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Acute Toxicity and Etho-toxicity of Three Insecticides Used for Mosquito Control on Amphibian Tadpoles

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Abstract Among the measures used to manage mosquito populations and prevent human diseases, the application of pesticides is the global strategy mostly employed. To investigate the lethal and sublethal effects of insecticides used to control mosquitoes on amphibians (*Rhinella arenarum*, *Rhinella fernandezae*, and *Physalaemus albonotatus*), tadpoles were exposed to commercial formulations of temephos (Abate®), *Bacillus thuringiensis* var. *israelensis* (Introban®), and permethrin (Depe®). Their acute toxicity in terms of median lethal concentration (LC_{50}) and no- (NOEC) and lowest-observed-effect concentrations (LOEC) was evaluated. To assess the sublethal effects on behavioral endpoints, tadpoles were exposed to the NOEC-24-h value of each insecticide. After that, tadpoles were recorded and video-streaming data were processed by Smart® software. Based on LC_{50}, permethrin was the most toxic insecticide, followed by temephos and *B. thuringiensis* var. *israelensis*. Also, intraspecific and interspecific susceptibilities of tadpoles to insecticides were observed. Regarding behavior, the exposure of *R. arenarum* to the three insecticides had a significant effect on all behavioral endpoints. Two of the three swimming parameters evaluated for *R. fernandezae* were affected by permethrin, and in the end, only one behavioral pattern was altered in *P. albonotatus* after exposure to temephos. These results showed that tadpoles' species were affected differently depending on the chemical properties of the pesticide and on a dose-response effect of the insecticides. Overall, our study suggests that further research is needed to quantify the potential damage of pyrethroid insecticides used for mosquito control on non-target aquatic organisms, mainly due to etho-toxic effects.

Keywords Adulticides · Amphibian larvae · Behavior · Larvicides · Mosquito management

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1 Introduction

In Argentina, since the largest epidemic of dengue virus occurred in summer 2009 (Zambrini 2011; Tarragona et al. 2012), mosquito control actions have been intensified by the Ministerio de Salud de la Nación (MSN) in almost all Northern and Central provinces of Argentina. Unfortunately, after 7 years of interrupting control actions to mosquitoes, the reestablishment of dengue in Argentina became now an epidemic problem (Hilbert 2016). Currently, the Programa de Control de Vectores recommends the use of three different insecticides that are worldwide employed for controlling mosquito populations: temephos and *Bacillus thuringiensis* var.

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israelensis as larvicides and permethrin as adulticide (MSN 2011).

The anti-cholinesterase (anti-ChE) organophosphate temephos is applied as 1% granules (Abate®) into household water containers, including those used for storing drinking water and other breeding foci for larval control (World Health Organization (WHO) 2008). Temephos is lethal to fish and non-target invertebrates (Selvi et al. 2004; Crivelenti et al. 2010), but little has been published about its toxicity on amphibians. Sparling et al. (1997) reported that only 0.00189 mg l^{-1} of temephos were lethal for half of the tadpoles of *Lithobates clamitans* exposed. While for Harischandra et al. (2011), the median lethal concentration (LC_{50})-24 h value was 16.56 mg l^{-1} for tadpoles of *Duttaphrynus melanostictus* exposed to Abate®. Temephos is also used in combination with *B. thuringiensis* var. *israelensis* to control larvae of *Aedes aegypti* (the main dengue vector) due to the emergence of mosquito resistance to temephos in Argentina (Biber et al. 2006; Seccacini et al. 2008). *B. thuringiensis* var. *israelensis* is a bioinsecticide widely used against mosquitoes, chironomids, and blackflies. Its larvicidal activity is a consequence of δ-endotoxins, which are synthesized during sporulation. A large number of toxicity studies with non-target aquatic species showed that *B. thuringiensis* var. *israelensis* has a low probable risk (e.g., Boisvert and Boisvert 2000; Caquet et al. 2011; Lagadic et al. 2014). For this reason, it is safe to be used in waterbodies and container-breeding mosquitoes and can also be applied in drinking water (WHO 2009). Nevertheless, a recent study (Maletz et al. 2015) confirmed the estrogenic activity of *B. thuringiensis* var. *israelensis*, particularly of the active substance (technical power) and the formulation VectoBac® water dispersible granule. Besides, Lajmanovich et al. (2015) found that a commercial liquid formulation of *B. thuringiensis* var. *israelensis* (Introban®) was relatively toxic for anuran tadpoles of *Leptodactylus latrans* ($LC_{50-48\,h}$=22.45 mg l^{-1}) and also altered enzymes of oxidative stress, induced genotoxicity, and caused intestine damage at sublethal concentrations. Finally, the ultra-low-volume (ULV) aerosol application of 10% permethrin (Depe®) is used to manage adult mosquitoes and is applied by a truck-mounted ULV sprayer. Permethrin is a pyrethroid insecticide (type I) that acts on the axons in the peripheral and central nervous systems by interacting with sodium channels in mammals and/or insects (Davies et al. 2007). This compound has good safety margin in terms of acute toxicity for warm-blooded animals, but it is toxic to non-target invertebrates and aquatic organisms (Schleier and Peterson 2010; Nkya et al. 2013).

