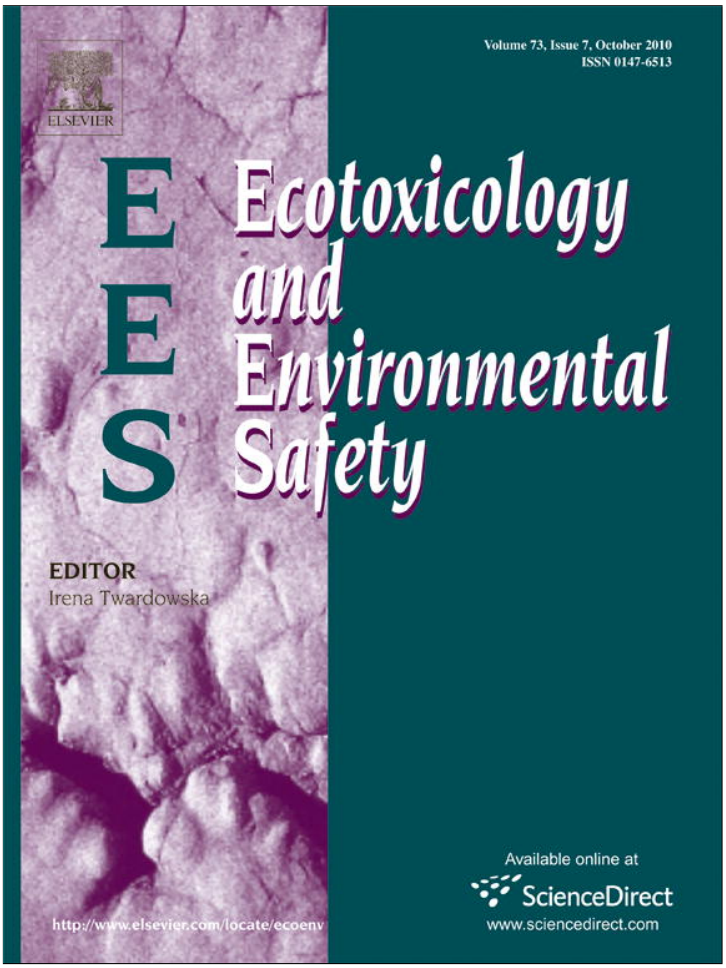

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Activity levels of B-esterases in the tadpoles of 11 species of frogs in the middle Paraná River floodplain: Implication for ecological risk assessment of soybean crops

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ABSTRACT

Soybean fields provide habitats for many species of amphibians. However, the persistence and health of amphibian populations may be at risk from the increasing use of pesticides and other agricultural chemicals. We examined the activities of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterases (CbEs) in 11 syntopic species of larval anurans. In vitro effects of malaoxon causing 50% BChE inhibition (IC_{50}) were also studied. In addition, we calculated a relative risk index (RI) based on the geographic distributions of the anurans, the phenology of soybean cultivation, and basal enzymatic values related to potential pesticide detoxification. Among the 11 species, AChE activity varied from 17.5 ± 1.6 to 68.2 ± 4.7 nmol min^{-1} mg^{-1} protein (PT). BChE activity also varied significantly, ranging from 3.3 ± 0.4 to 7.5 ± 0.4 nmol min^{-1} mg^{-1} PT. Both measures of CbE activities varied widely (CbE α-NA: 2.1 ± 0.5–12.4 ± 1.1 nmol min^{-1} mg^{-1} PT; CbE-4NPV: 21.8 ± 1.8–102.6 ± 7.9 nmol min^{-1} mg^{-1} PT). We also corroborate that lower BChE activity levels for the tadpoles were associated at minor IC_{50} values. The results of this study demonstrate significant variation in enzymatic levels among several tadpole species and intermediate to high RI values for 7 species. Based on these results, it appears that a conversion of native ecosystems to soybean crops may lead to increased ecological risk for anuran amphibians.

1. Introduction

Anuran species differ in their ability to tolerate chemical contaminants (e.g. Hall and Henry, 1992; Bridges and Semlitsch, 2000; Rowe et al., 2001; Christin et al., 2004; Relyea and Jones, 2009; Jones et al., 2009). These differences, in part, are related to the concept of allostatic capacity (ability of organisms exposed to environmental change to maintain homeostasis) (Navas and Otani, 2007).

