total protein concentrations in the supernatants were determined according to the Biuret method (Kingsley, 1942). Enzyme kinetics assays were performed in triplicate. GST activity was determined spectrophotometrically using the method described by Habig et al. (1974) and adapted by Habdous et al. (2002) for mammal serum GST activity. The enzyme assay was performed at 340 nm in 100 mM Na phosphate buffer (pH 6.5), 2 mM CDNB, and 5 mM GSH. Enzyme kinetic assays were performed at 25 °C, and whole GST activity was expressed as μmol min^{-1} mg^{-1} of protein using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. CAT activity was measured using the method described by Aebi (1984), and was expressed as μmol H_2O_2 min^{-1} mg^{-1} of protein using a molar extinction coefficient of H_2O_2 40×10^{-3} L/mol cm. The reaction medium was 50 mM phosphate buffer (pH=7.2) and 30 mM H_2O_2 and absorbance was read at the spectrophotometer at a wavelength of 240 nm at 25 °C (quartz cuvette). Data of enzymatic activity was expressed as the mean ± SE.

2.3. Genotoxicity

One smear was prepared on clean slides with blood samples obtained, then fixed and stained using the May-Grünwald-Giemsa method (Dacie and Lewis, 1991; Lajmanovich et al., 2014). Genotoxicity was tested via the presence of erythrocyte nuclear abnormalities (ENAs) in mature peripheral erythrocytes, according to the procedures of Guilherme et al. (2008). The frequency of the following nuclear abnormalities: micronuclei (MN), nuclear bud (NB), pycnotic nuclei (PN), kidney shaped nuclei (K), and lobed nuclei (L) were calculated. Coded and randomized slides were scored blind by a single observer.

2.4. Histological studies

For light microscopy examination, coil intestine of tadpoles were fixed in Bouin's fixative for 24 h, dehydrated in ethanol, cleared in xylol, and embedded in paraffin. Fixed tissues were then serially sectioned (5 μm) and stained with hemotoxylin-eosin. Sections of all intestines were entirely examined under light microscope and photographed.

2.5. Data analyses

LC_{50} values and their respective 95% confidence intervals were estimated using the Trimmed Spearman-Karber method (Hamilton et al., 1977). Mortality data were statistically evaluated using the Dunnett's test for post-hoc comparison of means to determine NOEC and LOEC (U.S. EPA, 1989). The influence of *Bti*-AS concentrations on the activity of both enzymes was analyzed using general linear models (GLMs) followed by Dunnett post-hoc comparisons. Data were tested for variance homogeneity and normality (Kolmogorov–Smirnov test and Levene test). Data of ENAs were analyzed using binomial proportion test (Margolin et al., 1983). All statistical analyses were performed using SPSS 10.0 (SPSS, Chicago, IL) and BioStat software 5.0 (Ayres et al., 2008). A significance level of 0.05 was used in all analyses and descriptive statistics were expressed as mean ± SE.

3. Results

3.1. Acute toxicity tests

The calculated 48 h acute LC_{50} value (95% confidence limits) of *Bti*-AS to *L. latrans* tadpoles was 22.45 mg/L (19.59, 25.73). No mortality was observed in the control treatment. NOEC value was 2.5 mg *Bti*-AS/L and LOEC was 5 mg *Bti*-AS/L. The highest concentration (40 mg *Bti*-AS/L) killed all tadpoles, whereas mortality