Behavioral assays provide biologically relevant endpoints that can be directly related to organismal health, as behavior links the physiological function with the ecological processes (Scott and Sloman 2004). Quantifiable behavioral alterations in organisms linked with short-term and sublethal exposures to pesticides provide valuable information that cannot be obtained from traditional toxicological methods (e.g., LC_{50}, and no-(NOEC) and lowest-observed-effect concentrations (LOEC)). In recent years, the development of video-tracking systems has allowed the accurate quantification of locomotor behavior of tadpoles, suggesting their usefulness as powerful tools to characterize environmental risk and analyze the toxicological impact of pesticides in amphibians (Brunelli et al. 2009; Denoël et al. 2012; Peltzer et al. 2013). Consequently, there has been increasing interest among researchers in evaluating the behavioral alterations by applying video-tracking technique in response to low-level exposures of pesticides. For instance, Lavorato et al. (2013) found that tadpoles of *Rana dalmatina* exposed to 10 and 50 μg of endosulfan l^{-1} exhibited several abnormalities in swimming patterns, such as shorter distance moved, swirling, resting, and unusual use of space. In addition, a recent investigation (Preud'homme et al. 2015) showed behavioral disorders (feeding and locomotion patterns) in *Xenopus laevis* tadpoles at lower environmental concentrations of endosulfan (0.1 and 1 μg l^{-1}) than those reported by Lavorato et al. (2013). Thus, in order to investigate the effect of insecticides massively employed worldwide for mosquito control on non-target organisms, the objective of the current study was to assess the lethal and sublethal effects of two larvicides (temephos and *B. thuringiensis* var. *israelensis*) and an adulticide (permethrin) on three amphibian larvae of *Rhinella arenarum*, *Rhinella fernandezae*, and *Physalaemus albonotatus*, through traditional ecotoxicological methods and behavioral endpoints (etho-toxicology).

2 Material and Methods

2.1 Test Chemicals

Three insecticides recommended and provided by the Coordinación Nacional de Control de Vectores, through the Programa Provincial de Zoonosis y Vectores for the control of adult and larvae mosquitoes that were used are (1) the insecticide organophosphate temephos (IUPAC name: *O*,*O*,*O*′,*O*′-tetramethyl *O*,*O*′-thiodi-*p*-phenylene bis(phosphorothioate)) Abate® 1SG containing 1% of active ingredient (a.i.; Basf Argentina, CAS no. 3383-96-8); (2) the commercial liquid (aqueous suspension (AS)) formulation of *B. thuringiensis* var. *israelensis* (Introban®, 1200 International Toxic Units (ITU) mg^{-1} formulation) produced by Valet BioSciences Corporation (USA) and imported by Chemotecnica S.A. (Argentina); and (3) the emulsifiable concentrate (10% a.i.) of permethrin (IUPAC name: 3-phenoxybenzyl (1RS)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanecarboxylate), Depe® (Chemotecnica S.A., Argentina; CAS no. 52645-53-1).