B-type esterases participate in the detoxification of pesticides, such as pyrethroids (PYs), carbamates (CBs), and organophosphates (OPs) (Sánchez-Hernández, 2007; Wheelock et al., 2008). Amphibians B-esterases may actually be better biomarkers to identify amphibians pesticide exposure (e.g. Sparling et al., 2001; Lajmanovich et al., 2004; Attademo et al., 2007; Sparling and Fellers, 2009). Aldridge (1953) classified these hydrolase enzymes as esterases that are inhibited by anticholinesterase compounds. In vertebrates, there are two types of B-esterases, cholinesterases (ChEs) and carboxylesterases (CbEs). The former includes acetylcholinesterase (AChE, EC 3.1.1.7) that breaks down the neurotransmitter acetylcholine at the nerve synapse and butyrylcholinesterase (BChE, EC 3.1.1.8) preferentially acts on butyrylcholine, but also hydrolyzes acetylcholine. CbEs (EC 3.1.1.1), however, consist of multiple isozymes that vary with both the tissue and organism and play significant roles in the metabolism and subsequent detoxification of many agrochemicals (Wheelock et al., 2008).

In vitro inhibition of BChE activity by OP pesticides can be an important determinant of the acute toxicity of OP compounds by playing a protective role in sequestering circulating OPs thereby decreasing the toxic effect of these compounds on brain AChE (Sánchez-Hernández et al., 2004). BChE is toxicologically important, because it hydrolyzes ChEs inhibitors including potent OP nerve agents before they reach their synaptic targets (Adresi, 2003). In this sense, it is very important to note that

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malaoxon, a biological and environmental metabolite, is up to 100 times more toxic than the initial, or parent, compounds of the OP malathion in vivo (Shao-Non and De-Fang, 1996; Sparling and Fellers 2007) and can inhibit tadpole BChE in vitro (e.g. Caballero de Castro et al., 1991).

In Latin America, countries such as Argentina, Brazil, Bolivia, Paraguay, and Uruguay have recently shown great expansions of genetically modified (GM) soybean cultivations with a simultaneous increase in the application of pesticides. These expansions are driven by prices of crops, government and agro-industrial support, and demand from importing countries, especially China, the world's largest importer of soy products (Pengue, 2005). Brazil and Argentina experienced the biggest expansion in soybean planting in 2005 (Altieri and Pengue, 2006). Argentina, in particular, planted 16 million ha of soybean for the 2006/2007 season (Harris, 2007), 90% of which were GM "Roundup Ready" soy that encouraged increased use of glyphosate-based herbicides. This herbicide and its surfactant were especially lethal to most species of frogs (Mann and Bidwell, 1999; Relyea, 2005 a,b). Exposure to these herbicides induced sublethal effects, such as malformation (craniofacial and mouth deformities, eye abnormalities, and bent or curved tails) in the South American species *Scinax nasicus* after exposure to glyphosate (Lajmanovich et al., 2003). In addition, inhibition of ChE activity in teleostean fishes (Glusczak et al., 2006, 2007) and amphibian tadpoles (Lajmanovich et al., 2010) exposed to glyphosate has recently been determined.

In the soybean-growing regions of central Argentina, the main agrochemical application period is November to March (Lorenzatti et al., 2004), generally coinciding with the reproductive periods of the amphibians (Peltzer and Lajmanovich, 2007). In these months, short but heavy rainfalls are very common and can cause intensive pesticide runoff to non-target compartments such as aquatic ecosystems (Jergentz et al., 2005). These authors detected endosulfan, chlorpyrifos, and cypermethrin in sediments, suspended particles, and water of some intensively cultivated regions in Argentina. At times concentrations of these pesticides exceeded environmentally safe levels (CASAFE, 2004), making them potentially dangerous to aquatic fauna. In addition, Peltzer et al. (2008) reported a concentration of endosulfan residues that exceeds the value suggested to protect aquatic fauna in Argentinean water bodies (NGNCA, 2004) in agricultural ponds where anuran reproduction frequently occurs, and verified the inhibition of ChEs in tadpoles collected from regions of soybean agriculture. Furthemore, endosulfan has been implicated in the decline of frog populations in California, possibly by depressing ChEs activity in tadpoles (Sparling et al., 2001). Similarly, it has been demonstrated that chlorpyrifos and cypermethrin inhibit ChEs enzymes in amphibians (e.g. Khan et al., 2003; Widder and Bidwell, 2006).