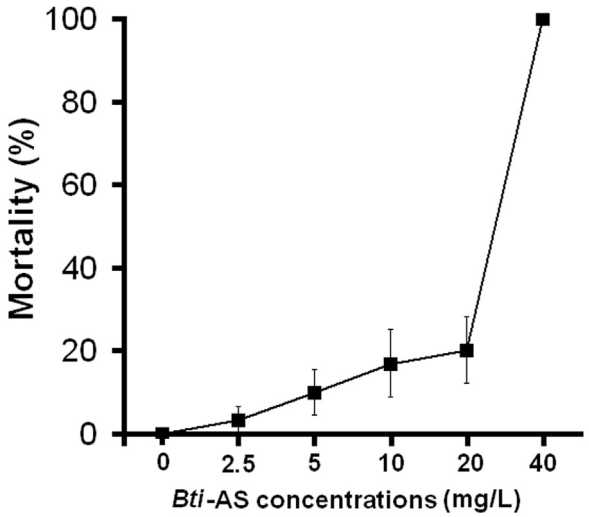

Fig. 2. Mortalities of *L. latrans* tadpoles exposed to different *Bti*-AS concentrations in 48 h. Data are expressed as mean ± SE ($n=126$).

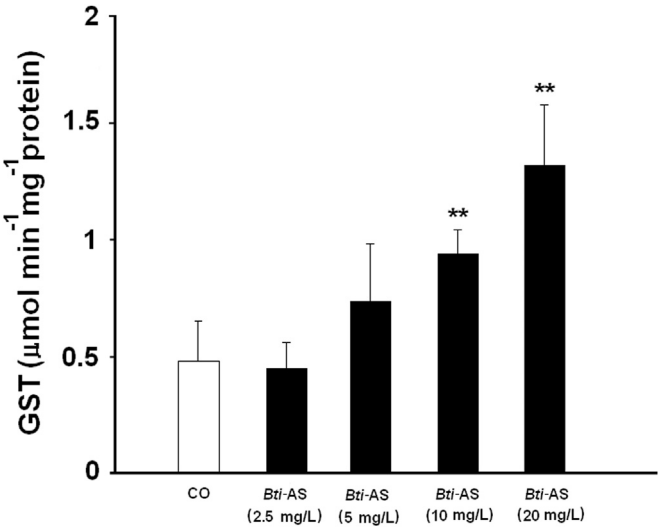

Fig. 3. Glutathione S-transferase (GST) activity in *L. latrans* tadpoles exposed to *Bti*-AS for 48 h. Data are expressed as mean ± SE, $n=10$. Significant differences were $^{*}p<0.05$ and $^{**}p<0.01$ with respect to the control (Dunnett's post-hoc test).

at the lowest concentration (2.5 mg *Bti*-AS/L) was on average 3.5% (Fig. 2).

3.2. Effect of Bti-AS on enzymatic activity

The mean value of the GST activity in control tadpoles was 0.49 ± 0.03 µmol min^{-1} mg^{-1} protein at 48 h. The commercial formulation assayed affected GST activities significantly (GLM $F=17.71$; df=4; $p<0.01$, $r^2=0.65$), differing significantly at 10 and 20 mg *Bti*-AS/L from the control GST activity (Dunnett's post-hoc test $p<0.01$, Fig. 3). Control CAT activity was 18.66 ± 0.92 µmol $min^{-1}mg^{-1}$ protein at 48 h. Likewise, CAT activity was affected significantly by *Bti*-AS (GLM $F=12.84$; df=4; $p<0.01$, $r^2=0.55$). CAT activity differed significantly from the control only at 20 mg *Bti*-AS/L (Dunnett's post hoc test $p<0.01$, Fig. 4).

3.3. Effect of Bti-AS on MN and ENAs

The mature erythrocytes of *L. latrans* tadpoles were oblong-oval shaped, with a centric nucleus. The nucleus was clearly structured and had a well-defined boundary, which facilitated the recognition of fragments in their cytoplasm. MN observed were spherical nuclear fragments separated from the parent nucleus. Single MN was dominant in the erythrocytes observed. However, some erythrocytes presented other clear morphological alterations, such as nuclear bud, pycnotic nuclei, kidney-shaped nuclei, and lobed nuclei, induced by exposure to *Bti*-AS commercial formulation.