2.2 Test Organisms

Prometamorphic tadpoles of *R. arenarum*, *R. fernandezae* (Bufonidae), and *Physalaemus albonotatus* (Leptodactylidae) were collected with dip net from temporary ponds of natural floodplains of Middle Paraná River (31° 38′ 25″ S–60° 40′ 18″ W, Reserva Ecológica de la Ciudad Universitaria (UNL) "El Pozo," Santa Fe province, Argentina) where no pesticides were used. These common anurans have an extensive distribution in the Neotropical America (International Union for Conservation of Nature (IUCN) 2015) and are frequently occurring on the margin of efimeral ponds and agricultural and urban waterbodies (Peltzer et al. 2006).

Tadpoles were acclimated on glass recipients that contained dechlorinated tap water, under laboratory conditions (12/12-h light/dark cycle and at 23 ± 2 °C). Larvae were fed ad libitum with boiled lettuce up to reaching Gosner stage 33 (Gosner 1960), time when the experiments were carried out. At this Gosner stage, tadpoles are active swimmers, with long tails, present hindlimb buds with interdigital indentations (between third and fourth and fourth and fifth toes), and no forelimb buds (McDiarmid and Altig 1999). The size of tadpoles used in the experiments was as follows (ten randomly sampled; mean total length ± SD): *R. arenarum*, 23.9 ± 1.2 mm; *R. fernandezae*, 13.1 ± 3.9 mm, and *P. albonotatus*, 15.8 ± 1.4 mm. Tadpoles used in this research have been treated according to ASIH (2004) criteria and with the approval from the animal ethics committee of the Facultad de Bioquímica y Ciencias Biológicas, FBCB Res. CD no: 388/06.

2.3 Acute Toxicity Tests

Acute static toxicity tests were performed following standardized methods proposed by the USEPA (2002) and ASTM (2007). To determine the concentrations used in the acute toxicity tests, preliminary tests were performed. The nominal concentrations ranged from 2 to 35 mg l^{-1} a.i. for temephos, between 1.50 and 40 mg l^{-1} a.i. for *B. thuringiensis* var. *israelensis*, and from 0.003 to 0.50 mg l^{-1} a.i. for permethrin. All test solutions were prepared immediately before use from appropriate dilution of stable stock solutions (1 g l^{-1}) of each insecticide (USEPA 1975). Laboratory toxicity tests were conducted in glass aquariums (12.5 cm in diameter and 13.5 cm in height) containing 1 l of test solutions at a temperature (mean ± SD) of 23 ± 2 °C, and 12:12 h photoperiod for a 48-h period. Negative control (dechlorinated water) and test concentrations of each insecticide were triplicated with seven tadpoles per aquarium. Larval mortality was monitored once every 24 h, and dead larvae were removed every 24 h. Animals were not fed during toxicity trials. After 48 h of exposure, the LC_{50}, LOEC, and NOEC were estimated separately for the three species and for each insecticide.

2.4 Behavioral Endpoints

Because of the lack of environmental concentration data deposited on waterbodies and uncertainties associated with the fate of the products used for mosquito management (Schleier and Peterson 2010), we performed our study using as sublethal concentration of exposure the NOEC-24 h value for each insecticide and species (see Table 1) based on results of acute toxicity tests (Wagner and Løkke 1991; Shao 2000). At the end of the exposure (24 h), one larva was carefully released at the center of a Petri dish (15 cm diameter, 2 cm height) filled with 200 ml of dechlorinated tap water. After 30 s of acclimation, behavioral variables were recorded during 5 min using a digital video camera (Motic®, 10.0 M pixel) placed just above the dish. Each tadpole was treated as independent experimental units (Van Buskirk and McCollum 2000), and ten replicates were conducted for each concentration of exposure, including the control. Behavioral parameters associated with locomotor performance of anuran larvae and considered sensitive indicators of toxicity (Denoël et al. 2010, 2013; Winandy and Denoël 2011) were quantified in tadpoles: total distance moved (the total distance covered by the tadpole during the 300 s; cm s^{-1}), immobility (the time tadpole spent in resting state; s), and global activity (during the experiment, the sum of the differences between two consecutive frames of the image source. This accumulated difference is then divided by two as a punctual movement of the tadpole generates a change in both the new place and in the space left in the image; cm^2). Video data were afterward analyzed using appropriate video-tracking software (Smart 3.0.02, Panlab Harvard Apparatus®) to obtain both X and Y coordinates of the central point of each tadpole (center-of-mass tracking detection mode) across the time. In our setup, the immobile threshold was 10%, and below this value, tadpoles were considered immobile.

