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Combined endosulfan and cypermethrin-induced toxicity to embryo–larval development of *Rhinella arenarum*

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ABSTRACT

The combined effects of two widely used pesticides, endosulfan and cypermethrin, on survival of embryo–larval development of the South American toad (*Rhinella arenarum*) were examined. The toxicity bioassays were performed according to the AMPHITOX test. Embryos and larvae were exposed to mixtures of these pesticides at equitoxic ratios from acute or chronic exposure to evaluate interaction effects. The results were analyzed using both Marking's additive index and combination index (CI)–isobologram methods. Acute (96-h) and intermediate (168-h) toxicity of endosulfan–cypermethrin mixtures remained almost constant for larvae and embryos, but when exposure duration was increased, there was a significant elevation in toxicity, obtaining chronic (240-h) no-observed-effect concentrations (NOEC) values of 0.045 and 0.16 mg/L for embryos and larvae, respectively. These are environmentally relevant concentrations that reflect a realistic risk of this pesticide mixture to this native amphibian species. The toxicity increment with the exposure duration was coincident with the central nervous system development on embryos reaching the larval period, the main target organ of these pesticides. The interactions of the pesticide mixtures at acute and chronic exposure were antagonistic for embryo development (CI > 1), and additive (CI = 1) for larvae, while chronic exposure interactions were synergistic (CI < 1) for both developmental periods. Data indicated that endosulfan–cypermethrin mixtures resulted in different interaction types depending on duration and developmental stage exposed. As a general pattern and considering conditions of overall developmental period and chronic exposure, this pesticide mixture usually applied in Argentine crop fields is synergistic with respect to toxicity for this native amphibian species.

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Argentina is one of the main agricultural producers globally, with currently approximately 25 million ha of extensive crops. This leads to the application of significant amounts of pesticides, approximately 317.17 million kg/L/yr (CASAFE, 2012), affecting environmental quality. The simultaneous presence of agrochemicals in rural environments is the most frequent scenario. However, only single toxic effects of pesticides have been usually studied disregarding eventual interaction effects deriving from additive, synergistic, or antagonistic actions on nontarget species (Loureiro et al., 2009). Moreover, methods of risk analysis usually focus on the toxicity assessment of single chemicals, underestimating the risk associated with the exposure to mixtures of compounds (DeRosa et al., 2004). However, in the last years there have been an increasing number of studies concerning toxic mixtures of chemicals in various species (Bernal et al., 2009; Bjergager et al., 2012; Gomez-Eyles et al., 2009; Harabawy and Ibrahim, 2014; Jin-Clark et al., 2002; Munkegaard et al., 2008; Pascotto et al., 2015; Rodea-Palomares et al., 2010; Wei et al., 2014).

Several models are available to study the interaction of compounds in mixtures. Rodea-Palomares et al. (2010) reported an environmental application of the median–effect/combination index (CI)–isobologram originally developed by Chou (2006), which enables quantitative determinations of chemical interactions at different concentrations and effect levels. This method was recently used to determine interactions among pollutants from different sources (Boltes et al., 2012; González-Pleiter et al., 2013; Rodea-Palomares et al., 2010, 2012; Rosal et al., 2010;

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Yang et al., 2015). The additive index uses a more quantitative approach (Marking, 1977). In this method, the additive index represents simple additive (additivity), greater than additive (synergism), and less than additive (antagonism) effects by zero, positive, and negative values, respectively. The advantages over existing methods include linearity for all index values and a procedure for determining significance of indices. For these reasons, various investigators selected this method to evaluate interaction effects of agrochemical mixtures on aquatic organisms (Green and Abdelghani, 2004; Howe et al., 1998; Key et al., 2007; Wei et al., 2014).

The aim of the present study was to assess mixture toxicity of endosulfan and cypermethrin, two widely used pesticides in Argentina, on embryos and larvae of the common South American toad *Rhinella arenarum*. Endosulfan is an organochlorine (OC) pesticide considered a persistent organic pollutant (POP) because of its high level of toxicity to living organisms, persistence in the environment, high bioaccumulation potential, and capacity for long-distance migration from its application site. For these reasons, the United Nations Association in 2011 banned use of endosulfan globally (United Nations, 2011). However, despite regulations and restrictions, it is still largely used, particularly in some developing countries such as Argentina, where it has been phased out since July 2013 (SENASA, 2013). Endosulfan levels ranging from 0.1 to 100 µg/L were reported in ground and surface waters in intensive agricultural areas (Dalvie et al., 2003; Leonard et al., 2001), with exceptional high levels of up to 500 µg/L occurring after runoff events (U.S. Environmental Protection Agency [EPA], 2001). The mechanism of action underlying toxicity is the overstimulation of the central nervous system (CNS), with inhibition of calcium and magnesium ATPase (Paquette and Liem, 1999).