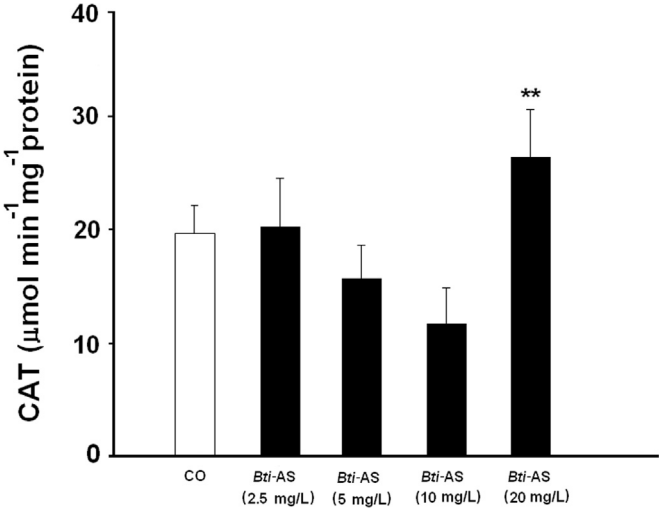

Fig. 4. Catalase (CAT) activity in *L. latrans* tadpoles exposed to *Bti*-AS for 48 h. Data are expressed as mean ± SE, $n=10$. Significant differences were $^{*}p<0.05$ and $^{**}p<0.01$ with respect to the control (Dunnett's post-hoc test).

Table 1
Frequencies (‰) of micronuclei (MN) and other erythrocyte nuclear abnormalities (ENAs) in peripheral blood erythrocytes of *L. latrans* tadpoles exposed to different *Bti*-AS concentrations.

Concentration (mg/L)	MN	ENAs			
		NB	PN	K	L
CO	0.82	1.43	0.11	2.7	0.94
2.5	2.21*	2.48*	4.89**	2.79	0.93
5	1.2	0.44	2.95*	4.7*	1.11
10	2.74*	1.47	1.46	5.4*	2.63*
20	0.42	0.06	0.19	0.21	0.00

CO: negative control.
NB: nuclear bud.
PN: pycnotic nuclei.
K: kidney shaped nuclei.
L: lobed nuclei.
$^{*}p<0.05$.
$^{**}p<0.01$; significant differences with respect to control values (Binomial Proportion's Test).

After 48-h exposure, all tested concentrations of *Bti*-AS showed a significant increase in MN and ENAs frequency in relation to the negative control group; however, the frequency was lower at 20 mg *Bti*-AS/L (Table 1).

3.4. Histology effect on intestine

No anomalies were observed in the intestine of tadpoles in the control group. For this group, light micrographs revealed that the intestine at premetamorphic stages is a long, simple tube with a single layer of cuboidal epithelial cells, the primary epithelia are surrounded by thin layers of muscles with little intervening connective tissue, and the submucosa is further enclosed by a thin serosa (Fig. 5A). By contrast, intestines of the treated tadpoles with *Bti*-AS showed inflammatory infiltration in the connective tissue underlying the epithelium and dilation of blood vessels (Fig. 5B).

4. Discussion

The culture rate obtained (spores–crystals, 2:1) could be associated with culture condition during fermentation process. Accordingly, the association between medium formulation and these rates is common and well documented in *Bt* culture (e.g. Kim et al., 2013; Vu et al., 2012).

