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Gabriela Svartz, Carolina Aronzon & Cristina Pérez Coll

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RESEARCH ARTICLE

Comparative sensitivity among early life stages of the South American toad to cypermethrin-based pesticide

Gabriela Svartz[1,2] · **Carolina Aronzon**[1,2] · **Cristina Pérez Coll**[1,2]

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Abstract Cypermethrin is one of the most widely used pesticides due to its low mammalian and bird toxicity, but it is extremely toxic to aquatic organisms. The aim of the present study was to evaluate the toxicity of a commercial formulation of cypermethrin on the embryo–larval development of *Rhinella arenarum*. An ecological risk assessment based on the hazard quotient (HQ) approach was performed. The results showed that cypermethrin toxicity was stage-dependent and dramatically increased during the larval period. Thus, larvae were more sensitive than embryos, obtaining at the end of the experiment a 336-h median lethal concentration (LC50) of 0.65 μg cypermethrin/L. Cypermethrin exposure caused morphological abnormalities such as general underdevelopment, edema, gill malformations, and behavioral alterations as hyperkinesia and spasmodic contractions. The 168-h teratogenic index was 5, implying a high risk for embryos to be malformed in the absence of significant embryonic lethality. Based on the results of the toxicity effects and the ecological risk assessed (HQ for chronic exposure>level of concern), this pesticide should be considered as a direct (effects on survival) or indirect (severe sublethal effects) risk for conservation purposes of this amphibian in agroecosystems.

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✉ Cristina Pérez Coll
cris060957@yahoo.com.ar; perezcoll@unsam.edu.ar

[1] Instituto de Investigación e Ingeniería Ambiental and Escuela de Ciencia y Tecnología, Universidad Nacional de San Martín, Av. 25 de Mayo y Francia (1650), San Martín, Buenos Aires, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Técnicas-CONICET, San Martín, Argentina

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Introduction

Cypermethrin, (*R*,*S*)-alpha-cyano-3-phenoxybenzyl (1RS)-cis, trans-3-(2, 2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate, is a synthetic pyrethroid, similar to natural pyrethrins but more stable in the environment. Its structure is based on pyrethrum, an extract from plants of the genus *Chrysanthemum* (Kamrin 2000). Cypermethrin-based formulations containing 25 % of a mixture of cis and trans isomers of cypermethrin are used as non-systemic insecticides to control a wide variety of insect pests associated to cotton, cereal, vegetable, and fruit crops. Pyrethroids, including cypermethrin, are replacing other insecticides as organochlorines and organophosphates due to their low persistence in the environment and low toxicity to mammals and birds (Berrill et al. 1993), so the increasing presence of cypermethrin in the environment is of concern. Cypermethrin is one of the most widely used insecticides in Argentina, where application rates are between 60 and 280 g active ingredient/ha, and over 3.5 million liters per year are consumed in this country (CASAFE 2010). Cypermethrin concentration levels in runoff and stream water of agricultural streams in the main soybean area of Argentina were reported to be ranging from 0.2 to 194 μg/L (Jergentz et al. 2005; Marino and Ronco 2005).

Cypermethrin is stable to hydrolysis with a half-life greater than 50 days and to photodegradation with a half life greater than 100 days (USEPA 1989). Despite relative insolubility in water, all pyrethroids are sufficiently soluble to cause adverse effects to aquatic organisms. Their lipophilicity allows them to be readily absorbed by biological membranes and tissues