2.5 Statistical Analysis

The LC_{50} (confidence interval 95%) at 24 or 48 h not only for insecticide but also separately for the three species was estimated by the trimmed Spearman-Karber (TSK) analysis for lethal tests (Hamilton et al. 1977). The criterion of non-overlapping 95% confidence intervals (CI) was used to determine significant difference among LC_{50} values (Wheeler et al. 2006). NOEC and LOEC values for each time of exposure were estimated by analysis of variance (ANOVA) followed by the Dunnett's test for post hoc comparison of means. ANOVA was also applied to assess the effects of temephos, *B. thuringiensis* var. *israelensis*, and permethrin on behavioral endpoints. Analyses were performed for each of the three species separately. When ANOVA results were significant ($p < 0.05$), a posteriori pairwise comparisons were made using Dunnett's test. Assumptions of normality and homogeneity of variance were confirmed with Kolmogrov-Smirnov and Levene's tests. SPSS 17.0 software was used for statistical analyses.

3 Results

3.1 Acute Toxicity Tests

Estimated LC_{50} nominal values and 95% confidence limits for 24 and 48 h of exposure to the insecticides temephos, *B. thuringiensis* var. *israelensis*, and permethrin are listed in Table 1, as well as the values for NOEC and LOEC. Acute toxicity tests indicated that

Table 1 Summary of median lethal concentrations (LC_{50}), lowest-observed-effect concentrations (LOEC), and no-observed-effect concentrations (NOEC) (mg l^{-1}) of insecticides on anuran tadpoles after 24- and 48-h exposure

	24 h			48 h		
	LC_{50} (95% CI; mg l^{-1})	NOEC (mg l^{-1})	LOEC (mg l^{-1})	LC_{50} (95% CI; mg l^{-1})	NOEC (mg l^{-1})	LOEC (mg l^{-1})
Temephos						
Rhinella arenarum	19.76 (17.86–21.85)a	16.0[a]	18.8	16.79 (15.32–18.41)a	13.60	16.00
Rhinella fernandezae	4.43 (4.03–4.88)b	3.10[a]	3.65	4.08 (3.76–4.42)b	3.10	3.65
Physalaemus albonotatus	6.48 (5.65–7.44)c	3.65[a]	4.30	5.88 (5.19–6.65)c	3.65	4.30
Bacillus thuringiensis var. *israelensis*						
R. arenarum	20.51 (18.63–22.58)a	13.88[a]	15.43	19.25 (17.36–21.34)a	12.50	13.88
R. fernandezae	10.73 (7.45–15.46)b	3.0[a]	5.0	10.73 (7.45–15.46)b	3.0	5.0
P. albonotatus	11.87 (8.89–15.85)b	5.0[a]	8.30	11.87 (8.89–15.85)b	5.0	8.30
Permethrin						
R. arenarum	0.047 (0.024–0.090)b	0.006[a]	0.012	0.035 (0.017–0.072)b	0.003	0.006
R. fernandezae	0.056 (0.035–0.089)b	0.012[a]	0.025	0.028 (0.018–0.044)b	0.006	0.012
P. albonotatus	0.009 (0.006–0.013)a	0.003[a]	0.006	0.007 (0.005–0.010)a	0.006	0.012

[a] Sublethal concentrations used to evaluate effects on behavior. Different letters (a–c) indicate significant differences in LC_{50} among species