Acute toxicity tests are a simple approach to assess and compare the toxicity of a compound in terms of its effect on a given species, and give the first lines of exposure evidences for ecotoxicological risk assessment. The 48 h-LC_{50} value of *Bti*-AS obtained in this study for *L. latrans* tadpoles (22.45 mg *Bti*-AS/L) is considerably lower than values published for other non-target organisms, for example for fish *Fundulus heteroclitus* with a 96 h LC_{50} of 980 mg/L (Lee and Scott, 1989). In our study, the mortality percentage shows a slow rise up to 20 mg/L and then a major spike in lethality to 40 mg/L. While this may be a signal a tipping point in the tadpoles's metabolism it could also may indicate a methodological artifact. At this point it is important to note that literature falls into controversies about the *Bti* risk on non-target

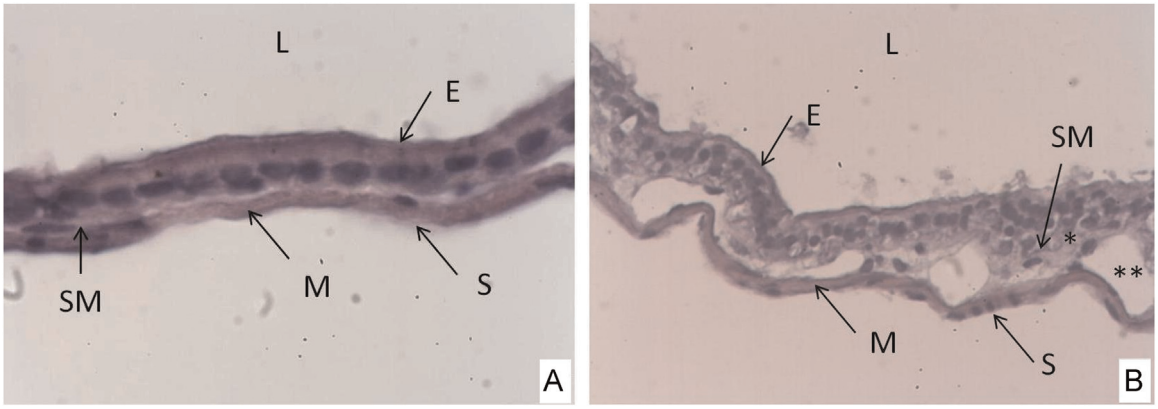

Fig. 5. Light micrographs of intestinal walls of *L. latrans* tadpoles at premetamorphic stages. (A) Control; (B) 48- h exposure to *Bti*-AS (2.5 mg/L). L: lumen; E: layer of cuboidal epithelial cells; M: muscle layer; SM: submucosa; S: serosa. (*) Infiltrate in the connective tissue underlying the epithelium. (**) Dilation of blood vessels. Hemotoxylin-Eosin, 100 ×.

aquatic organisms. Karmrin (1997) found that *Bti* is practically non-toxic to rainbow trout (*Oncorhynchus mykiss*) and bluegills (*Lepomis macrochirus*) exposed for 96 h at concentrations of 560 and 1000 mg/L. According to Becker and Margolit (1993), freshwater cnidaria of the genus *Hydra* were not affected by *Bti* in laboratory tests at a concentration of 100 mg/L, and the oligochaetes of the genus *Tubifex* were also not affected by *Bti* at 180 mg/L. Regarding amphibians, a revision (WHO, 1999) of several laboratory and field studies that examined the impact of *Bti* on frogs (*Pseudacris regilla* and *Rana temporaria*) and toads (*Bufo* species) found no records of adverse effects; however, the tested concentrations were not indicated. Laboratory toxicity tests reported 0% mortality at 1 mg/L of *Bt* for *Anaxyrus boreas* tadpoles (Miura and Takahashi, 1973).

LC_{50} values obtained in the laboratory are useful to compare the biopesticide effects among non-target aquatic organisms, although it is known that field concentrations used for mosquito control strategies tend to be higher than the LC_{50} values obtained in the laboratory. For example, application rates recommended for mosquito control ranged from 8 to 40 mg/L of *Bti* (water-dispersible granule VectoBac® WDG and extruded pellet VBC Valent BioSciences®, respectively) (Farajollahi et al., 2013). According to the LC_{50} value calculated in our study (22.45 mg *Bti*-AS/L), this scenario would result in more than 50% of the dead tadpoles, or even higher if we consider that *Bti* has a short duration of toxic action, usually 48 h, and must therefore be applied at frequent intervals (Poopathi and Abidha, 2010).