(Oros and Werner 2005). Moreover, cypermethrin formulations are combined with coadjuvants, decreasing its superficial tension and increasing solubility, leading to high toxicity also on non-target organisms. Particularly, amphibians play a key role in food webs, living near or in water reservoirs affected indirectly by pesticides. Amphibians are extremely sensitive because they have permeable skin and eggs that readily absorb chemicals from the environment (Boyer and Grue 1995). Moreover, many species complete their life cycle in ponds and streams adjacent to agricultural fields where pesticides are applied, and these applications often coincide with breeding and larval development (Hayes et al. 2003). Not only studies on lethal but also sublethal effects caused by pesticides are highly valuable due to the indirect effects that increase the vulnerability to predation and fitness reduction, which could eventually affect the projection of amphibian populations (Little et al. 1990). Sublethal effects were registered at environmentally relevant concentrations. In *Hypsiboas pulchellus* larvae exposed to 0.3–3.4 μg cypermethrin/L, axial flexures and multiple malformations of eyes, head, and intestines as well as behavioral alterations were observed (Agostini et al. 2010). Also, *Rana temporaria* exposed to concentrations as low as 1 μg cypermethrin/L at early developmental stages exhibited underdevelopment, reduced body size, and tail flexures, whereas at latest developmental stages, the pesticide caused the inhibition of metamorphosis (Paulov 1990).

Pyrethroids act primarily on the nervous system, and its commonly accepted mechanism of action is the prolongation of the open state of voltage-dependent sodium channels (Vijverberg and van den Bercken 1990; Narahashi 2000; Soderlund et al. 2002). It has been shown that cypermethrin inhibits ATPase enzymes, particularly critical to aquatic organisms because these provide the energy for active transport and are especially important at sites of oxygen exchange. ATPase inhibition and disruption of active transport possibly affect the ion movement and the ability to maintain ion balance and disrupt respiratory surfaces, indicating that cypermethrin is inherently more toxic to aquatic organisms (Siegfried 1993). Then, it is not surprising that cypermethrin is considered as highly toxic to aquatic biota (USEPA 2008).

Toxicity bioassays represent useful tools to assess the risk of ecosystems to exposure to different physicochemical agents. In this sense, AMPHITOX test is a battery of bioassays that use embryo–larval stages of the common South American toad, *Rhinella arenarum* (Herkovits and Perez-Coll 2003). Previously, we reported the effects of cypermethrin on *R. arenarum* early larvae (Svartz and Perez Coll 2013) by means of the AMPHICHRO test (Herkovits and Perez Coll 1999; Herkovits et al. 2002). This test, included in the AMPHITOX battery, offers the advantage of evaluating toxicity by means of endpoints that just need a quick and easy analysis such as mortality that does not need too much observer expertise, but sublethal effects, which are subtle in many cases, would be beyond this analysis. The evaluation of sublethal effects is particularly important because these usually occur at environmentally relevant concentrations, as in the case of agroecosystems where pesticides are applied. The AMPHIEMB, also included in the AMPHITOX battery, is an early life stage toxicity test that also allows assessment of effects on both morphogenesis (teratogenesis) and behavior but requires a more careful and detailed analysis of an expert researcher. It also highlights the relevance to dispose a toxicity profile of a xenobiotic as completely as possible. Although the pulse-exposure toxicity tests are performed at high levels, simulate concentrations were reached immediately after field pesticide application, before dilution by rain or irrigation or concentrations which can occur during environmental emergencies as spilling pesticide during transport or deficiency of the application equipment. This model allows to identify the most sensitive developmental stage and to establish the maximum threshold level of the xenobiotic in the environment where it is intentionally applied, focusing on conservation purposes of the native species. The aim of this study was to assess lethal and sublethal effects (morphological and behavioral alterations) of a commercial formulation of cypermethrin (Glextrin 25®) on *R. arenarum* embryos and larvae by means of acute (96 h), short-term chronic (7 days), chronic (14 days), and 24-h pulse exposure toxicity bioassays. The results were discussed within an ecological risk assessment of cypermethrin based on the hazard quotient approach (USEPA, 1998) on this native species and its toxicity mechanism.