CI confidence interval

permethrin was the insecticide most toxic for the three tadpole species, followed by temephos and *B. thuringiensis* var. *israelensis*. Also, intraspecific differences in the susceptibility of species to temephos, *B. thuringiensis* var. *israelensis*, and permethrin were observed, and based on the CIs, these differences were significant ($p < 0.05$) among the three insecticides for *R. fernandezae* and *P. albonotatus* at 24 and 48 h, but not between temephos and *B. thuringiensis* var. *israelensis* for *R. arenarum*. The species analyzed separately (*R. arenarum*, *R. fernandezae*, and *P. albonotatus*) showed different sensitivities to insecticides. Interspecific differences were significant ($p < 0.05$, Table 1) for the three tadpole species when were exposed to temephos for each time of exposure, with *R. fernandezae* being the most sensitive species ($LC_{50–24\ h} = 4.43$ mg l^{-1} and $LC_{50–48\ h} = 4.08$ mg l^{-1}) and *R. arenarum* the most tolerant ($LC_{50–24\ h} = 19.76$ mg l^{-1} and $LC_{50–48\ h} = 16.79$ mg l^{-1}). For *B. thuringiensis* var. *israelensis*, significant interspecific differences were observed between *R. arenarum* (the most tolerant specie, $LC_{50–24\ h} = 20.51$ mg l^{-1} and $LC_{50–48\ h} = 19.25$ mg l^{-1}) and the other two species for both times of exposure. As regards permethrin, interspecific differences were found at 24 and 48 h between the most sensitive species (*P. albonotatus*, $LC_{50–24\ h} = 0.009$ mg l^{-1} and $LC_{50–48\ h} = 0.007$ mg l^{-1}) and the bufonids (*Rhinella* spp. tadpoles).

3.2 Behavioral Endpoints

Sublethal exposure to temephos, *B. thuringiensis* var. *israelensis*, and permethrin caused alteration on swimming endpoints. For *R. arenarum*, the exposure to the insecticides had a significant effect on each of the three swimming endpoints: distance moved ($F_{3,\ 40} = 15.819$; $p < 0.001$), immobility ($F_{3,\ 40} = 13.813$; $p < 0.001$), and global activity ($F_{3,\ 40} = 16.975$; $p < 0.001$). Post hoc tests showed that tadpoles exposed to temephos moved less, spent more time immobile, and had less global activity than controls (all $p < 0.001$) (Fig. 1). The same effects were found on the tadpoles exposed to permethrin ($p < 0.001$) and to *B. thuringiensis* var. *israelensis* ($p < 0.01$) (Fig. 1).

Exposure of *R. fernandezae* to the insecticides had a significant effect on the distance moved ($F_{3,\ 40} = 5.912$; $p < 0.05$), and global activity ($F_{3,\ 40} = 4.893$; $p < 0.01$), but immobility was unaffected ($F_{3,\ 40} = 2.614$; $p > 0.05$). Dunnett's post hoc tests showed tadpoles exposed to permethrin moved more than controls ($p < 0.05$) and also increased their global activity ($p < 0.05$) (Fig. 2). No significant effects were found among tadpoles exposed to temephos and *B. thuringiensis* var. *israelensis* ($p > 0.05$) with respect to controls (Fig. 2).

Finally, tadpoles of *P. albonotatus* exposed to insecticides exhibited significant effects only on the distance moved ($F_{3,\ 40} = 3.538$; $p < 0.05$). Also, this difference was observed in tadpoles exposed to temephos ($p < 0.05$) compared with the control group (Fig. 3). *B. thuringiensis* var. *israelensis* and permethrin had no significant effects on the three behavioral endpoints (all $p > 0.05$) (Fig. 3).

4 Discussion

The present study provides relevant information on the hazardous potential of three widely used insecticides for mosquito control on anuran tadpoles. Permethrin exhibited the highest toxicity ($LC_{50–48\ h}$) to all tadpole