Cypermethrin is a synthetic pyrethroid used as nonsystemic pesticide that acts by contact and ingestion to control a wide range of insects associated with cotton, cereal, vegetable, and fruit crops. Concentrations ranging from 0.2 to 150 µg/L cypermethrin were detected in streams near agroecosystems (Garforth and Woodbridge, 1984; Jergentz et al., 2005; Marino and Ronco, 2005). Pyrethroids act primarily by extending the open state of voltage-dependent sodium channels in nervous tissue (Narahashi, 2000; Soderlund et al., 2002; Wolansky and Tornero-Velez, 2013). In our previous studies both severe lethal and sublethal effects were examined in *Rhinella arenarum* embryos and larvae exposed to environmentally relevant concentrations of endosulfan (Svartz et al., 2014) and cypermethrin (Svartz and Pérez-Coll, 2013; Svartz et al., 2015).

Toxicity bioassays represent useful tools for assessing the environmental risk to different physicochemical agent exposures. In this sense, the AMPHITOX test is a battery of bioassays using embryonic and larval stages of *R. arenarum* (Herkovits and Perez-Coll, 2003). Usually, toxicity bioassays are performed utilizing organisms at only one life-cycle stage, typically the easiest to handle. However it is important to determine the toxicity profile of a xenobiotic, as completely as possible, in view of objectives of nature conservation. The bioassays with larvae offer the advantage of evaluating toxicity by means of endpoints that just need a rapid and easy parameter such as mortality that does not require observer expertise, but sublethal effects, which are subtle in many cases, go beyond this type of analysis. The evaluation of sublethal effects is particularly important because generally these occur at low and environmentally relevant concentrations, that is, in the most common scenarios, as in the case of agroecosystems where pesticides are applied. The sublethal effects of pesticides on amphibians may be valuable in the assessment of the sensitivity to contaminants that may produce detrimental effects such as increased vulnerability to predation and a reduction in fitness, which ultimately affect amphibian populations (Little et al., 1990). Toxicity tests with embryos (Early Life Stage test) enable one to assess effects not only on survival but also on morphogenesis (teratogenesis) and behavior, but require a more careful and detailed analysis of an expert researcher. The aim of the present study was to assess the combined effects of endosulfan and cypermethrin on *R. arenarum* embryos and

larvae by means of the AMPHITOX test (Herkovits and Pérez-Coll, 2003).

Materials and methods

Acquisition of rhinella arenarum *embryos and larvae*

To examine the potential effects of endosulfan-cypermethrin mixture on the embryo-larval development of *R. arenarum*, 3 mating pairs of adults weighing approximately 200–250 g per animal were acquired in a non-pesticide-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 (ip) injection of a suspension of 1 homogenized toad pituitary gland in 1 ml 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. Oocytes were fertilized in vitro using a testicular macerate homogenate suspended in AMPHITOX solution, resulting in a spermatozoid suspension of 10%. The sperm viability was confirmed by observing spermatozoid morphology and movements under an optical microscope. Eggs were inspected for quality and fertility and considered acceptable if the fertility rate was greater than 75%, and embryo survival at the neurula stage was greater than 70%. For embryo treatment, the jelly coat was dissolved by immersing egg ribbons in a solution of 2% thioglycolic acid at pH 7.2 containing 1.35 ml saturated sodium hydroxide (NaOH) solution in 100 ml AS. This step was followed by a thorough wash of embryos. For larvae experiments, embryos were kept in AS and maintained at 20 ± 2°C. AS was replaced entirely every 3 d and monitored weekly to ensure that pH 7 was maintained.

Preparation of test solutions

An endosulfan stock solution was performed using technical-grade endosulfan (PS81; Supelco) with a purity of 99%. A primarily stock solution containing 1 g endosulfan/L was prepared by dissolving endosulfan in analytical-grade acetone. The exposure concentrations were obtained by diluting the stock solution with AS. Maximum acetone concentration in test solutions was 0.5 mL/L, lower than 1.1% (ASTM, 1993). The concentration of endosulfan in stock solution was analyzed by high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC-EIMS) (negative mode), the identity of the compound was confirmed by scan detection, and the ion m/z = 405 and m/z = 407 were used for quantification (Chusaksri et al., 2006). The solution was analyzed daily and found to be stable over the exposure period.

Cypermethrin test solutions were obtained using a commercial formulation with 25% active ingredient (Glextrin 25), which is commercialized in Argentina by Gleba S.A. Company. The commercial formulation is an emulsifiable concentrate and contains a mixture of *cis* and *trans* isomers of cypermethrin. Test solutions were prepared from a stock solution containing 1 g cypermethrin/L. The cypermethrin concentration in stock solution was verified by chromatographic methods using a gas chromatograph (GC)/mass spectrometer (MS) (Agilent 5975C equipment) with a DB5-MS 30 × 0.25 mm column and 0.25 μm film thickness, helium as a carrier, and a programmed temperature of 150°C with 8°C/min to 280°C. The error between nominal and measured concentrations did not exceed 5%. Endosulfan–cypermethrin

Table 1. Conditions of Endosulfan (ES)–Cypermethrin (CY) Equitoxic Mixture (1:1) Toxicity Bioassays

Developmental stage	Exposure (h)	Mixture stock solution (mg/L)	Dilution (%)	Exposure concentration (mg/L)
Embryos	240 h	45 mg/L (67% ES, 33% CY)	0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100	0.0045, 0.0225, 0.045, 0.225, 0.45, 2.25, 4.5, 11.25, 22.5, 45
Larvae	240 h	32 mg/L (6% ES, 94% CY)	0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25, 50, 100	0.0032, 0.016, 0.032, 0.16, 0.32, 1.6, 3.2, 8, 16, 32

mixture toxicity bioassays were performed under the conditions summarized in Table 1.