Materials and methods

Acquisition of *R. arenarum* embryos and larvae

To examine the potential effects of the commercial formulation of cypermethrin on the embryo–larval development of *R. arenarum*, three mating pairs of adults weighing approximately 200–250 g per animal were acquired in a non-impacted site, Lobos (Buenos Aires province, Argentina: 35°11′ S, 59°05′ W). Toad care, breeding, embryo acquisition, and analysis were conducted according to the methods described in the AMPHITOX protocols (Herkovits and Pérez-Coll 2003). Briefly, ovulation of females was induced by means of an intraperitoneal injection of a suspension of one homogenized toad pituitary gland in 1 mL of AMPHITOX solution (AS) per female, preserved according to Pisanó (1956), plus 2500 IU human chorionic gonadotropin (hCG). The composition of AS was NaCl 36 mg/L, KCl 0.5 mg/L, $CaCl_2$ 1 mg/L, and $NaHCO_3$ 2 mg/L, prepared in distilled water. Eggs were fertilized in vitro using a testicular macerate homogenate suspended in AS, resulting in a spermatozoid suspension of 10 %. The sperm viability was confirmed by observing the spermatozoid morphology and movements under an optic microscope. The egg quality and fertility were inspected and

considered acceptable if the fertility rate was greater than 75 % and embryo survival at the neurula stage was greater than 70 %. Embryos were staged according to Del Conte and Sirlin (1951) as follows: early blastula (S.4), neural plate (S.13), muscular activity (S.18), gill circulation (S.20), opercular folds (S.23), and complete operculum (S.25) stages. For embryos used before hatching (S.18), the jelly coat was dissolved by immersing egg ribbons in a solution of 2 % thioglycolic acid neutralized at pH 7.2–7.4 with 1.35 mL of saturated sodium hydroxide (NaOH) solution every 100 mL in AS. This step was followed by a thorough wash of the embryos. Embryos were kept in AS and maintained at 20±2 °C. The AS was replaced entirely every 3 days and monitored weekly to ensure that the pH was at acceptable levels (7±0.5).

Preparation of test solutions

Toxicity tests were performed using a commercial formulation with 25 % active ingredient of cypermethrin (Glextrin 25®, Gleba S.A.). The formulation is an emulsionable concentrate and contains a mixture of cis and trans isomers of cypermethrin. The nominal concentration of the stock solutions was 250 mg cypermethrin/L. The cypermethrin concentration in stock solution was verified by chromatographic methods using a GC/MS (Agilent 5975C equipment) with a DB5-MS 30×0.25-mm column and 0.25-μm film thickness, helium as a gas carrier, and a programme temperature of 150 °C 8 °C/min–280 °C.

Toxicity bioassays

Ten embryos or larvae were randomly placed in triplicate 10-cm-diameter glass Petri-dishes containing 40 mL of test solution. Cypermethrin concentrations tested ranged between 0.0005 and 30 mg/L. The toxicity bioassays were performed under the following conditions:

1. Continuous exposure of embryos from early blastula (S.4) up to complete operculum stage (S.25) for acute (96 h), short-term chronic (168 h), and chronic (240 and 336 h) periods.
2) Continuous exposure of larvae from early complete operculum stage (S.25) up to late complete operculum stage (S.25) for acute (96 h), short-term chronic (168 h), and chronic (240 and 336 h) periods.
3. 24-h pulse exposure of embryos at different embryonic stages: blastula (S.4), neural plate (S.13), muscular activity (S.18), gill circulation (S.20), opercular folds (S.23), and complete operculum (S.25). After 24-h pulse treatments, embryos were thoroughly washed and kept in AS until 336 h post-exposure.

Organisms were maintained at 20±2 °C and 12:12 h light/dark photoperiod. Larvae were fed with three granules (6± 0.5 mg) of balanced fish food TetraColor® per Petri-dish. Test solutions were entirely replaced every 48 h. Control groups were simultaneously maintained in AS without additions.

Lethal and sublethal effects were evaluated each at 24 h, comparing the alterations with the normal development and behavior of controls. Morphological effects such as developmental delay, cellular dissociation, irregular surface, persistent yolk plug, underdeveloped gills, microcephaly, wavy tail, and edema were evaluated. Neurotoxicity endpoints included spasmodic contractions, alterations in swimming, and narcosis. Smooth movements of the Petri dishes, followed by stimulation with a light source, were done. In case of no response, soft mechanic stimulation with a glass rod was made, and finally heartbeat was checked. Also, feeding behavior was qualitatively assessed. Abnormalities and neurotoxic effects were observed under a binocular stereoscopic microscope (Zeiss Stemi DV4), photographed and recorded with a Sony DSC-S90 digital camera, and identified according to Bantle et al. (1998). Embryos with significant adverse effects and controls were fixed in 4 % formalin, dehydrated in a gradient of ethanol, prepared for scanning electron microscopy (SEM) by means of the critical point drying technique, and observed in a Philips XL-30 operated at 10 kW for ultrastructure evaluation.

