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RESEARCH ARTICLE

Lethal and sublethal effects of the natural and healthy spinosad-based formulation Tracer™ on tadpoles of two neotropical species

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Abstract

This paper presents the first acute toxicity data of the natural insecticide spinosad in amphibians. The sensitivity of two neotropical sympatric anuran species, *Boana pulchella* and *Rhinella arenarum*, to spinosad-based formulation Tracer™ was evaluated. Lethal effects are reported in tadpoles of *B. pulchella* stage 25 between 2.81 and 35.44 mg spinosad/L, while for the same concentration range no lethal effects were detected in tadpoles of *R. arenarum* of the same stage. In addition, Tracer™ produced sublethal effects at the individual level on the swimming activity, morphology (growth and presence of abnormalities), and development of *B. pulchella* from 2.81 to 5.78 mg spinosad/L, while in *R. arenarum* effects were only detected in the swimming activity and growth from 2.78 and 6.22 mg/L, respectively. At the biochemical level, Tracer™ produced inhibition of different enzymatic activities, among them, catalase activity at 2.81 mg spinosad/L, glutathione S- transferase activity from 2.81 to 2.98 mg spinosad/L, and acetylcholinesterase activity at 2.81 mg spinosad/L. These findings allow us to conclude that *B. pulchella* is more sensitive than *R. arenarum* to spinosad-based formulation Tracer™. The effects demonstrated here are not consistent with those expected since spinosad is supposed to be an environmental healthy alternative. This paper provides useful and necessary information to implement regulations on the use of new compounds entering the market and its associated risks.

Keywords Spinosad · Tracer™ · Mortality · Swimming activity · Morphology · Biochemical effects · Anuran species sensitivity

Introduction

Amphibians are among the non-target organisms affected by pesticides. They are particularly sensitive to pollutants due to their biological and ecological characteristics, among them, in general terms, the presence of bare skin, exposed embryogenesis, fully aquatic larval phase, and reproduction in shallow water bodies (Stebbins and Cohen 1995). In this context, they have been proposed as bioindicators (Blaustein et al. 1994; Blaustein and Kiesecker 2002). Numerous studies have evidenced the numerical decrease in amphibian populations and proposed chemical contamination among the possible causes (Blaustein et al. 2003, 2011; Beebee and Griffiths 2005; Hayes et al. 2010). In addition, some studies have demonstrated the association between the decline in amphibian populations and their proximity to agroecosystems (Davidson 2004; Peltzer et al. 2006; Smalling et al. 2015; Suarez et al. 2016). In summary, a wide variety of effects of pesticides have been reported on amphibian larvae, including alterations in enzymatic and swimming activity, alterations in feeding, inhibition of growth and development, increased susceptibility to diseases, manifestation of abnormalities, and reduction in survival (Boone and Bridges 2001; Relyea 2004; Peltzer et al. 2008; Agostini et al. 2010; Vera Candioti et al. 2010; Bernabò et al.

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2011; Ruiz De Arcaute et al. 2012; Brodeur et al. 2014; Attademo et al. 2017; Natale et al. 2018). However, a key limitation is the need to know the species sensitivity distribution since some species are more sensitive than others to a stress factor.

Around 70 years ago, crop pests began to be controlled with organochlorine pesticides, which are toxic, bioaccumulative, and persistent. These were replaced by less persistent pesticides such as organophosphates, carbamates, and pyrethroids (Casida and Quistad 1998). In the last few years, there has been a growing interest in the design of new generation pesticides with more specific action toward pests, faster degradation in the environment, and low toxicity for non-target biota (Isman 2006; Devine and Furlong 2007; Dayan et al. 2009). Among the naturally occurring compounds proposed as a replacement for synthetic pesticides is spinosad, a macrocyclic lactone is produced by the soil actinomycete *Saccharopolyspora spinosa*, which is toxic to insects. It is composed by the natural mixture of two active components (85% spinosyn A and 15% spinosyn D) and two metabolites of lower importance (spinosyn B and spinosyn K) (Mertz and Yao 1990; Kirst et al. 1992). Spinosad was registered under the Reduced Risk Pesticide program of the United States Environmental Protection Agency (USEPA) and won the Presidential Green Chemistry Challenge in 1999. It has been registered in more than 50 countries and used in crops such as soybeans, cotton, corn, fruits, and vegetables against pests of the orders Lepidoptera, Thysanoptera, Diptera, Coleoptera, and is also used for the control of *Ctenocephalides* sp. in dogs and cats (Thompson and Sparks 2002). It reaches water bodies by drift or surface runoff after the rains. Its application rate in crops varies between 48 and 360 mg active ingredient/L depending on the pest and the crop (Miles and Dutton 2003). Its half-life varies between 1 and 10 days being rapidly degrade by microbial action and photolysis (Saunders and Bret 1997; Sharma et al. 2007; Adak and Mukherjee 2016). The expected concentration of spinosad in water is between 0.0003 and 0.00215 mg/L (USEPA 2009). It is a neurotoxic compound that enters the insect body through contact or intake and acts at all life stages (White et al. 2007). It is fixed on the postsynaptic receptor, at a different site from acetylcholine, producing a synergistic effect on its activity. It generates the continuous entry of cations, causing hyperexcitation of the central nervous system, involuntary contractions of the muscles, and tremors followed by paralysis and death (Salgado 1998; Watson 2001; Orr et al. 2009). It is classified as a product of low environmental and toxicological risk, moderately or slightly toxic to fish and almost non-toxic to birds and mammals (Borth et al. 1996a, 1996b; Bret et al. 1997; Breslin et al. 2000). Although there are no contraindications or warnings about the environmental risks, it is known that some non-target aquatic invertebrates are sensitive to the exposure (Deardorff and Stark 2009; Duchet et al. 2011). It has also been reported as highly toxic for bees, with an acute LD50 of 5×10^{-5} mg/bee (Mayes et al. 2003). Among vertebrates, lethal and sublethal effects have been reported only in fishes (Borth et al. 1996a, 1996b; Piner and Üner 2012). There is no further available information on the effects it may cause in other non-target organisms.