Embryos and larvae were exposed to equitoxic mixtures, indicating that both pesticides were combined in equal proportions of their respective toxicity. Thus, ratios were expressed as the minimum entire relation of toxic units (TU). This concept, which was first described by Sprague (1970), assigns a value of 1 TU to a toxicant concentration that elicits a particular response, in the case of present study 50% mortality (LC_{50}). More explicitly, if test mixture has a value of 1 TU, it contains 0.5 TU of endosulfan (half of the LC_{50}) and 0.5 TU of cypermethrin (half of the LC_{50}); that is, the combined total toxicity is 1 TU. Binary mixtures were combined using different ratios, based on double their corresponding 168-h LC_{50} of each chemical, obtained independently and simultaneously for each clutch. Test solutions were prepared by diluting the corresponding volume of stock solutions in AS, maintaining the compound proportions.

Toxicity experimental protocols

Rhinella arenarum embryos and larvae, obtained from three different clutches, were continuously exposed to endosulfan and cypermethrin independently and in mixtures from early blastula stage (embryos) and complete operculum stage (larvae) onward for acute (96 h), intermediate (168 h), and chronic (240 h) periods. Developmental stages were identified according to Del Conte and Sirlin (1951).

For each experimental condition, triplicate batches of 10 embryos or larvae were placed in covered 10-cm-diameter glass petri dishes containing 40 ml test solution. Controls, both AS and acetone, were simultaneously maintained and did not differ markedly from one another, so the term "control" in the rest of the article represents the means of both controls. Lethality was observed and dead individuals were removed every 24 h. Test solutions were renewed every other day and temperature was maintained at 20 ± 2°C. Test solutions were analyzed daily and found to be stable over the exposure duration. Each time test solutions were changed, a stock solution was prepared for each of the pesticide being tested. Larvae were fed with 3 granules of balanced fish food TetraColor for 24 h every other day. 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 (LC_{50}) at different times were statistically estimated by the U.S. EPA Probit program (U.S. EPA, 1988). To examine statistical differences between lethal concentration values, a comparison was made, considering the difference statistically significant when the higher lethal concentration/lower lethal concentration ratio exceeded the critical value (95% confidence interval) established by the American Public Health Association et al. (2005). A two-way analysis of variance was conducted to evaluate the effect of concentration and exposure time on survival and also to determine the no-observed-effect concentration (NOEC). Dunnett's tests were used to compare treatment means at a significance level of $p < .05$. For this analysis, GraphPad Prism version 6.03 was used. The teratogenic index (TI) was calculated as lethality NOEC divided by sublethality NOEC, establishing a TI >1.5 as a high risk for embryos to be malformed in the absence of significant embryonic lethality (American Society for Testing and Materials [ASTM], 1993).

To evaluate joint toxicity, Marking's additive index of co-effects for aquatic toxicology was used (Marking, 1977; Xiu et al, 1994):

$$S = (Am/Ai) + (Bm/Bi) \quad (1)$$

where S is the sum of the toxicity of pesticides A and B; Ai is the LC_{50} of single pesticide A; Am is the LC_{50} of pesticide A in mixture; Bi is the $LC_{50}$of single pesticide B; and Bm is the LC_{50} of pesticide B in mixture.

$$\text{When} S \leq 1, \quad AI = 1/S - 1 \tag{2}$$

where AI is the additive index When S > 1,

$$AI = -S + 1 \tag{3}$$

If upper and lower limits of the additive index (AI) were <0, co-effects were considered antagonistic, and when they were >0, the co-effects were considered synergistic. Finally, if zero was included between upper and lower limits of AI, the co-effects were considered additive (Zhang et al., 2011).

The isobolograms were also plotted to illustrate additivity, synergism, or antagonism, indicating the equipotent combinations of different pesticide concentrations. An isobologram consists of classical plots of each single chemical, at a fixed concentration with a variable concentration of the other chemical. From these classical curves, iso-effects (e.g., LC_{50} values) are estimated. These values are used to generate the isobologram (Figure 1). When the chemical combination is synergistic, data points from the combination are depicted on the left side of the curve, while the combination appears antagonistic if these points lie on the right of the curve.

The results were also analyzed using the median-effect/combination index (CI)–isobologram equation according to Chou (2006) and Chou and Talalay (1984). This method is based on the median-effect principle (mass-action law) (Chou, 1976) that demonstrates that there is a univocal relationship between concentration and effect independently of number of substances and mechanism of action or inhibition. The computer program CompuSyn (Chou and Martin, 2005; Compusyn, Inc., USA) was used for the calculation of CI values at different effect levels (Fa), with Fa = 1 – (%lethality/100), for different exposure times, where CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism, respectively.