Data analysis

Median lethal concentrations (LC50s) were statistically estimated by the USEPA Probit Program (USEPA 1988). To compare LC50 values, differences were considered to be statistically significant when the higher LC50/lower LC50 ratio exceeded the corresponding critical value established by the American Public Health Association (2005). The Teratogenic Index (TI) was calculated as NOEC (no observed effect concentration) lethality/NOEC sublethality at 168 h. TI>1.5 implies a high risk for embryos to be malformed in the absence of significant embryonic lethality (ASTM 1993). We conducted a one-way analysis of variance (ANOVA) to evaluate the NOEC value, and Tukey's tests were used to compare treatment means where significant ($P<0.05$). For this analysis, the GraphPad Prism software version 6.03 was used.

Ecological risk evaluation

The ecological risk was assessed using the hazard quotient (HQ) approach, which was calculated as EEC/NOEC of lethality (USEPA 1998). The EEC is an estimated (or maximum) environmental contaminant concentration at the site, with 0.194 mg cypermethrin/L being the maximum level reported in Argentina (Marino and Ronco 2005). After the risk quotient was calculated, it was compared to the USEPA level of concern (LOC). The LOC is a policy tool that the Agency

uses to interpret the HQ and to analyze the potential risk to non-target organisms and the need to consider regulatory action. The LOC value for risk is 1. If the HQ>1, harmful effects are likely due to the contaminant in question.

Results

Continuous exposure of embryos and larvae for 336 h

Acute lethal effects of cypermethrin for *R. arenarum* embryos were just observed from 30 mg/L, reaching 100 % lethality before 48 h exposure, whereas the remaining concentrations caused more gradual lethality over time. On the other hand, cypermethrin toxic effects on larvae were very dramatic just from the first hours; significant mortality from 96 h at concentrations above 2.5 mg/L ($p<0.05$) was registered.

Figure 1 highlights the significant increase of cypermethrin toxicity over time both for embryos and larvae; at all exposure times, larvae were more sensitive than embryos. So, for embryos, at acute and short term-chronic exposure, the LC50 at 96, 168, and 240 h were 11.20, 6.44, and 3.50 cypermethrin/L, respectively, whereas for larvae these were 6.43, 2.74, and 1.82 mg cypermethrin/L respectively. Moreover, for short-term chronic period, the lethality 168-h NOEC for embryos was 2.5 mg cypermethrin/L, whereas for larvae, it was as low as 0.5 mg/L. During chronic exposure, embryo lethality persisted much longer, obtaining a 336-h LC50 of 1.85 mg cypermethrin/L, while for larvae lethality was even more severe, reaching a 336-h LC50 of as low as 0.65 μg/L.

Sublethal effects of cypermethrin on *R. arenarum* embryos consisted of delayed development, underdeveloped gills, microcephaly, edema, axial flexures, and wavy tail (Fig. 2). Moreover, persistence of a relevant number of ciliated cells in specific regions of the epithelium was observed by SEM in cypermethrin exposed embryos. This observation confirms