Several studies have shown that the conversion of forests and grasslands to croplands and the consequent changes in land cover alters the regional climate, increasing air temperature near the ground's surface, and decreasing evapotranspiration and precipitation (e.g. Nobre et al., 1991; Bonan, 2001). For these reasons, soybean cropland expansion in eastern Amazonia led to critical changes in local climate (Malhi et al., 2008). In addition, the accelerated changes in land cover in central Argentina have produced climatic changes, principally in annual rainfall (Zak et al., 2008). Reduced precipitation and increased summer evaporation could have dramatic effects on the duration or occurrence of seasonal wetlands, which are critical habitats for many species of amphibians (Corn, 2005). Due to the combined effects of temperature and water stress, the extinction of some amphibians and other aquatic species is predicted in Costa Rica, Spain, and Australia (Pounds et al., 2006).

Classical risk assessment depends on the description of toxicological effects and expression of the toxicity of a compound in quantifiable terms (e.g. LD_{50} and LC_{50}, the dose or concentration that is lethal to 50% of the test population; lowest observable effects level, LOEL; Lowest observable effects concentration, LOEC; Selck et al., 2002). Other methodologies (e.g. predicted environmental concentration—PEC/toxicity) assess the potential ecological impact of pesticide residues have after being applied to agricultural systems (Sánchez-Bayo et al., 2002). The use of biomarkers is also important in ecotoxicology (Walker, 1995) because they provide a measurement endpoint, which is an aim of environmental monitoring and risk assessment (Forbes et al., 2006). One criticism that biochemical biomarkers have to face is the consequence of individual changes on a higher ecological order (i.e., at the population level) (Solé et al., 2008). To detect the impacts of pesticides in different species of amphibian, comparative physiology and multi-biomarkers (e.g. ChEs, glutathione-S-transferase, endogenous polyamine levels, molecular biomarkers, genetic response, alterations in hormone signaling) have been used as effective tools in assessing environmental risk (Venturino et al., 2003). Such studies, especially in biogeographic zones with high amphibian diversity, have been largely neglected in the ecotoxicological literature (Schiesari et al., 2007).

The purpose of our work was to characterize the normal activities of total AChE, BChE, and CbEs across multiple species of larval amphibians and investigate the interspecies variation in these enzymes. In addition, we determined the inhibitory effect of malaoxon on BChE activity. Moreover, considering that most of anuran breeding and feeding coincide with soybean cropping in Argentina (see: Attademo et al., 2005; Peltzer et al., 2006), we carried out a characterization of ecological risk based on spatial and temporal overlap of cultivation and anuran geographical distribution, and basal enzymatic values.

2. Materials and Methods

2.1. Anuran tadpoles

We selected 11 sympatric species of larval anurans: *Rhinella fernandezae* (Bufonidae), *Leptodactylus ocellatus*, *L. mystacinus* (Leptodactylidae), *Physalaemus albonotatus*, *P. santafecinus* (Leiuperidae), *Odontophrynus americanus* (Cycloramphidae), *Hypsiboas pulchellus*, *Scinax nasicus*, *S. squalirostris*, *Pseudis limellum* (Hylidae), and *Elachistocleis bicolor* (Microhylidae). These anurans have extensive neotropical distributions (IUCN, 2008), and are frequently found in vegetated areas, wetlands, agricultural land, and urban regions (Peltzer et al., 2006). Moreover, the reproduction in these species generally takes place in agricultural ponds and temporally overlaps with soybean cultivation (Attademo et al., 2005; Peltzer et al., 2006).

A total of 105 prometamorphic larvae (stages 36–41; Gosner, 1960), were collected during January and February 2009 from temporary pond in one site of the natural parafluvial forests of the Paraná River Boundary (31°11′31S/60°09′29W, Argentina). Despite the wide distribution of soybean crops in the region, all the tadpoles were collected in non-agricultural areas, so they likely had minimal exposure to pesticides. The tadpoles were acclimated for 24 h to a 12:12 h light–dark cycle in glass tanks (12.5 cm diameter and 13.5 cm high) filled with dechlorinated tap water (pH = 7.4 ± 0.05; conductivity = 175 μmhos cm^{-1}; dissolved oxygen = 6.5 ± 1 mg L^{-1}; hardness = 50.5 mg L^{-1} of CO_3Ca at 24 ± 2 °C). During acclimation, the tadpoles were not fed in order to purge the gut.