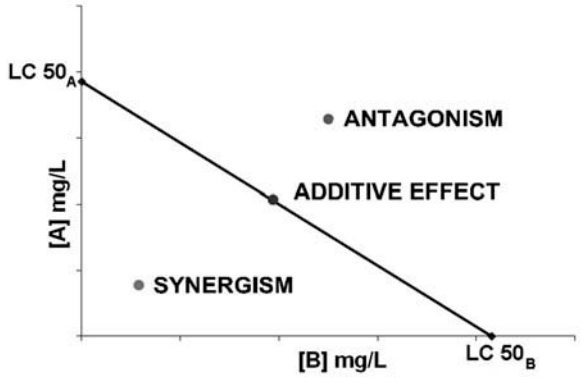

Figure 1. Example of an isobologram showing the isobole for additivity and the regions that represent synergism (to the left of the additivity isobole) and antagonism (to the right of the additivity isobole).

Results

Concentrations greater than 22.5 mg/L endosulfan–cypermethrin mixture produced acute lethal effects on embryos at 48 h, with 100% mortality of embryos exposed to the highest concentration (45 mg/L). Subsequently, lethality remained relatively constant during embryonic period up to 192 h, with a 168-h LC_{50} of the mixture (LC_{50}m) of 19.06 mg/L (Figure 2) and a NOEC value of 11.25 mg/L. However, at chronic exposure, mixture toxicity was exacerbated almost 100-fold, with a 240-h LC_{50}m of 0.18 mg/L and a 240-h NOEC value as low as 0.045 mg/L.

Regarding toxicity of the pesticides (ES: endosulfan, CY: cypermethrin) in the mixture (LC_{50} ESm, LC_{50} CYm) versus single-pesticide toxicity (LC_{50} ES, LC_{50} CY), there were no significant differences at acute and intermediate exposure. However, at the end of chronic exposure, the toxicity of the pesticides in mixture was significantly higher than single pesticide toxicity (at 240 h: LC_{50} ES = 0.49; LC_{50} ESm = 0.12; LC_{50} CY = 6.08; LC_{50} CYm = 0.06).

The toxicity of cypermethrin in mixture (LC_{50} CYm) was almost twofold higher than for endosulfan (LC_{50} ESm) for embryos at all exposure durations, whereas single acute and intermediate toxicity of cypermethrin (LC_{50} CY) was higher than endosulfan (LC_{50} ES), reversing this toxicity pattern at chronic exposure.

Regarding larvae, the acute lethality following exposure to endosulfan–cypermethrin mixtures just from 8 mg/L was significant, reaching 80% mortality at 96 h. Survival remained relatively constant until intermediate exposure, in which there was another increment of toxicity with a 168-h NOEC value of 3.2 mg/L. Finally, larval mortality again increased markedly after 192 h as a consequence of exposure to concentrations above 0.32 mg/L, with a 240-h NOEC value of 0.16 mg/L.

Figure 3 shows the slow rise in mixture toxicity with exposure time until 192 h, with 72-h and 168-h LC_{50}m

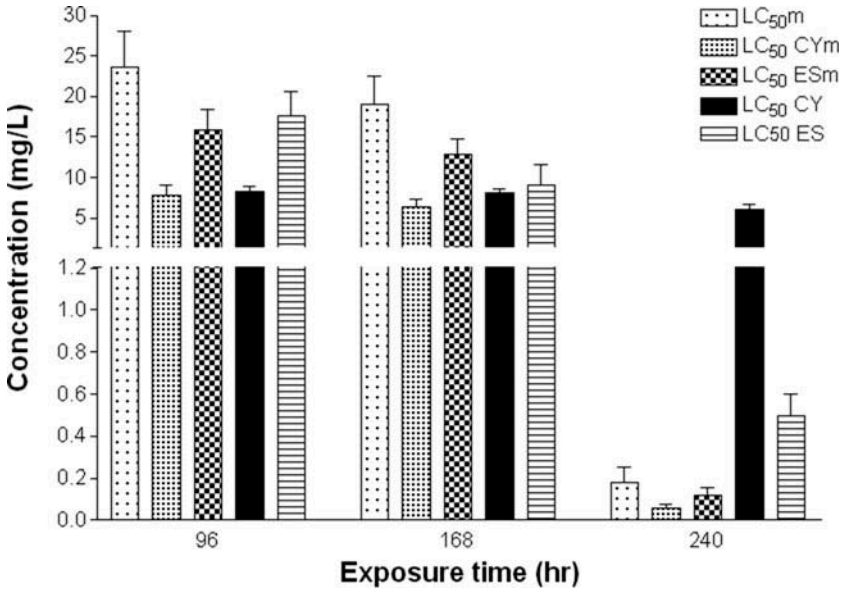

Figure 2. Comparison of LC50 with confidence intervals of endosulfan (ES) and cypermethrin (CY) mixture (LC50m) about LC50 of each pesticide in the mixture (LC50 ESm, LC50 CYm) and individual pesticides (LC50 ES, LC50 CY), for *Rhinella arenarum* embryos at different exposure times.