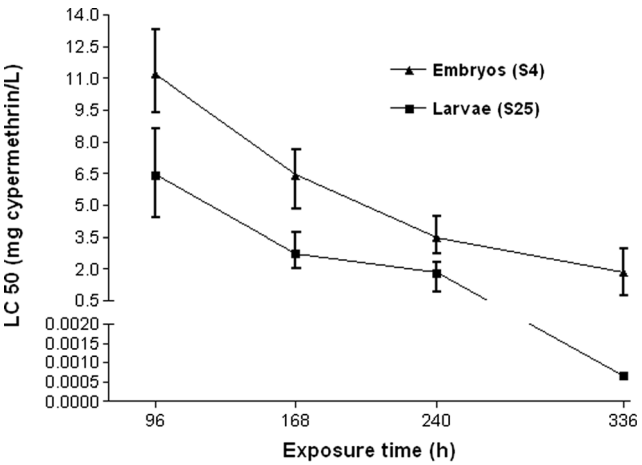

Fig. 1 Toxicity profile (TOP curves) of cypermethrin for *R. arenarum* embryos and larvae continuously exposed to the pesticide

the delayed development of exposed embryos with respect to controls. At muscular response stage (S.18), embryos began to develop behavioral alterations, as hyperkinesia, spasmodic contractions, abnormal and erratic swimming, evolving to general weakness and finally lethargy and non-feeding behavior. The sublethal 168-h NOEC was 0.5 mg/L, so the TI was 5.

The sublethal effects on larvae were mainly associated with behavioral alterations. The neurotoxicity was expressed as hyperkinesia, spasmodic contractions, erratic swimming, lack of correct equilibrium, non-feeding behavior, and reduced movements up to narcosis. These effects were observed from the first hours and even at the lowest tested concentrations (0.5 μg cypermethrin/L).

24-h pulse exposure at different developmental stages

Figure 3 shows the LC50 values for embryonic stages at different post-exposure times. S.18 was the most sensitive stage at all observation times, obtaining 96- and 336-h LC50 of 1.08 (0.47–3.04) and 0.33 (0.15–0.91) mg cypermethrin/L respectively. At a few hours post-exposure, embryos at early stages (S.4, S.13, and S.18) were significantly more sensitive than later stages (S.20, S.23, and S.25), while toward the end of the observation period, this pattern was inverted. So, at acute period the order of sensitivity from most to least sensitive to cypermethrin was as follows: S.18>S.13–S.4>S.20–S.23-S.25. Finally, at chronic period, the order of sensitivity was as follows: S.18>S.20–S.23–S.25>S.4–S.13. The sensitivity of embryos exposed at S.4 remained almost constant up to 240 h but dramatically increased four times at subsequent hours, obtaining 240- and 336-h LC50 of 13.63 (12.69–14.65) and 3.42 (1.71–6.84) mg cypermethrin/L. In contrast, the toxicity of the pesticide during late embryonic stages significantly increased from the first hours of post-exposure, obtaining for S.20 96- and 168-h LC50 of 17.08 (13.09–22.3) and 7.29 (3.55–14.99) mg cypermethrin/L, respectively.

Teratogenic effects on pulse-exposed embryos at early stages were observed just during the first hours post-exposure at higher concentrations (from 10 mg cypermethrin/L), and these were cellular dissociation, delayed development, and persistent yolk plugs. As the development advanced, reduced body size, underdeveloped gills, microcephaly, axial flexures, wavy tail, and edema were also observed.

From the beginning of the neuromuscular activity (S.18), alterations in behavior were the most important events which extended during the larval period. In this case, from the first hours after exposure, the alterations were hyperkinesia, erratic swimming, spasmodic contractions that evolved to reduced movements until absence of both spontaneous and mechanically/light induced movements. Also, reduced food intake in exposed larvae was observed. Embryos which were 24-h pulse-exposed at late stages (S.20, S.23, and S.25) at lower concentrations (up to 0.5 mg cypermethrin/L) recovered their normal behavior after 10 days post-exposure.