2.2. Enzymatic determinations

Tadpoles were euthanized with approval of Faculty of Biochemistry and Biological Sciences animal ethics committee following ASIH et al. (2004) guidelines. Whole tadpoles were homogenized (still on ice) in 0.1% t-octylphenoxypolyethoxy ethanol (triton X-100) in 25 mM tris(hydroxymethyl) aminomethane hydrochloride (pH 8.0) using a polytron. The homogenates were centrifuged at 14,000 rpm for 15 min at 4 °C and the supernatant was collected (called crude extract). All assays were performed using three or more replicate extracts. AChE and BChE activities were measured according to Ellman et al. (1961). The reaction mixture consisted of 0.01 ml of extract, 2 mM dithio bis 2-nitrobenzoic acid

(DTNB), 20 mM acetylthiocholine, and butyrylthiocholine iodide (AcSCh and BuSCh, respectively), 25 mM Tris–HCl, and 1 mM $CaCl_2$ (pH 7.6). The variation in optical density was recorded at 410 nm for 1 min at 25 °C using a JENWAY 6405 UV–vis spectrophotometer. Protein (PT) concentrations in the supernatants were determined according to the Biuret method (Kingbley, 1942). Enzyme activity was expressed as nmol min^{-1} mg^{-1} of PT using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}. CbEs activities were also assayed spectrophotometrically using two substrates α-naphthyl acetate (α-NA) and 4-nitrophenyl valerate (4-NPV), and specific enzyme activity was expressed as nmol min^{-1} mg^{-1} of PT. CbEs activity using α-NA was measured by the Gomori method (1953) as adapted by Bunyan and Jennings (1968). The reaction solution contained 25 mM Tris–HCl, 1 mM $CaCl_2$ (pH=7.6), and the sample. The reaction was initiated with the addition of α-naphthyl acetate (1.04 mg/ml in acetone) after a preincubation period of 5 min at 25 °C. The formation of naphthol was stopped after 10 min by addition of 2.5% sodium dodecyl sulphate (SDS) and subsequently 0.1% Fast Red ITR in 2.5% Triton X-100 in water (freshly prepared). The samples were left in the dark for 30 min to develop, and the absorbance of the complex was read at 530 nm (using a molar extinction coefficient of 33.225×10^3 M^{-1} cm^{-1}). Hydrolysis of 4-NPV by CbE was determined as described by Carr and Chambers (1991). Samples were preincubated in 50 Mm Tris–HCl (pH=7.5) for 5 min at 25 °C, and the reaction was initiated by the addition of 4-NPV (5×10^{-4} M, final concentration). After 10 min, the reaction was stopped by the addition of a solution of 2% (w/v) SDS and 2% (w/v) Tris base. The p-nitrophenol liberated was read at 405 nm and quantified by a calibration curve (5–100 mM).

The data were analyzed statistically using the non-parametric Kruskal–Wallis ANOVA; pairwise comparisons between samples from the 11 species were tested by the Dunn test for post-hoc multiple comparisons. Correlations between tadpole body size and specific enzyme activities were tested using the Spearman's correlation test. Statistical significance was held at $\alpha=0.05$. Analyses were performed with GraphPad InStat®.

2.3. BChE inhibition by malaoxon

We calculated the molar (M) concentration of malaoxon causing 50% enzyme inhibition (IC_{50}) to determine the tolerance to OP pesticides. Insecticide solutions were initially prepared in dimethylsulfoxide and the solvent concentration in the reaction medium was kept below 0.1%. Extract samples were incubated for 30 min at 25 °C at seven malaoxon concentrations (9.4×10^{-5}–9.4×10^{-11} M) to yield a range of esterase inhibition between 10% and 90%. The percentage of BChE inhibition was calculated by comparison with control samples, which received an equal volume of deionized water. All incubations were run in duplicate. The IC_{50} M concentration of malaoxon was estimated using the best fits found with the potential equation $y=ax^b$. A correlation analysis was performed to describe the relationship between BChE activities and the IC_{50} values, outliers were detected using the 97.5% quantile with two degrees of freedom and removed from pairwise correlation analyses (Rousseeuw and Leroy, 2003).