Bt based products are distributed in different types of formulations in many countries. In these products, spores and δ-endotoxin obtained from fermentation are mixed with the additives, wetting agents, stickers, sunscreens and synergists (Burges and Jones, 1999; Gašić and Tanović, 2013). These additives listed as "inert" ingredients on product labels remain steeped in trade secret by the manufacturers of the products. *Bt*-formulations contain ingredients other than *Bt* which are potentially the most toxic components of the formulations (Swadener, 1994). The use of a surfactant (i.e. organosilicone surfactants, fluorochemical surfactants such as fluoroalkyl quaternary ammonium iodides, ammonium perfluoroalkyl sulfonates, and others) at an effective concentration serves to potentiate *Bt* pesticide activity (e.g. Salama et al., 2009; Shapiro et al., 1998). These antecedent reveal that toxicity of *Bti*-AS larvicide may be strengthened by the presence of "hazardous components" (e.g., surfactants) contained in the so-called inert ingredients incorporated in pesticide formulations, rather than by the active ingredient.

Since tadpole's exposure to *Bti*-AS not only produced mortality but also multiple sublethal effects, the use of biomarkers appears particularly suitable to assess impacts of mosquito control programs on these non-target vertebrates. The analysis of antioxidant enzymes (GST and CAT) revealed significant variations in tadpoles from *Bti*-AS treatments compared to the control group, suggesting the influence of specific treatment conditions. Although, we consider that these enzymes activities could varied between total tadpole homogenate and those without the intestine; it has been demonstrated that in amphibian tadpoles, antioxidant enzyme activity in the liver is higher than in other tissues (Jones et al., 2010). Oxidative stress may occur if the equilibrium between oxidants and antioxidants is interrupted either by reduction of antioxidant defences or by excessive increase of reactive oxygen species (ROS) (Valavanidis et al., 2006). Furthermore, *Bt* is known to produce oxidative stress, with biological effects such as alteration in the diverse antioxidant enzymes GST and CAT (Dubovskiy et al., 2008; Dzuy et al., 2004; Mansouri et al., 2013). Hence, GST and CAT provide valuable information about amphibian health status (Ezemonye and Tongo, 2010; Jones et al., 2010; Stefani Margarido et al., 2013).

On the other hand, in the present study, the antioxidant enzyme CAT showed an increased activity after 48 h exposure to 20 mg *Bti*-AS/L but showed a quite tendency to decrease activity at lower concentrations, suggesting that at higher concentration an increase in antioxidant defense to eliminate reactive oxygen species (ROS), mainly formed during the metabolism of this biocides (Vieira et al., 2014). In contrast, the decline at lower concentration of *Bti* may be explain in relation to its consumption during the scavenging process for the oxidation induced by this biocide; and it is related to finding in fishes with intestinal inflammation by soybean feed allergens (Zhang et al., 2013) and fishes with erythrocyte nuclear aberration and damage at environmental concentration pesticides (Vieira et al., 2014). This enzyme is essential to promote the degradation of H_2O_2, a precursor of hydroxyl radical which induces DNA damage in peripheral erythrocytes (Galindo et al., 2010). Actually, the protective role of CAT on DNA has been demonstrated in different studies (Cemeli et al., 2009; Halliwell and Gutteridge, 1999). GST activity also presented a significant increase after the experimental periods. Thus, it seems that the increased CAT and GST activity is related to genotoxic effects (Galindo et al., 2010; Gravato et al., 2004), probably caused by the exposure to the *Bti* commercial formulation. However, further studies are necessary to elucidate the role of oxidative stress on cytogenetic endpoints in *Bti*-induced toxicity.