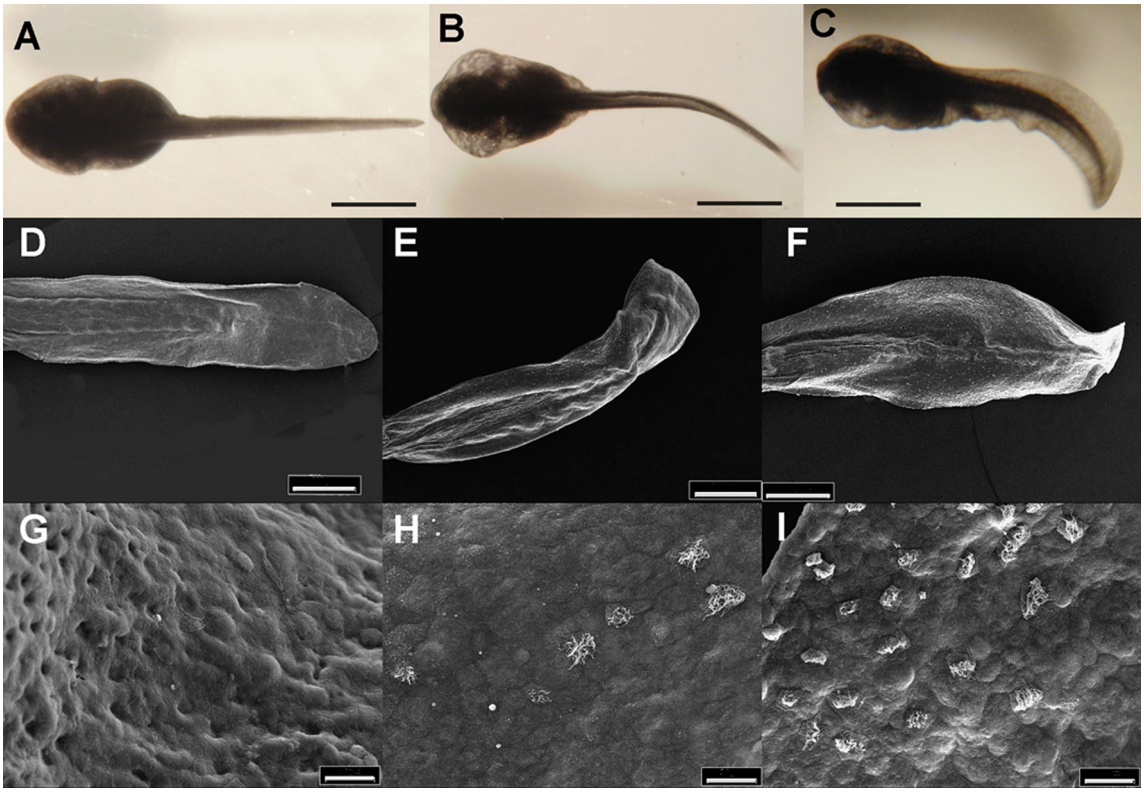

Fig. 2 Stereoscopic and scanning electron microscopy pictures of *R. arenarum* embryos continuously exposed to cypermethrin from blastula stage (S.4) fixed at 168 h. **a**, **d**, **g** Control embryo at right operculum closed stage (S.24): **a** panoramic view, **d** tail detail, **g** epithelium surface. **b**, **e**, **h** Embryo exposed to 0.05 mg cypermethrin/L: **b** edema and axial flexure, **e** axial flexures and folded tail, **h** persistence of scarce ciliated cells in specific regions of the epithelium. **c**, **f**, **i** Embryo exposed to 0.5 mg cypermethrin/L: **c** general underdevelopment, edema, wavy tail and axial flexure, **f** folded tail and axial flexure, **i** persistence of relevant number of ciliated cells in specific regions of the epithelium. *Scale bars* **a**–**c** 2 mm, **d**–**f** 500 μm, **g**–**i** 20 μm

Ecological risk assessment

Risk evaluation analysis for embryos and larvae continuously exposed to cypermethrin (Section 3.1) were performed. Hazard quotients (HQ) for embryos and larvae were calculated as EEC/NOEC of lethality (USEPA 1998). Based on local reported values, the EEC (estimated environmental concentration) of 0.194 mg cypermethrin/L was considered as the maximum environmental water level (Marino and Ronco 2005). NOEC of lethality values of cypermethrin for *R. arenarum* were obtained for embryos and larvae at different exposure times. After the risk quotients were calculated, they were compared to the USEPA level of concern (LOC=1).