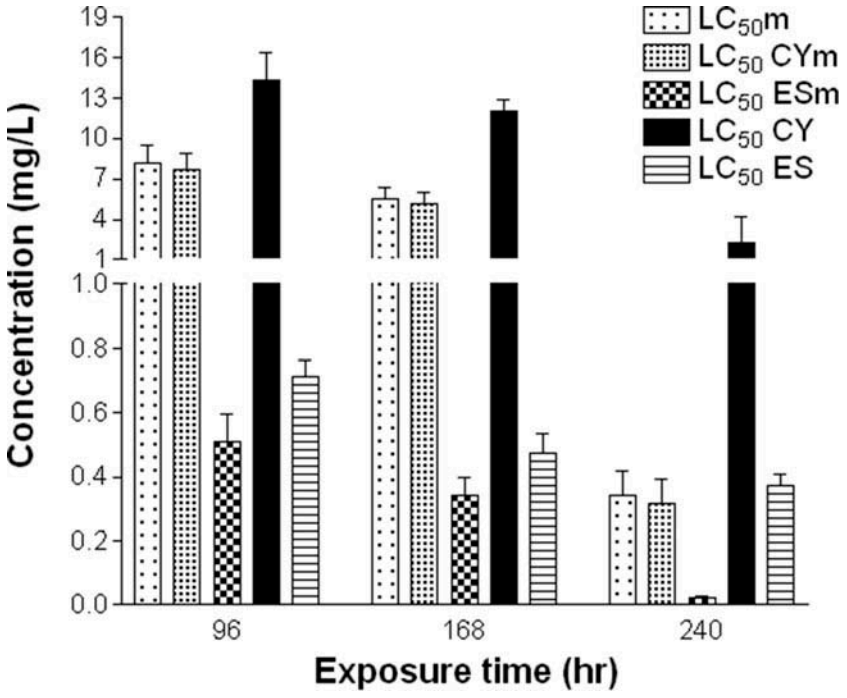

Figure 3. Comparison of LC50 with confidence intervals of endosulfan (ES) and cypermethrin (CY) mixture about LC50 of each substance in the mixture (ESm, CYm) and individual pesticides, for *Rhinella arenarum* larvae at different exposure times.

of 9.14 and 5.49 mg/L, respectively. At chronic exposure, mixture toxicity increased almost eightfold, with 240-h LC_{50}m of 0.34 mg/L. This time-dependent toxicity was also reflected in endosulfan and cypermethrin toxicity in the mixture (CYm and ESm).

Regarding the toxicity of pesticides in the mixture (LC_{50} ESm, LC_{50} CYm) versus single-pesticide toxicity (LC_{50} ES, LC_{50} CY), there was no marked difference between ESm toxicity and individual toxicity until intermediate exposure, while at chronic levels, ESm toxicity was 18.5-fold higher than individual toxicity. CYm toxicity was always greater than individual toxicity, reaching a sevenfold difference with chronic exposure.

Table 2. Sum and Additive Indexes Values With Respective Upper and Lower Confidence Limits Obtained by Analyzing the Joint Toxicity Data From Endosulfan–Cypermethrin Mixtures on *Rhinella arenarum* Embryos and Larvae (Marking, 1977; Xiu et al, 1994)

Stage	Exposure time (h)	Sum index	Additive index	Toxicity interaction
Embryos	96	1.83	−0.83	ANT
		(1.38; 2.48)	(−1.48; −0.38)	
	168	2.20	−1.20	ANT
		(1.56; 3.14)	(−2.14; −0.56)	
	240	0.25	2.94	SYN
		(0.15; 0.54)	(0.87; 5.62)	
Larvae	96	1.25	−0.25	ADD
		(0.97; 1.75)	(−0.75; 0.03)	
	168	1.16	−0.16	ADD
		(0.88; 1.68)	(−0.68; 0.14)	
	240	0.20	0.80	SYN
		(0.10; 0.41)	(1.44; 8.88)	

Note. The additive index limits allow a decision rule based on statistical analysis. If zero was included between upper and lower limits of AI, the coeffects were considered additive (ADD); if zero was above this range was considered antagonism (ANT); and if it was below these values the interaction was considered synergistic (SYN).