Although amphibians have been proposed as bioindicator species and may potentially be exposed to spinosad in the field, no study examined its toxicity yet, to our knowledge. In this study, the lethal and sublethal effects of spinosad-based formulation Tracer™ were evaluated on stage 25 larvae (Gosner 1960) of the frog *Boana pulchella* and the toad *Rhinella arenarum*, using acute toxicity bioassays and evaluating the effects at the individual level (mortality, swimming activity, growth, development, and presence of morphological abnormalities). In *B. pulchella*, biochemical effects were also examined by the enzymatic activity of catalase (CAT), glutathione S-transferase (GST), and acetylcholinesterase (AChE).

Materials and methods

Test species

B. pulchella is a tree frog with Neotropical distribution from southern Brazil, southeastern Paraguay, Uruguay, to the province of La Pampa in Argentina (Cei 1980; Kwet et al. 2004a). It inhabits wetlands, flooded lowland meadows, grasslands, and agroecosystems, near urbanized sites (Natale and Ronco 2003; Peltzer et al. 2006; Agostini et al. 2009). It reproduces twice a year in coincidence with the rainiest seasons (March–April and August–September) and lays their eggs, which hatch in a week, in submerged masses associated with aquatic vegetation (Cei 1980). The larvae reach 70 mm in length, and are easy to maintenance in the laboratory (Natale 2006). The species has been proposed as a good model for ecotoxicological studies (Natale et al. 2018) since its body size and the duration of the larval period up to 6 months allow the evaluation of effects at different individual and subindividual levels (Natale 2006; Agostini et al. 2010; Pérez-Iglesias et al. 2014, 2017).

R. arenarum is a species of toad with a wider neotropical distribution from southeastern Brazil, Uruguay, Bolivia, and to the south of the province of Chubut in Argentina (Kwet et al. 2004b). It reproduces from August to April, in small ponds with stagnant water and lagoons, being the period associated with the availability of water. It lays up to 40,000 eggs, which hatch in approximately 2 or 3 days, in long gelatinous cords at the bottom of the water body (Cei 1980; Natale 2006). The larvae reach up to 25 mm in length and are also easy to maintenance in the laboratory (Cei 1980). It is a widely studied species at the biochemical, physiological,

and cytogenetic level, and it is frequently used in toxicity bioassays as a model species of ecotoxicological studies (Ferrari et al. 2008; Pérez-Coll et al. 2008; Brodeur et al. 2009; Salinas et al. 2015; Soloneski et al. 2016; Attademo et al. 2017).

Source of organisms

The eggs were collected from a temporary pond located on the outskirts of the city of La Plata, Buenos Aires, Argentina (34° 59′ 14″ S, 57° 51′ 9″ W), with low degree of anthropogenic disturbance and no detected pesticides in sediment (Sansiñena et al. 2018). For each bioassay, 10% of different clutches (n = 4 per species) were collected in order to guarantee the representativeness of the samples, and transferred to the laboratory in polyethylene bags with water from the site. The rest of the clutches were left in the pond for conservation purposes. Once in the laboratory, the eggs were acclimatized under controlled conditions (25 °C ± 1, photoperiod 16L: 8D) in trays with dechlorinated tap water (pH = 7.6–8.3, hardness = 155 mg CO_3Ca/L) and continuous aeration until reaching Gosner stage 25 (Gosner 1960). Tadpoles were fed with commercial fish flakes twice a week. Egg masses were collected under a license from the Direction of Flora and Fauna of the Province of Buenos Aires (File 22500-41820/17). All procedures for the care and use of laboratory animals agree with local guidelines for vertebrate animal welfare (Protocol No. 023-22-15) as well as with US Public Health Service and/or European Union policy.