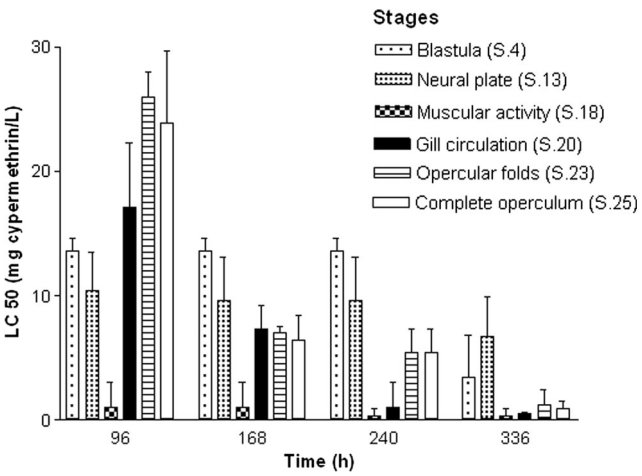

Fig. 3 Median lethal concentration and its corresponding confidence limit (95 %) in cypermethrin 24-h pulse exposure in *R. arenarum* embryos at different developmental stages

Table 1 shows the HQ values obtained for embryos and larvae exposed to cypermethrin at different times. It highlights that HQ values for both stages at acute and short-term chronic exposure periods were below the LOC value, but the HQ was above the LOC value for chronically exposed larvae (336 h) to the pesticide.

Discussion

The results obtained in the present study highlight the toxicity of a commercial formulation of cypermethrin on *R. arenarum* embryo–larval development. This study confirms and extends our previous results on the comparative toxicity of this

Table 1 Toxicity and hazard quotients of cypermethrin for *R. arenarum* exposed from embryo and larval stages

	Exposure time (h)							
	96		168		240		336	
	NOEC[a]	HQ	NOEC	HQ	NOEC	HQ	NOEC	HQ
Embryos (S.4)	5	0.04	2.5	0.08	1	0.19	0.5	0.39
Larvae (S.25)	1	0.19	0.5	0.39	0.5	0.39	<0.0005	>388[b]

[a] No observed effect concentration (mg cypermethrin/L) and HQ values for acute (96 h), short-term chronic (168 h), and chronic (240 and 336 h) exposures

[b] HQ>1, estimates harmful effects from cypermethrin exposure

commercial formulation and the active ingredient of cypermethrin showing the much higher toxicity of the formulated product (Svartz and Pérez Coll 2013), which was a very relevant fact considering that it is really applied to control pests. This differential sensitivity was also shown for most pesticides (Mann and Bidwell 1999; Relyea 2009; Aronzon et al. 2011) and could be explained due to the effects of the coadjuvants, causing additional toxicity and a magnification of the active ingredient activity. So, this work was extended to assess the toxicity of this commercial formulation of cypermethrin on different early stages of *R. arenarum*.

The toxicity of cypermethrin on the embryo–larval development of *R. arenarum* revealed that larvae are much more sensitive than embryos at all exposure times. Since pyrethroids mainly act on sodium and calcium channels of nervous tissues, the highest sensitivity of late stages could be related to the nervous system maturation. So, cypermethrin toxicity on larvae was almost 2800 folds higher than embryos, obtaining 336-h LC50 of 1.85 and 0.00065 mg cypermethrin/L for embryos and larvae, respectively. A similar pattern was observed in the case of 24-h pulse exposure treatments, in which muscular activity stage (S.18) was the most sensitive, and at chronic exposure time the toxicity was higher for late stages than for early stages. This stage-dependent sensitivity was also reported in other amphibian studies, where the larval stages were more sensitive than the embryonic ones (Berrill et al. 1993; Biga and Blaustein 2013). Moreover, embryo recovery observed in this study after 24-h pulse exposure was also reported for *Rana clamitans* larvae exposed to cypermethrin for 96 h that exhibited behavioral alterations and significantly smaller body size than non-exposed larvae, which were reverted 25 days post-exposure (Berrill et al. 1993). However, despite the fact that embryos could recover from the toxic effects, few hours of exposure may represent vulnerability to developing amphibians while recovery is underway.