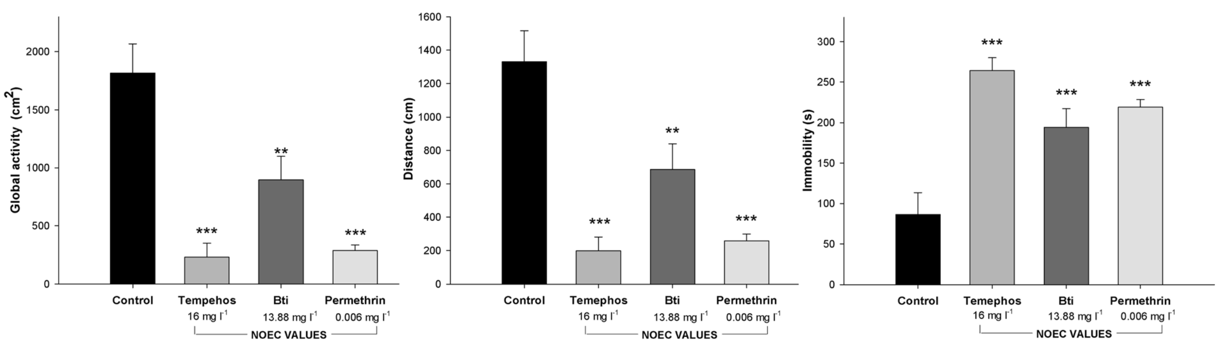

Fig. 1 Global activity, distance moved, and immobility of *R. arenarum* tadpoles after 24 h of exposure to NOEC concentrations of insecticides and of the control group. The *bars* show mean ± SD, and *asterisks* show the treated groups that differ from the control; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (post hoc test, Dunnett)

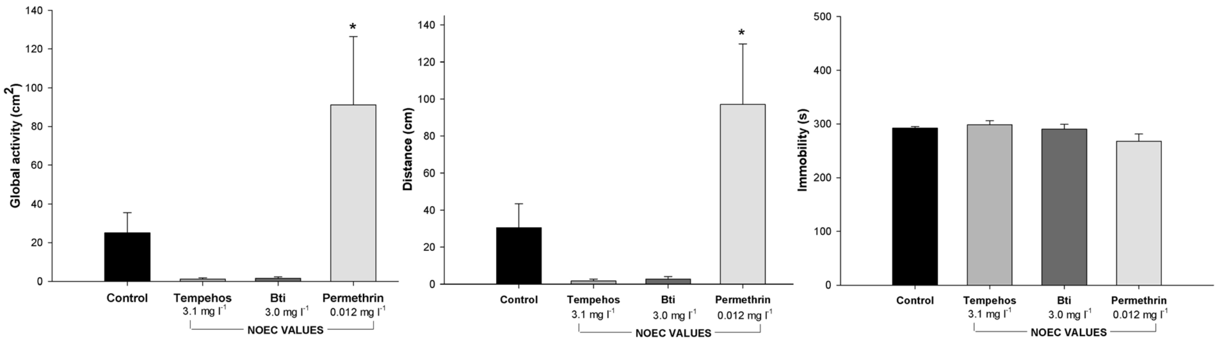

Fig. 2 Global activity, distance moved, and immobility of *R. fernandezae* tadpoles after 24 h of exposure to NOEC concentrations of insecticides and of the control group. The *bars* show mean ± SD, and *asterisks* show the treated groups that differ from the control; **p* < 0.05; ***p* < 0.01; ****p* < 0.001 (post hoc test, Dunnett)

species: *R. arenarum* (0.035 mg l^{-1}), *R. fernandezae* (0.028 mg l^{-1}), and *P. albonotatus* (0.007 mg l^{-1}). The toxicity for *P. albonotatus* is high, since these were approximately four and five times more sensitive to permethrin exposure than the other two species of toads. These results reinforced the hypothesis that amphibian species vary in their susceptibility to pesticides (Relyea and Jones 2009; Moore et al. 2012) due to intrinsic factors such as the capacity of contaminant detoxification via enzymes (Lajmanovich et al. 2010; Attademo et al. 2014). The observed difference between results of LC_{50} may also be attributed to differences in species-specific sensitivity. Another studies available also suggested that permethrin can be classified as highly toxic to amphibians, with LC_{50} values ranging from of 0.0025 mg l^{-1} (for *Rana temporaria*, Johansson et al. 2006) to 0.693 mg l^{-1} (for *Lithobates catesbeianus*, Fort et al. 1999).