Experimental design

All tests were performed under controlled laboratory conditions previously mentioned and following USEPA recommendations to accomplish with test procedures (USEPA 2002; ASTM 2007), with minor modifications proposed by Natale (2006) which were implemented several times for both test species (Nikoloff et al. 2014; Natale et al. 2018). Glass test chambers of 1000 ml were used, in which 500 ml of test solution and five 25-stage larvae were placed. The bioassays were semi-static and without food, with renewal of the media every 24 h. The different endpoints were recorded prior to the renewal and dead organisms were removed.

Three acute 96-h experiments were carried out: two experiments (experiments I and II) to assess lethal and sublethal effects at the individual level in *B. pulchella* and *R. arenarum* tadpoles at stage 25; the third (experiment III) was conducted only with *B. pulchella* tadpoles due to the greater sensitivity observed in previous bioassays, so as to examine biochemical effects, namely the enzymatic activity levels of CAT, GST, and AChE. All experiments include two negative control groups (dechlorinated tap water). Experiments I and II were performed by quadruplicate with 9 and 12 different concentrations ranging between 5 and 500 mg/L of spinosad for *B. pulchella* and *R. arenarum*, respectively. At the end (96 h), tadpoles were anesthetized with MS 222, fixed and stored in 10% formaldehyde to later examine growth and morphological abnormalities. Experiment III was performed with eight replicates and eight different concentrations of spinosad ranging between 5 and 500 mg/L of spinosad. After 96 h of exposure, tadpoles were anesthetized with MS 222 and individually preserved at − 80 °C until enzymatic determinations were performed.

Test chemical and quality controls

The test substance employed was the commercial formulation Tracer™, which is produced and sold by Dow AgroSciences®. The formulation Tracer™ is a suspension concentrate and contains spinosad in a proportion of 44.20%, equivalent to 480 g spinosad/L. A stock solution of 1000 mg spinosad/L was prepared by mixing 2.2711 g of product in 1 L of water from which test solutions were made. Samples of 2 ml of test solutions (n = 7) from experiments I and II (from 5 to 500 mg/L) were taken by triplicate immediately after preparation (0 h) and after 24 h of exposure. They were stored at − 20 °C until analyzed by analytical methods. Samples were filtered by 0.45 μm and the concentration of the water-soluble fraction was analyzed by LC-MS. An Agilent model 1100 liquid chromatograph coupled to an Agilent quadrupole mass spectrometer model VL was used. A C18 Zorbax Agilent column was used for the analytical separation, with a gradient of acetonitrile and ultrapure water both with 0.1% formic acid at 0.5 ml/min. A source of electrospray (ESI) was used in positive mode with nitrogen as a drying gas at 350 °C. The energy of the fragmenter was set at 150 eV in full scan mode. The extraction of the ionic chromatograms (EIC) was applied at m/z 128, 142, 718, 732, and 746 corresponding with molecular ions of the different isomers of spinosyn (A, D, K, and B) (Benincasa et al. 2011). Their chromatographic responses were summed for quantification and did not show significant differences from the standard. The Tracer ™ formulation extracted by sonication in methanol was used as a standard to develop a 1 mg/ml stock solution, from which intermediate dilutions were made in methanol and water. The limit of quantification was 0.5 mg/L and no matrix effect was observed in the samples as ionic suppression (Taylor 2005).

Evaluated endpoints

Mortality

Mortality (M) was determined every 24 h. Individuals were considered dead when immobility and rapid decomposition of the body were corroborated by visual observation. Also,

absence of cardiac activity was complementary checked under a Wild Heerbrugg M8 binocular stereoscope. Dead individuals were registered, extracted from the test chamber, and fixed in 10% formaldehyde for later examination of possible abnormalities and growth/development inhibition.

Effects on swimming

Swimming alterations were evaluated every 24 h after gently swirling the water with a glass rod and observing swimming activity of each individual tadpole for 1 min. The effects on swimming were classified according to the descriptions made by Brunelli et al. (2009) with minor modifications as suggested by Agostini et al. (2010) and Bach et al. (2016) in three categories: (1) regular swimming (RS), which corresponds to normal swimming; (2) irregular swimming (IS), corresponding to delayed normal swimming after stimulation; and (3) immobility (IN) which is illustrated by body twisting and convulsions, or complete stillness with slight movements which are observed after gently prodding each tadpole with a glass rod.