2.4. Soybean's land and phenology cover amphibian overlap

We used GIS package ArcView 3.3 to store and manage the data sets, and the INTA digital soil atlas of Argentina, scale 1:500,000, as a base map (INTA, 1995) for determining the Soybean Land Cover Amphibian Overlap (SLCAO). The distributions of 11 anuran species were derived from the IUCN Red List (2008), and soybean land cover was based on an existing map (SAGPyA, 2008). Initially, these transformed grid maps of each anuran species were superimposed onto the soybean land cover grids using the Map Calculate function in ArcView (ext. Spatial Analyst 2.0). The resulting areas over which both maps (anuran/soybean cover) overlapped were expressed as overlapping area percentage, defined as the area where each species distribution overlapped that of soybean cover divided by the total area of that species' distribution. Additionally, the degree of temporal overlap of non-target species with soybeans was named as Soybean Phenology Overlap (SPO), and was determined by linking the reproductive period of each species with the phenology of soybean cultivation.

2.5. Developing a hazard index for amphibians associated with soybean cultivation

We used a modification of the qualitative ecological risk assessment (ERA) approach established by Foran and Ferenc (1999). The relative risk index (RI) was calculated based on a numerical ranking of the parameters analyzed (BChE and CbEs activities, SLCAO, SPO). For instance, each species was assigned a value (1–11) and ordered according to the normal value of enzyme activities (1: less enzyme activity, high risk; 11: high enzyme activity, low risk) and overlap at spatial and temporal levels (1: high overlap at both scales, high risk; 11: low overlap, low risk). Subsequently, we used transformed final data to a percent scale (taking into account 11 species × 4 variables), and a range between 0% to 100% was determined, where zero represents no risk and 100 the highest risk. A correspondence analysis (CA) was also performed aiming to corroborate the RI values and to identify which attributes were most relevant for each species' ordination (Mc Garigal et al., 2000). This multivariate analysis facilitates a graphic representation of the current data in a contingency table (Ter Braak, 1987). A matrix was developed with the numerical ranking assigned for each species from the enzyme and spatial and temporal variables. The numerical values were standardized and the CA was performed using MVSP software (Kovach, 1999).

3. Results

3.1. B-esterase activity levels

The basal whole B-esterases activities varied among tadpoles species (Table 1). Mean AChE activity differed significantly ($P<0.05$) among species, varying from 7.45 ± 1.62 nmol min^{-1} mg^{-1} PT (*R. fernandezae*) to 68.19 ± 4.65 nmol min^{-1} mg^{-1} PT (*P. albonotatus*). Similarly, mean BChE activity also differed significantly ($P<0.05$) among species (3.30 ± 0.35 nmol min^{-1} mg^{-1} PT in *P. santafecinus* to 9.45 ± 0.74 nmol min^{-1} mg^{-1} PT in *E. bicolor*). In addition, both CbEs activities were relatively low for *P. santafecinus* (CbE α-NA: 2.13 ± 0.47 nmol min^{-1} mg^{-1} PT; and *S. squalirostris* (CbE, 4-NPV: 21.80 ± 1.84 nmol min^{-1} mg^{-1} PT) and higher for *E. bicolor* (CbE α-NA: 12.35 ± 1.05; CbE, 4-NPV:102.6 ± 7.88 nmol min^{-1} mg^{-1} PT, respectively), significantly differing between substrates used ($P<0.05$). There were no significant effects of body size on activity of the four enzymes ($P>0.05$).

3.2. BChE inhibition

The IC_{50} values in the 11 tadpoles studied ranged between 10^{-11} and 10^{-6} M (Table 2). The tendency of reduction in IC_{50} values was related with the low average of BChE activity (Fig. 1). After removing outliers (i.e. *H. pulchellus*) BChE activity and IC_{50} values were correlated ($r^2=0.73$; $P<0.01$).