Regarding larvicide temephos (Abate®), LC_{50} values found in *R. arenarum* ($LC_{50-24\ h}$ = 19.76 mg l^{-1}; $LC_{50-48\ h}$ = 16.79 mg l^{-1}) were similar to the concentration reported by Harischandra et al. (2011) for *D. melanostictus* (Bufonidae) (LC_{50} = 16.56 mg l^{-1} ± 0.47). Comparatively, temephos seemed to be less toxic to *P. albonotatus* ($LC_{50-48\text{-}h}$ = 5.88 mg l^{-1}) than to *R. fernandezae* ($LC_{50-48\text{-}h}$ = 4.08 mg l^{-1}) but was similar to LC_{50} value (4.18 mg l^{-1}) found for *Rana chensinensis* (Agnelli et al. 2015). On the other hand, among the insecticides used in this study, *B. thuringiensis* var. *israelensis* (Introban®) was the less toxic pesticide to tadpoles because it showed the less toxicity levels. Nevertheless, when this larvicide is used for mosquito control according to recommendations of the MSN (2011), the *B. thuringiensis* var. *israelensis*-aqueous suspension is applied within a range of volume (19 drops) of formulated product per square meter, regardless of the depth of waterbodies to be treated. Because water depths, even within a single wetland, can vary greatly, field concentrations of *B. thuringiensis* var. *israelensis* can also vary

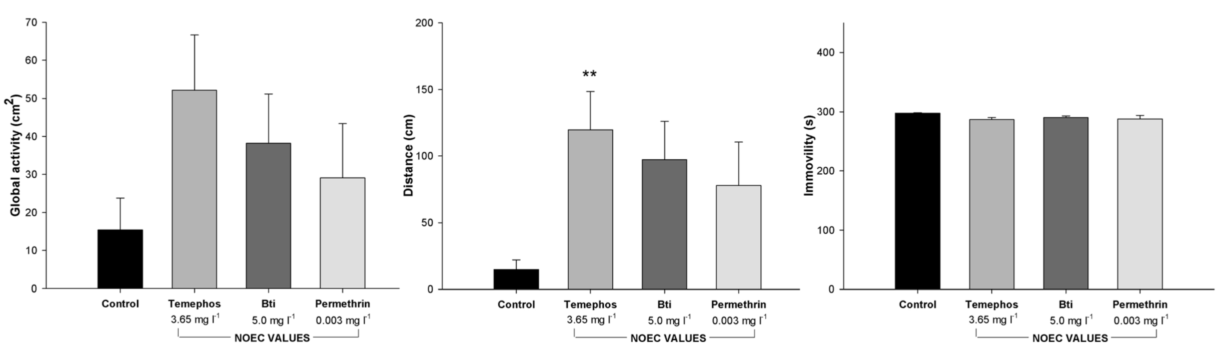

Fig. 3 Global activity, distance moved, and immobility of *P. albonotatus* tadpoles after 24 h of exposure to NOEC concentrations of insecticides and of the control group. The *bars* show mean ± SD, and *asterisks* show the treated groups that differ from the control; **p* < 0.05; ***p* < 0.01; ****p* < 0.001 (post hoc test, Dunnett)

widely, reaching a concentration greater than necessary to kill mosquito larvae. Such a scenario occurs also when temephos is applied on the aquatic habitats for larval mosquito control since the MSN (2011) recommended the application of 1 mg of temephos l^{-1} of water that the recipient is capable to store, regardless of the volume of water contained. Under this assumption, both larvicides could be more toxic to tadpoles and other non-target aquatic organisms in "real-life" situations.

Because most adulticides can be applied over or near water when used for mosquito control, there are risks to aquatic organisms from direct deposition and runoff of the pesticides. This could be important given that permethrin (Depe®) is applied at concentrations ranging between 200 and 5400 mg l^{-1} (operational dose recommended by the MSN 2011). This range is 10,000 times higher than the $LC_{50-48\ h}$ values of 0.007, 0.028, and 0.035 mg l^{-1} of permethrin for *P. albonotatus*, *R. fernandezae*, and *R. arenarum*, respectively. Permethrin is considered lipophilic because of their high octanol-water partition coefficient (log $K_{ow} > 7$, Ecological Structure Activity Relationships, ECOSAR-USEPA software) and has low water solubility, implying a possible high bioconcentration in biota (Miyamoto et al. 2015) and high ecotoxicological risk for anuran larvae.