Effects on growth, development and morphology

Growth was assessed at the end of acute (96 h) exposure by measuring total length (TL), body length (BL), and body width (BW) after digital photograph with the ImageJ® program version 1.46r (Rasband, USA), with a precision of 0.01 cm. Photographs were taken with a digital camera Samsung WB850F. The inhibition of development and the presence of morphological abnormalities were also assessed at the end of acute exposure (96 h) by visual observation under an Ahecro ZTX-3EI stereoscopic binocular microscope. The stage of development was determined according to the classification proposed by Gosner (1960). Morphological abnormalities were classified after the categories proposed by Bantle et al. (1996).

Biochemical effects

Enzymatic activities of CAT, GST, and AChE were measured. Those activities respectively serve as biomarkers of oxidative stress, activation of pesticide biotransformation, and alteration of acetylcholinesterase (Brodeur et al. 2011; Colin et al. 2016). They were measured in whole tadpoles (Brodeur et al. 2011, 2017) as follows: four tadpoles from the same concentration treatment (one from each replicate) were processed jointly in order to achieve detectable levels of enzymatic activities (= 10 replicates per concentration). Briefly, tadpoles were homogenized in ice-cold 50 mM tris (hydroxymethyl) aminomethane buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid and 0.25 M of sucrose using a Teflon-glass Potter-Elvehjem homogenizer. Homogenates were centrifuged at 12,000 *g* for 5 min at 4 °C to remove debris, and the resulting supernatant was used for enzymatic determinations.

CAT activity was determined by measuring the decrease in absorbance resulting from hydrogen peroxide (H_2O_2) consumption using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}. The reaction mixture consisted of 300 μL of PBS (100 mM, pH 7), 10 μL of 10% H_2O_2, and 10 μL of diluted sample. The change in absorbance was recorded at 240 nm and 25 °C. GST activity was measured in a reaction mixture containing 300 μL of PBS (100 mM, pH 7) with added reduced glutathione, 10 μL of 1-chloro-2,4-dinitrobenzene (0.1 M), and 10 μL of diluted sample. The change in absorbance was recorded at 340 nm and 25 °C. Enzymatic activity was calculated using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Finally, AChE activity was determined by the method of Ellman (Ellman et al. 1961). The reaction mixture consisted of 200 μL of phosphate-buffered saline (PBS) (100 mM, pH 8), 10 μL of acetylthiocholine iodide (1 mM), 10 μL of 5,5′-dithiobis-(2-nitrobenzoic acid) (0.5 mM), and 100 μL of diluted sample. The change in absorbance was recorded at 25 °C and 412 nm. Enzymatic activity was calculated using a molar extinction coefficient of 14,150 M^{-1} cm^{-1}.

Statistical analysis

Homogeneity of variances and normality were corroborated with Bartlett's and Shapiro-Wilk's test, respectively. In cases where the assumptions were not fulfilled, a non-parametric Kruskal-Wallis test was carried out followed by Dunn's posteriori test. A paired *t* test was performed to compare the concentrations of the solutions immediately after preparation (0 h) and after 24 h. A Wilcoxon test was performed to compare nominal and real (= measured) concentrations. With the data of nominal and water-soluble fraction concentrations from experiments I and II (5–500 mg/L), a simple linear correlation analysis and a regression analysis were performed to predict the concentrations of the treatments in which no measurements were made. The LC50/EC50 and 95% confidence limits for mortality and swimming alterations were obtained by Probit analysis version 1.5 and following the method of linear intersection to estimate concentration-response curves. The highest non-observable effect concentrations (NOEC) and lowest observable effect concentrations (LOEC) were estimated by the LC/EC-1 and LC/EC-10, respectively. The NOEC and LOEC values for growth and morphological abnormalities were calculated by ANOVA followed by Dunnett's test.

Results

Quality controls

No significant differences ($t = 0.17$; $p = 0.864$) were found between spinosad concentrations at 0 h (initial time) and 24 h of exposure (maximum time before renewal of the media). However, significant differences were found between nominal and soluble fraction concentrations ($Z = 2.26$; $p < 0.05$). Since data showed a positive and very highly significant correlation ($r = 0.9970$; $p < 0.005$), all nominal concentrations were corrected with the concentrations predicted by the regression line equation: $2.4901 + 0.0659 \times X$ ($r^2 = 0.9941$; $p < 0.005$).

Lethal effects

Table 1 shows the results of Tracer™ lethal effects on the larvae of *B. pulchella* and *R. arenarum*. Mortality of *B. pulchella* increased significantly with the increment of the time of exposure. Tadpoles of *R. arenarum*, for their part, showed absence of lethal effects at the maximum tested concentration (= 35.44 mg spinosad/L).