References on the genotoxicity of *Bt* is controversial, depending on the genetic system or the assay used. In a safety testing of *Bt* using the *Salmonella* mutagenicity assay, Carlberg et al. (1995) conclude that δ-endotoxins are non-mutagenic. However, using

grasshoppers (*Oxya chinensis*) as animal model for chromosome aberration assays, Ren et al. (2002) demonstrate genotoxicity of *Bt* to *O. chinensis*. In addition, in a genotoxic evaluation through a series of tests in vivo in rodents, including erythrocyte MN assay and bone marrow of chromosomal aberration test, Curbelo Valiente et al. (2013) did not find an increase genetic damage. Nevertheless, our results indicate that *Bti* mixed with inert ingredients of the evaluated commercial formulation is potentially genotoxic. Accordingly, ISO (2006) recommendations for amphibian larvae point out if at least one of the test concentrations be statistically significant response respect to negative control, indicate the positivity of MN test.

ENAs frequencies in peripheral blood of *L. latrans* tadpoles were significantly higher than those observed in the negative control group, except for 20 mg *Bti*-AS/L, in which ENAs frequency was lower than the in control group. This result is likely to be related to the obtained LC_{50}. At higher toxic doses, the rate of cell division decreased (Lajmanovich et al., 2014); consequently, ENAs values (including the MN) may have decreased to 20 mg *Bti*-AS/L in response to experimental stress. In agreement with our results, a study in Brazil showed genotoxicity as well as embryo toxic effects of *Bt* on Zebrafish (*Danio rerio*) (Grisolia et al., 2009). This study also demonstrated the increase of ENAs frequency (i.e. MN) in peripheral erythrocytes. Remarkably, these genotoxic effects occurred at 100 mg/L of δ-endotoxins in absence of fish mortality. Therefore, we postulate that *L. latras* tadpoles are sensitive organisms for assessing genotoxic effects of *Bti* formulations.

Many histophatological investigations in insect larvae infected with *Bt* demonstrated morphological lesions in the midgut epithelium, which exhibited swollen cells, degenerated brush borders, disorganized nuclei, enlargement of intercellular spaces and cell lysis (e.g. Lacey and Federici, 1979; Rey et al., 1998). However, the lack of deleterious effects of δ-endotoxins on non-target organisms (e.g., mammals) has been explained by stomach acidification and by the absence of specific binding sites for Cry toxins in the intestine (McClintock et al., 1995). The present study shows several histological alterations of the intestinal epithelium in the treated tadpoles. Two histological symptoms were distinguished at all *Bti*-AS concentrations: infiltration in the connective tissue underlying the epithelium and dilation of blood vessels. Both epithelial alterations are regarded as defense mechanisms of the organisms against a stressor. This interpretation is supported by observations on surviving tadpoles, which showed only moderate forms of intestinal dysplasia. However, in many cases the defense reactions of the larvae were insufficient and epithelia erosion occurred. It remains unclear at the moment whether intestinal damage is reversible in tadpoles.

5. Conclusions

The current study provides relevant results of the effects of *Bti* commercial formulation on neotropical tadpoles, showing that *Bti*-AS is toxic to *L. latrans*. Acute and sublethal exposures ranging between 2.5–40 mg/L of *Bti*-AS induced genotoxic endpoints such as nuclear abnormalities in tadpole erythrocytes. Moreover, *Bti*-AS exposure induced antioxidant enzymes and caused histopathological changes in the intestine. In summary, the results of the present work indicate that exposure to commercial *Bti* formulation, even at low concentrations and for a relatively short period, can induce genotoxicity and intestinal damage. The extensive global use of different *Bt* formulations in control programs of mosquito-borne diseases underlines the importance of the finding. Finally, given that amphibian tadpoles and mosquito larvae often co-occur, more studies and data of biosafety on native species are necessary to elucidate the ecological risk posed by massive use of *Bti*-formulations on amphibian populations and human health.

Conflict of interest statement

The authors declare that they have no competing interests.

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