Behavioral endpoints associated with locomotion of amphibians, and computed from video tracking, were affected after a short-term exposure to insecticides. Tadpoles of *R. arenarum* exposed to permethrin (0.047 mg l^{-1}), *B. thuringiensis* var. *israelensis* (13.88 mg l^{-1}), and temephos (16 mg l^{-1}) changed different traits related to activity and space use. Exposed tadpoles spent less time swimming, traveled shorter distances, and spent longer time motionless than the control. However, at a sublethal concentration (0.012 mg l^{-1}) for tadpoles of *R. fernandezae*, permethrin appears to have a stimulatory effect rather than a sedative one, since tadpoles spent more time swimming and traveled greater distances than the control individuals. A similar effect was observed for temephos (3.65 mg l^{-1}) in tadpoles of *P. albonotatus* that traveled greater distances. Data confirm that the exposure of tadpoles to smaller concentrations than those that cause significant mortality (NOEC-24 h value) affects their swimming performance. As a consequence of sublethal exposure, tadpoles can react in different ways either decreasing (Brunelli et al. 2009; Denoël et al. 2012, 2013) or increasing (Mandrillon and Saglio 2007; Peltzer et al. 2013) their swimming activities. These differences in behavioral swimming could vary depending on the chemical properties of insecticides (type, concentration, modes of action) and the time of exposure. In the case of *R. arenarum*, all ethological responses were significantly affected by the three insecticides. Here, it appears that the effects observed on the behavior may be due to the higher concentrations of exposure (0.047 mg l^{-1} permethrin, 13.88 mg l^{-1} *B. thuringiensis* var. *israelensis*, and 16 mg l^{-1} temephos) with respect to the NOEC of other species (*R. fernandezae* = 0.012 mg l^{-1} permethrin; *P. albonotatus* = 3.65 mg l^{-1} temephos). It is possible that significant behavioral alterations observed in *R. arenarum* can be attributed to a dose-response effect of insecticides. Since environmental concentration data are scarce and unreliable for many substances (Smetanová et al. 2014), usually NOEC data are used, which may not be a "safe" no-effect concentration due to the fact that a biologically significant effect may occur at a test concentration (Jager 2012). However, the reported data indicate that behavior is a parameter considered "ecologically relevant" and, thus, more research is needed to obtain ecotoxicity data from these powerful tools.

To summarize, because the range of application of permethrin is 10,000 times higher than the $LC_{50-48\ h}$ values for tadpoles, it is strongly recommended to avoid insecticides reaching the water phase for the conservation of aquatic non-target organisms, including amphibians. The application of larvicides to treat the larval habitats of mosquitoes should be considered only in case of real need and focused on well-defined target areas, since in a real scenario larvicides the could be exposed to concentrations similar or higher than NOEC and LOEC values reported in this work. Moreover, the use of chemicals for mosquito control should be considered a complementary method of environmental management. For example, taking into account the role of tadpoles in biological control of mosquitoes (Bowatte et al. 2013; Sarwar 2015), the development and implementation of conservation programs to protect amphibians can minimize the use of pesticides for mosquito control and thus, decrease vector-borne disease risks to humans, mostly malaria, lymphatic filariasis, encephalitis, zika, chikungunya fever, dengue, and other deadly diseases (Hagman and Shine 2007; Rubbo et al. 2011). Finally, we demonstrated that the swimming patterns of tadpoles were altered at concentrations below those causing mortality (NOEC values). Assuming that these concentrations could occur in a real scenario, tadpoles and other non-target aquatic organisms could have severe implications for survival if the performance of normal behavior was altered. For this reason, it is needed to integrate behavioral endpoints at the standard toxicological assays and increase the significance and usefulness of these parameters as indicators of aquatic toxicity, mainly when there are no publicly available data concerning the deposition of insecticides in waterbodies after applications.

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Compliance with Ethical Standards

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

Ethical Approval All procedures performed in this study involving animals were in accordance with the ASIH (2004) criteria and ethical standards of the animal ethics committee of the Facultad de Bioquímica y Ciencias Biológicas, FBCB Res. CD no. 388/06.

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