Sublethal effects

Swimming activity

The three categories of swimming alterations (RS, IS, IN) were considered as a single one (effects on swimming activity, ES) since they behaved complementary and one was dependent on the other. Table 1 shows the results of Tracer™ sublethal effects on the swimming activity of *B. pulchella* and *R. arenarum* larvae. When comparing EC50 values at 96 h, *B. pulchella* is 5.42 times more sensitive than *R. arenarum*.

Table 1 Effects of spinosad-based formulation Tracer™ on mortality and swimming activity of *Boana pulchella* and *Rhinella arenarum* tadpoles

	Boana pulchella	*Rhinella arenarum*
Mortality		
LC50-96 h	3.5 (3.21–3.78)[a]	> 35.44
NOEC	1.99	> 35.44
LOEC	2.56	> 35.44
Swimming activity		
EC50-96 h	1.709 (0.26–6.27)[b]	9.26 (6.7–18.3)[b]
NOEC	0.37	0.64
LOEC	0.76	2.13

[a] *LC50*, lethal concentration 50 and their 95% confidence limits. Values are expressed in mg spinosad/L

[b] *EC50*, effect concentration 50 and their 95% confidence limits

NOEC highest non-observable effect concentration; *LOEC*, *l*owest observable effect concentration

Growth, development, and morphological abnormalities

Tracer™ showed highly significant growth inhibition in both species (*B. pulchella*: $F(4, 145) = 16.49$; $p < 0.05$; *R. arenarum* = $F(9; 190) = 3.44$; $p < 0.05$) with NOEC and LOEC values for *B. pulchella* of < 2.81 and 2.81 mg spinosad/L, respectively, and NOEC and LOEC values for *R. arenarum* of 4.46 and 6.44 mg spinosad/L, respectively. The LOEC value also showed that *B. pulchella* is 2.3 times more sensitive than *R. arenarum*. Tracer™ showed no effects on the development of both species and caused no morphological abnormalities in *R. arenarum*, while in *B. pulchella* it caused tail flexure (a bending generated from the bottom or in the middle region of the tail by moving it side; $F(4, 43) = 6.83$; $p < 0.005$), absence of keratodonts (a significant lack of keratodonts of at least ten consecutive pieces, in one or more rows; $F(4, 43) = 74.18$; $p < 0.005$), and displaced intestine (an abnormal bowel, without the normal spiral shape; $F(4, 42) = 196.63$; $p < 0.005$) at 96 h of exposure (Fig. 1). Tail flexure was observed at 3.47 mg spinosad/L with 19% of affected individuals. Absence of keratodonts and displaced intestine were observed between 2.81 and 5.78 mg spinosad/L with 97% of affected individuals.

Sublethal effects at the biochemical level in *B. pulchella*

Catalase activity levels were significantly inhibited only in larvae exposed to 2.81 mg spinosad/L, with 28.6% lower activity than the control group (Fig. 2a). GST activity was significantly inhibited in larvae exposed from 2.81to 2.98 mg spinosad/L, with 25% lower activity than the control group (Fig. 2b). Finally, acetylcholinesterase activity was significantly inhibited in larvae exposed to 2.81 mg spinosad/L, with an activity 40% lower than the control group (Fig. 2c).

Discussion and conclusions

This study is the first to report acute toxicity data of spinosad in amphibians. Lethal and sublethal effects of the spinosad-based formulation Tracer™ are reported in larvae of two native species of anurans, *R. arenarum* and *B. pulchella*, showing significant differences in their sensitivity. While *R. arenarum* demonstrated tolerance to Tracer™, *B. pulchella* proved to be sensitive to lethal and sublethal effects at the individual level. This sensitivity was also revealed when evaluating biochemical endpoints (CAT, GST, and AChE activity) at low concentrations. Considering the toxicity categories proposed by USEPA (Carey et al. 2008), spinosad can be classified as moderately toxic for *B. pulchella*