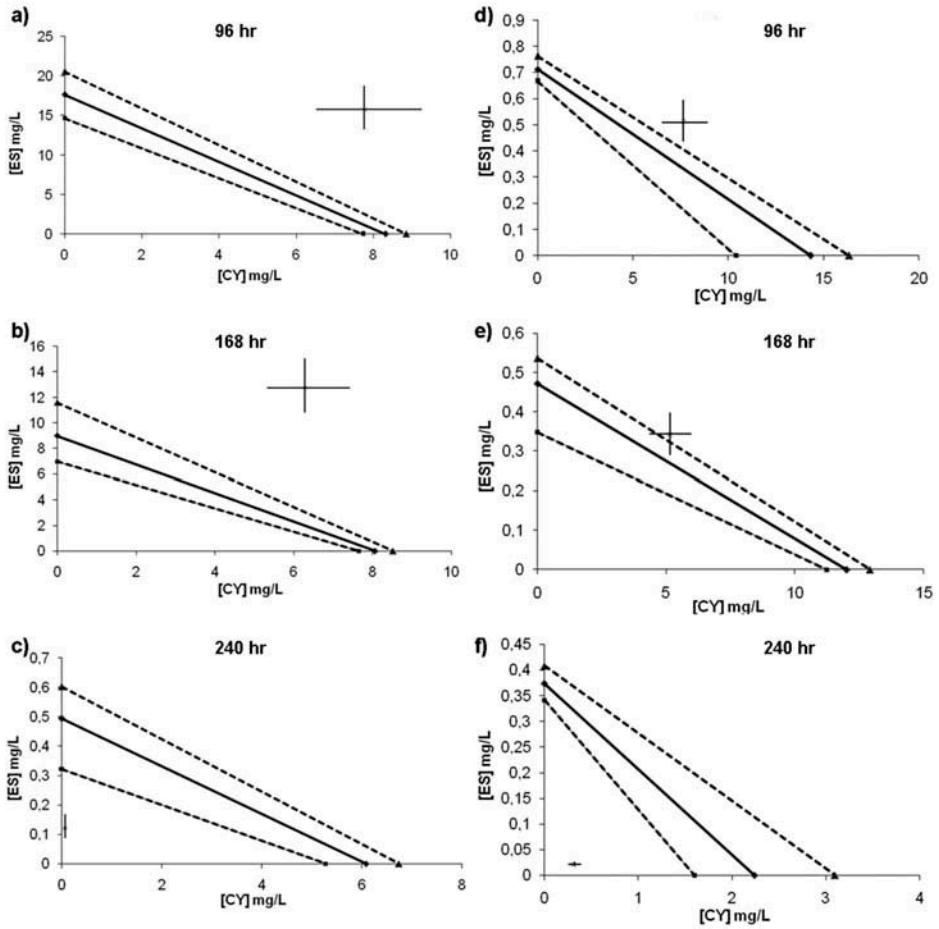

Figure 4. Isobolograms for *Rhinella arenarum* embryos (a, b, c) and larvae (d, e, f) exposed to endosulfan (ES)-cypermethrin (CY) mixtures up to 240 hr analyzed with Marking´s method (Marking, 1977; Xiu et al, 1994). The straight line represents the additivity effect curve and the dashed lines represent 95% confidence intervals. Interaction effects were synergistic when data points from combination lie at the left of the additivity curve, while combination was antagonistic when points were at the right of the curve.

Endosulfan, both singly and in mixture, was more toxic than cypermethrin at all exposure times, with 16- and 25-fold greater toxicity in mixture and single exposure respectively. Data reported in Table 2 and isobolograms (Figure 4) show endosulfan–cypermethrin mixture interaction effects for embryos and larvae at different exposure durations analyzed according to

Table 3. Combination Index (CI) Values With 95% Confidence Intervals for *Rhinella arearum* Embryos and Larvae Exposed to Endosulfan–Cypermethrin Mixtures at 96, 168, and 240 h of Exposure and the Type of Interaction at Different Effect Levels (Fa) Analyzed With the Combination Index–Isobologram Method (Chou, 1976; Chou and Talalay, 1984; Chou, 2006)

Embryos					Larvae		
Effect level (Fa)		96 h	168 h	240 h	96 h	168 h	240 h
0.1	CI	1.56 ± 0.52	3.26 ± 1.61	0.13 ± 0.35	0.94 ± 0.39	1.16 ± 1.00	0.39 ± 0.62
	Interaction	ANT	ANT	SYN	ADD	ADD	SYN
0.5	CI	1.61± 0.45	2.67 ± 0.43	0.28 ± 0.31	1.09 ± 0.22	1.43 ± 0.81	0.23 ± 0.09
	Interaction	ANT	ANT	SYN	ADD	ADD	SYN
0.9	CI	1.73 ± 1.27	2.56 ± 1.05	2.12 ± 1.44	1.31 ± 0.46	1.85 ± 0.70	0.19 ± 0.08
	Interaction	ADD	ANT	ANT	ADD	ANT	ANT

Note. CI < 1, CI = 1, and CI > 1 indicate synergism (SYN), additive effect (ADD), and antagonism (ANT), respectively.