Comparatively, the acute lethal effect of cypermethrin on *R. arenarum* was less severe than those obtained for other amphibians. So, the 96-h LC50 for *Hypsiboas pulchellus* larvae was 0.18 mg cypermethrin/L (Agostini et al. 2010) and for other bufonid species, such as *Bufo melanostictus* larvae, was 0.009 mg cypermethrin/L (Saha and Kaviraj 2008), whereas *R. arenarum* larvae at acute exposure was less sensitive, obtaining a 96-h LC50 of 6.43 mg cypermethrin/L. Although the acute lethal effects of this pesticide on *R. arenarum* exceed the environmentally relevant concentration range, sublethal and chronic effects were of great concern, highlighting the significance and relevance of evaluating anomalies and chronicity of exposure for risk assessment studies. Furthermore, the teratogenic potential of this pesticide represented by a 168-h TI of 5 was three times the threshold level to consider a substance as high risk for embryos to be malformed in the absence of significant lethality (ASTM 1993). The cypermethrin morphological alterations were similar as those observed in other amphibians, as well as the typical pyrethroid behavioral alterations (Agostini et al. 2010; Ghodageri and Pancharatna 2011; David et al. 2012). Embryos exhibited severe malformations as microcephaly and edema; other alterations reflecting delayed development as underdeveloped gills and persistence of ciliated cells were also observed. Some malformations as axial flexures and wavy tails interfere with the movements and the normal swimming of organisms, hindering the feeding and the predator avoidance behavior. The pyrethroid neurotoxicity reflected in hyperkinesia and spasmodic contractions has been explained by the apoptosis on the central nervous system induced by cypermethrin (Izaguirre et al. 2000; Casco et al. 2006).

As was previously mentioned, pyrethroids are particularly more toxic to aquatic biota than for mammals and birds probably due to its low efficiency of the detoxification systems. In fishes, a low biotransformation rate of pyrethroids was observed (Haya 1989), excreted mainly through the bile pathway (Glickman et al. 1982; Edwards et al. 1987). The disruption of the enzyme antioxidant activity caused by cypermethrin exposure was reported for *Duttaphrynus melanostictus* larvae, as well as the lipid peroxidation that may induce apoptosis (David et al. 2012). On the other hand, due to their lipophilicity, cypermethrin is able to penetrate through the lipid bilayer of cells, disrupting the phospholipid orientation and inducing changes in membrane fluidity (Vaalavirta and Tahti 1995). Moreover, it was reported that cypermethrin easily permeate through the gill, which is a contributing factor in the sensitivity on aqueous exposures (Mishra et al. 2005). Cypermethrin absorption in the gill region might inhibit the enzyme gill Na^+/K^+-ATPase from disrupting the cellular and ionic regulation and

salt uptake (Suvetha et al. 2010). In addition, the cypermethrin and other pyrethroids metabolism release cyanohydrins which are unstable at physiological conditions and further decompose to cyanide and aldehyde ions. These metabolites generate free radicals, inducing oxidative stress underlying the morphological and physiological effects (Grajeda et al. 2004).

Considering the results of the risk assessment (HQs) based on cypermethrin concentration reported for natural waters in Argentina which was 0.194 mg cypermethrin/L (Marino and Ronco 2005) and the lethality NOEC, the HQs calculated were above the level of concern (LOC=1) at chronic exposure, indicating that cypermethrin represents a potential risk in the environment for *R. arenarum* larvae. However, if the HQ is calculated based on the sublethality NOEC instead of lethality NOEC values, considered to be conservative estimates (i.e., based on worst-case scenarios), the potential risk to larvae would be due to acute exposure to cypermethrin (sublethal NOEC<0.5 μg cypermethrin/L). This fact would imply that evolving populations with a significant number of abnormal individuals unable to reach the adult or reproductive stage face the challenge of keeping the population size.

Taking into account the data obtained in this study and considering the risk assessment results based on the HQ approach, the contamination of the aquatic ecosystems with cypermethrin could cause severe adverse effects on *R. arenarum* embryo–larval development and potentially threaten this amphibian native population.

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