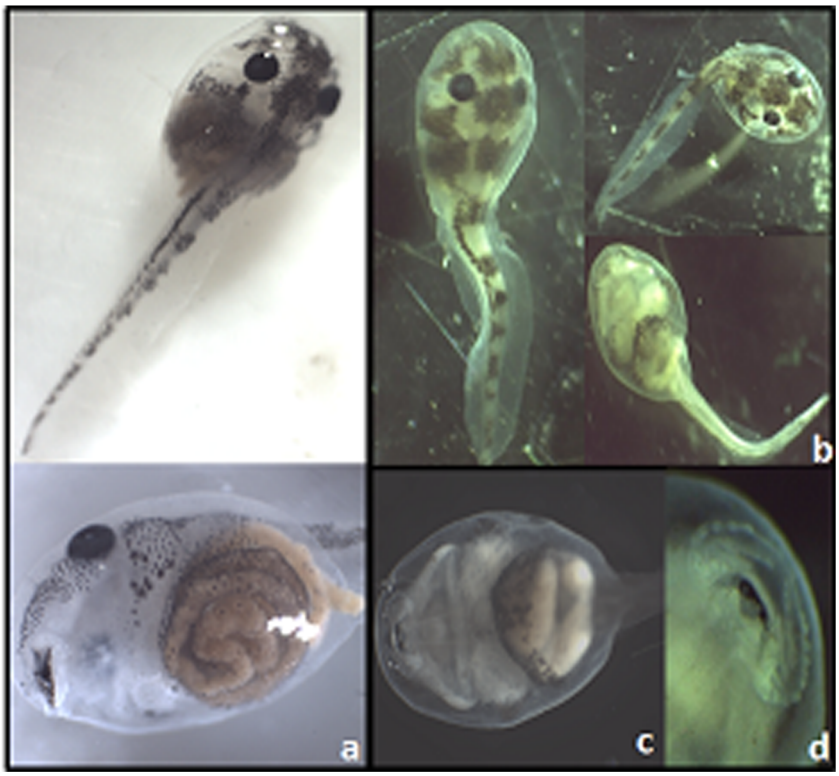

Fig. 1 Morphological abnormalities in tadpoles of *Boana pulchella*. **a** Normal tadpole without morphological abnormalities; **b** lateral flexure of the tail; **c** displaced intestine; **d** absence of keratodonts

(acute LC50 between 1 and 10 mg/L) and slightly or almost non-toxic for *R. arenarum* (acute LC50 between 10 and 100 mg/L or > 100 mg/L, respectively). The magnitude of the different sensitivity of both species is particularly important in the context of current ecotoxicology, especially for the environmental risk assessment of pesticides. It should also be considered when selecting test species for the evaluation of the toxicity of any substance, before authorizing its commercialization, and at the time of choosing native indicator species.

There are several factors which are responsible for the variability of the evaluated endpoints, among them breeding conditions, food type, source of organisms, and characteristics of the water (Rand 1995; ASTM 2007). This study eliminated those sources of variation using the same experimental conditions and performing the tests by the same operator. In addition, the organisms share the source and the ecological attributes: species are sympatric, cohabit the same ecosystem, and use the same reproductive site. All this allowed us to propose genetic distance as the factor that influences the differential sensitivity of both species (phylogenetic hypothesis proposed by Hammond et al. 2012).

The differential response of both species exposed to other insecticides is consistent with the results reported here, being *B. pulchella* more sensitive than *R. arenarum* when considering lethal effects of endosulfan (Agostini et al. 2009; Svartz et al. 2014), chlorpyrifos (Liendro et al. 2015; Barreto et al. 2020), cypermethrin (Agostini et al. 2010; Svartz et al. 2016), and pirimicarb (Vera Candioti et al. 2010; Natale et al. 2018). Although *R. arenarum* is the most frequently used test species in Argentina, reported data indicate that it is not the most sensitive one. In that sense, this paper provides evidence in favor of the usefulness of *B. pulchella* as a test species when assessing effects on aquatic ecosystems. Considering that there is no ecotoxicological information about more than 87% of the native anuran species, this study promotes the ecotoxicological investigation of native species with the intention of classifying them in terms of their sensitivity, either for use in bioanalytical tests or as indicator species.

If we compare the LC50 values available for aquatic organisms exposed to spinosad with those obtained in this study (Table 2), the sensitivity of the larvae of *B. pulchella* is in the 60th percentile. Despite the less sensitive response of this species compared with cladocerans, it is the most sensitive vertebrate species. In addition, a group of very tolerant species can be distinguished, not being able to determine the LC50: > 35.44 mg spinosad/L for *R. arenarum*, > 202 mg/L for *Poecilia reticulata* and *Xiphophorus maculatus*, and > 500 mg/L for *Oncorhynchus kisutch*.

Detecting sublethal effects at low concentrations is of great relevance since they can cause negative effects on the individual fitness and compromise the survival of larvae and adults. Several studies have reported alterations in swimming activity in larvae of *B. pulchella* and *R. arenarum* exposed to different pesticides such as azinphos methyl, carbaryl, cypermethrin, and pirimicarb (Ferrari et al. 2009; Agostini et al. 2010;