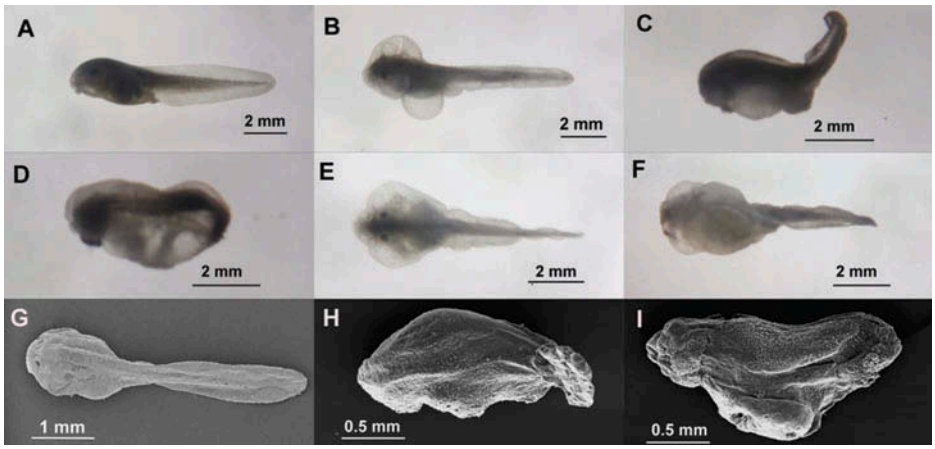

Figure 5. Stereoscopic and Scanning Electron Microscopy pictures of *Rhinella arenarum* larvae exposed from embryonic development to different concentrations of endosulfan and cypermethrin mixture and fixed at 168 hr: (A and G) control larvae, S.25; (B, C, D, H, I) 5% mixture (2.25 mg/L); (B) huge blisters; (C) developmental delay, axial curvature, edema, irregular borders; (D, H, I) general underdevelopment, microcephaly, edema; (E) 10% mixture (4.5 mg/L), edema, wavy tail; (F) 25% mixture (11.25 mg/L), edema, wavy tail. Magnification: (G): 18x; (H): 50x; (I): 46x.

Marking's additive index. For embryos, endosulfan–cypermethrin interaction toxicity was antagonistic up to 168 h of exposure. By extending exposure time, mixture toxicity increased significantly, and gave rise to synergism at 240 h. For larvae, endosulfan–cypermethrin mixture toxicity was additive up to 168 h, but extending exposure to 240 h, the pesticide mixture was also synergistic.

The combination index values presented in Table 3 indicate the interaction at different effect levels (Fa = 0.1, 0.5, 0.9). Thus, at Fa = 0.5, the interaction was the same as obtained by Marking´s method both for embryos and larvae. For embryos and larvae the influence of mixture was similar for 0.1 and 0.5. At 0.9 effect level the interaction was additive at 96 h, and antagonistic at 168 and 240 h for both embryos and larvae.

Sublethal effects on embryos exposed to pesticide mixtures were more severe than those found by single pesticides. During the first hour of exposure, embryos exhibited cell dissociation, irregular surface, persistent yolk plugs, and developmental delay. As development proceeded, embryos exposed to mixture concentrations from 0.225 mg/L showed reduced body size, underdeveloped gills, microcephaly, axial curvatures, wavy tails, and marked edema (Figure 5). Reaching the larval period, behavioral alterations such as spasmodic contractions, tremors, little spontaneous movements, or absence of movements were observed even at the lowest concentrations. The 168-h NOEC value for sublethal effects was below 0.0045 mg/L (the lowest concentration tested), while for lethal effects, NOEC was 2.25 mg/L, such that the 168-h TI of mixture was greater than 500.

Pesticide-exposed larvae exhibited mainly behavioral alterations from the first hour of exposure. Thus, at lower concentrations larvae showed

spasmodic contractions, tremors, and erratic swimming. Larvae exposed to higher concentrations (>3.2 mg/L) exhibited marked edema, huge blisters, and narcosis. During chronic exposure, larvae displayed reduced body size, edema, and behavioral alterations such as spasmodic contractions and nonfeeding behavior. Larvae exposed to concentrations from 1.6 mg/L had no spontaneous or induced movements.

Discussion

The results obtained in this study determined that endosulfan–cypermethrin mixtures were significantly toxic for larvae and embryos, and by extending exposure duration the severity of effects increased markedly for both developmental stages. Pesticide interaction was mainly additive for larvae, whereas for embryos it was antagonistic up to intermediate exposure. Chronically the interaction was synergistic for both stages (increasing embryo toxicity nearly 100-fold in 48 h). By observing the CI values at different effect levels, this indicates that there was the same type of interaction for Fa = 0.1 and 0.5. This is relevant because synergism may occur at environmentally relevant concentrations (Fa = 0.1 is equivalent to LC_{10}, a low concentration) when exposure is extended along the embryo–larval development. At Fa = 0.9, the interaction was additive at 96 h, and antagonistic at 168 and 240 h for both embryos and larvae. Further enhanced toxicity to embryos during the chronic exposure was also observed for single endosulfan/cypermethrin exposure. Since 168 h postfertilization embryos reached the larval stage (by evolving to a feeding and free-swimming tadpole), this toxicity increment was coincident with development of the CNS, the main target organ of these pesticides. It is interesting to point out that the toxicity of cypermethrin remained almost constant regardless of exposure duration, whereas toxicity of endosulfan significantly increased from 7 to 10 d as the mixture toxicity did. Evidence thus indicates the majority of mixture toxicity might be due to this pesticide. In addition, the synergism noted at chronic exposure might be due to bioaccumulation of endosulfan, as was reported previously (Svartz et al., 2015). Further studies need to be designed to investigate both the biochemical and the physiological basis of the synergy. Regarding sublethal effects, embryos exposed to the endosulfan–cypermethrin mixture displayed more severe malformations than those exposed to individual pesticides. Pesticide exposed larvae were sluggish and showed impaired locomotion and orientation. These effects may be problematic in natural environments because of increased liability of larvae to predation and reduced foraging success, resulting in decreased growth and delayed development. Subsequently, this might enhance the time when risk-prone size classes are exposed to predators, increase the time to metamorphosis (important in temporary ponds), and decrease size at metamorphosis and survival to reproduction (Howe et al., 1998).