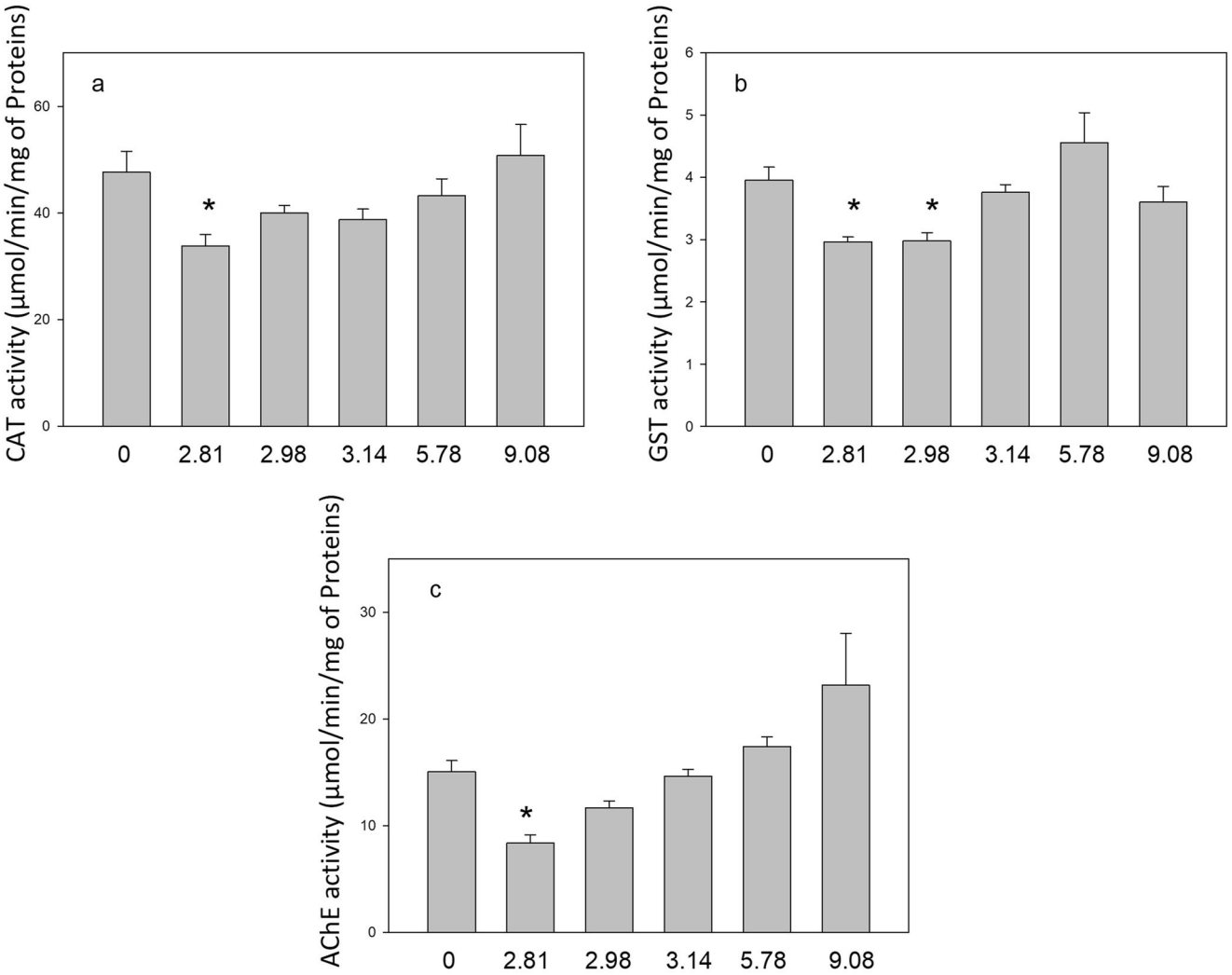

Fig. 2 Enzymatic activity (mean ± S.E.) of individuals of *Boana pulchella* exposed to Tracer™. **a** Catalase activity (CAT), **b** glutathione S-transferase activity (GST), and **c** acetylcholinesterase activity (AChE). A single asterisk indicates significant differences between treatments and control group. Values are expressed in mg spinosad/L

Svartz et al. 2016; Sansiñena et al. 2018). An individual presenting an alteration of swimming activity will have difficulties in finding and obtaining food (Horat and Semlitsch 1994), and will be more vulnerable to predation (Lehman and Williams 2010; Denoël et al. 2012). In addition, several studies have reported growth inhibition in larvae of both species exposed to azinphos methyl, chlorpyrifos, pirimicarb, and cypermethrin (Agostini, Natale and Ronco, 2010; Ferrari et al. 2011; Sotomayor et al. 2012; Natale et al. 2018). Growth inhibition during the larval period causes individuals to reach metamorphosis with a smaller size, and small juveniles to have a decreased fitness and consequently less chances of survival (Semlitsch et al. 1988; Boone and Bridges 2001; Altwegg and Reyer 2003).

Regarding morphological abnormalities reported here, tail flexure, the absence of keratodonts, and displaced intestine have been detected in larvae of *B. pulchella* and *R. arenarum* exposed to azinphos methyl, carbaryl, chlorpyrifos, cypermethrin, pirimicarb, and sediments from a pesticide use zone (Ferrari et al. 2009; Agostini et al. 2010; Lascano et al. 2011; Liendro et al. 2015; Natale et al. 2018; Sansiñena et al. 2018). Tail flexure has a negative effect on swimming and reduces the possibility of efficiently escaping from predators. It also affects the ability to search for food, decreasing the probability of survival as stated by Sansiñena et al. (2018). Alterations in the oral disc reduce feeding efficiency (Venesky et al. 2010; Tolledo et al. 2014) negatively, influencing growth and development. Intestine alterations may be associated with hypertrophy or hyperplasia, may be part of a defense response to pollutants, and may contribute reducing the entry of toxic substances into the epithelial cells (Barja-Fernández et al. 2013). We can conclude that the evaluated insecticide induces the expression of morphological abnormalities that have severe consequences on the individual survival.

Table 2 Spinosad LC50 values reported for different aquatic species

Species name	LC50 (mg spinosad/L)	Authors
Daphnia magna	0.0005[a]	Duchet et al. (2011)
Daphnia pulex	0.0005[a]	Duchet et al. (2011)
Ceriodaphnia dubia	0.0018[a]	Deardorff and Stark (2009)
Daphnia magna	0.0048[a]	Deardorff and Stark (2009)
Daphnia pulex	0.129[a]	(Stark and Banks 2001)
Daphnia pulex	0.129[a]	Deardorff and Stark (2009)
Boana pulchella	3.5[b]	This study
Cyprinus carpio	5[b]	Borth et al. (1996)
Lepomis macrochirus	5.9[b]	Borth et al. (1996)
Cyprinodon variegatus	7.9[b]	Borth et al. (1996)
Oncorhynchus mykiss	30[b]	Borth et al. (1996)
Rhinella arenarum	> 35.44[b]	This study
Poecilia reticulata	> 202[b]	Pereira et al. (2016)
Xiphophorus maculatus	> 202[b]	Pereira et al. (2016)
Oncorhynchus kisutch	> 500[b]	Deardorff and Stark (2009)

LC50, lethal concentration 50

[a] LC50 at 48 h

[b] LC50 at 96 h

Concerning the effects at the biochemical level, some studies have reported that the exposure of anuran larvae to endosulfan, chlorpyrifos, and glyphosate-based herbicides causes inhibition in the activity of CAT (Pandey et al. 2001) and GST (Lajmanovich et al. 2010; Sotomayor et al. 2015), while others reported an increased activity in CAT (Sotomayor et al. 2015) and GST (Pandey et al. 2001; Ferrari et al. 2009; Ezemonye and Tongo 2010). Also, some studies demonstrated inhibition of GST activity in brain and liver of *Oreochromis niloticus* (Orden Perciformes) exposed to 75 mg spinosad/L (Piner and Üner 2011, 2013). It should be noted that an inhibition of GST activity causes a failure in the detoxification system that involves modulating the conjugation of GSH with an electrophilic compound and its subsequent elimination (Hayes and Pulford 1995; Hayes et al. 2005). On the other hand, an inhibition of CAT generates an imbalance of the cellular redox state by not being able to hydrolyze H_2O_2 and causing cellular damage (Gil et al. 1987). For their part, AChE is involved in nerve impulse transmission. Several studies have reported their inhibition in larvae exposed to chlorpyrifos, lambdacialotrin, and diazinon (Colombo et al. 2005; Attademo et al. 2014). Piner and Üner (2012) reported inhibition in the liver and brain of *O. niloticus* exposed between 25 and 75 mg of spinosad/L. Its inhibition causes an accumulation of acetylcholine in the synaptic cleft, and consequently a continuous nerve transmission (Xuereb et al. 2009). Although spinosad's mode of action does not involve AChE as a site of action, this study shows an inhibition of its activity. Thereby, the entry of cations would be prolonged and generate the consequent hyperexcitation. The study of a battery of biomarkers is an important tool for detecting exposure to environmental pollution, with the effects evaluated being early warning signals (Shugart et al. 1992). This allows detecting the problem caused by certain substances before the damages are irreversible. In the present study, effects on the activity of CAT, GST, and AChE were detected at the lowest concentrations tested; therefore, we consider them to be very useful as biomarkers.

This paper provides useful and necessary information to implement regulations on the use of new compounds entering the market, and the evaluation of the ecotoxicological risks associated with their use. These precise data may also be useful when discussing the selection of sensitive species as bioindicators. Although the spinosad entered the world market in 1997 as a replacement for those highly toxic, bioaccumulative, and persistent insecticides, this study represents the first report of lethal and sublethal effects of this compound on amphibians. The effects demonstrated here are not consistent with those expected since spinosad is supposed to be an environmental healthy alternative.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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