The main agrochemical used particularly in Argentina is glyphosate, followed by endosulfan and cypermethrin; such levels of these pesticides are frequently found in areas with intensive agricultural practices (Bonansea et al., 2013; Jergentz et al., 2005; Marino and Ronco, 2005; Ronco et al., 2007). Aerial fumigations of mixtures of endosulfan (Thionex-L formulation, 35% w/v) and cypermethrin (Sherpa formulation, 25% w/v) at a proportion of 700 ml + 150 ml in 100 L water, approximately 7:1 ratio, are applied per hectare in the northeastern Buenos Aires Province, Argentina (Agostini et al., 2009). Agostini et al. (2013) also reported a high incidence of abnormalities (limb malformations, microcephaly, scoliosis, and pigment disruptions) in amphibian populations belonging to agroecosystems of the same study area. The uptake data for these two pesticides in aquatic biota were also noted (Grant et al., 2013; Svartz et al., 2015), but these studies evaluated the toxicity of individual pesticides and not toxicity interactions. Further, little is known regarding the toxicity of mixtures of endosulfan or cypermethrin with other pesticides on aquatic organisms. Brodeur et al. (2014) reported synergistic interactions between cypermethrin- and glyphosate-based pesticides during acute exposure of *Rhinella arenarum* larvae. This mixture produced toxicity two- to ninefold higher than that predicted by the concentration addition model. In fish, *Mystus vittatus*, exposed to heterotoxic mixtures of endosulfan, dichlorvos, and carbofuran, synergistic effects were recorded in tertiary mixtures when endosulfan and dichlorvos were kept constant and carbofuran

variable (Verma et al., 1980). By exposure to binary mixtures, antagonistic effects were observed both when dichlorvos remained constant and endosulfan variable and when carbofuran remained constant and endosulfan variable (Verma et al., 1980). Poletta et al. (2011) assessed the genotoxic effect of pesticide mixtures usually used in the field on caiman eggs (*Caiman latirostris*). Data demonstrated that mixtures of glyphosate, endosulfan, and cypermethrin formulations induced higher rates of genotoxicity (micronucleus frequency, DNA damage, and teratogenesis) and metabolic alterations than the glyphosate formulation alone. Further, Ramos-Chavez et al. (2015) found that a mixture of permethrin/allethrin induced genotoxicity and cytotoxicity in human peripheral blood lymphocytes. From a recent review of possible mechanisms resulting in synergism, it was concluded that interactions on metabolic processes affecting transformation of xenobiotics seem to be the most common mechanism of synergy. If the groups of chemicals that are likely to induce synergistic interactions could be identified, special precautions need to be taken in relation to risk assessment of these chemicals (Cedergreen, 2014).

The median drug effect analysis was originally described by Chou and Talalay (1984), and now it is the most widely used method in pharmacology for both in vitro and in vivo bioassays for analyzing chemical combinations. This method was selected to obtain the combination index (CI) values at different effect levels (Fa), whereas Marking´s method encompasses only the effect level at Fa = 0.5 (LC_{50}). In the present study CI values obtained at Fa = 0.5 indicated the same type of interactions as those obtained by Marking's method. The isobologram can also be generated by the computer program Compusyn (Chou and Martin, 2005); however, this has two main disadvantages: (i) It does not allow any calculations regarding the extent of synergism and (ii) it does not provide confidence intervals. Based on our results, the combination index (CI)–isobologram equation is a useful tool to determine potential toxic interactions of mixtures at different effect levels. Marking's method is also valuable because it helps to elucidate toxic interaction (but only at one effect level) by obtaining the isobologram with its respective confident intervals.

The sensitivity to these pesticides was concentration and time exposure dependent, producing a significant increase in lethal and sublethal effects following chronic exposure as previously observed in several studies. These results highlight the relevance of performing bioassays by extending the exposure time from an acute to a chronic period and exposing different developmental stages for assessing the single toxicity of compounds and potential toxic interactions in mixtures. It is interesting to point out the relevance of assessing sublethal effects, unlike other studies that are based only on lethality assessment, because while there may be no apparent synergistic effect on lethality, worsening of sublethal effects might occur, as in this case.

In conclusion, this study demonstrates that endosulfan and cypermethrin in mixtures exhibit synergistic interactions following chronic exposures to embryos and larvae of the common South American toad, *R. arenarum*. Given the widespread agricultural use of cypermethrin and high environmental persistence of endosulfan, it is likely that these pesticides may frequently coexist in agroecosystems, disrupting amphibian populations.

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