3.3. Soybean's land and phenology cover amphibian overlap

Geographic distributions of all amphibian species overlapped with the distribution of soybean cropland cover (Fig. 2). *Hypsiboas pulchellus* had the greatest overlap, comprising 83% of its distribution, whereas *P. santafecinus* had the lowest overlap with 49%.

The degree of temporal overlap between anuran reproduction and soybean phenology also showed a high degree of overlap in all the species studied. The SPO varied from 33.6% for *P. albonotatus* and 71.4% for *E. bicolor*; with intermediate values for *P. santafecinus* (35.6%), *H. pulchellus* (38.2%), *L. ocellatus* (45.2%), *S. nasicus* (50.3%), *O. americanus* (50.3%), *S. squalirostris* (54.7%), *L. mystacinus* (57.4%), *P. limellum* (62.5%), and *R. fernandezae* (67.9%) (Fig. 3).

3.4. Anuran relative risk associated with geographical and temporal patterns of soybean cultivation

Risk indices were elevated (> 50%) for 5 species (*S. squalirostris*, *R. fernandezae*, *L. mystacinus*, *O. americanus*, and *P. limellum*), intermediate (=50%) for 2 species (*P. santafecinus* and *E. bicolor*), and low (< 50%) for the rest (*S. nasicus*, *H. pulchellus*, *L. ocellatus*, *P. albonotatus*) (Fig. 4). Similar results were obtained in the CA. The ordination axis (Fig. 5) extracted the two first dimensions that explain 95.4% of the total variance (dimension 1=70.2% of inertia; dimension 2=29.7%). Mainly, the first dimension was positively related to enzymes level (ordered species with low RI values, excepted *E. bicolor*), and negatively with temporal and spatial anuran–soybean overlaps related to anurans with high RI values, except *P. santafecinus*.

Table 1
Basal levels of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CbE): α-naphthyl acetate (α-NA) and 4-nitrophenyl valerate (4-NPV) activities in eleven anuran tadpoles. Expressed as nmol min^{-1} mg^{-1} of protein (PT).

Amphibian species	N	Size[a]	AChE	BChE	CbE α-NA	CbE-4NPV
Bufonidae						
Rhinella fernandezae	11	18.5 ± 2.0	7.45 ± 1.62^{abcdef}	6.78 ± 1.43	4.59 ± 0.25^{abc}	28.01 ± 1.42^{abc}
Leptodactylidae						
Leptodactylus ocellatus	11	33.8 ± 4.3	30.3 ± 3.28^{ghijk}	5.67 ± 0.38	5.94 ± 0.52^{d}	85.8 ± 10.63^{ade}
Leptodactylus mystacinus	9	19.3 ± 4.4	44.46 ± 3.74	4.32 ± 0.48^{ab}	5.13 ± 0.48^{e}	57.65 ± 5.91
Leiuperidae						
Physalaemus albonotatus	10	15.4 ± 2.8	68.19 ± 4.65^{ag}	6.70 ± 0.89^{cd}	4.87 ± 0.37	80.35 ± 14.76^{gh}
Physalaemus santafecinus	10	19.8 ± 1.6	57.13 ± 2.66^{bh}	3.30 ± 0.35^{cefg}	2.13 ± 0.47^{d}	32.47 ± 3.73^{i}
Cycloramphidae						
Odontophrynus americanus	9	32.9 ± 2.0	44.01 ± 3.9	6.03 ± 0.79	4.63 ± 0.64	24.19 ± 3.79^{dgj}
Hylidae						
Hypsiboas pulchellus	10	42.5 ± 3.4	46.44 ± 3.04	7.54 ± 0.43^{aeh}	11.52 ± 1.02^{a}	89.77 ± 6.44^{bikl}
Scinax nasicus	11	33.5 ± 1.5	67.85 ± 6.94^{ci}	7.33 ± 1.27^{f}	4.97 ± 0.55	41.46 ± 8.23^{km}
Scinax squalirostris	8	28.6 ± 1.8	52.41 ± 4.69^{d}	3.47 ± 0.27^{dh}	3.93 ± 0.27	21.80 ± 1.84^{ehln}
Pseudis limellum	8	29.7 ± 4.7	61.43 ± 1.87^{ej}	4.97 ± 0.17	12.14 ± 1.25^{b}	45.68 ± 3.01
Microhylidae						
Elachistocleis bicolor	8	23.9 ± 1.7	63.86 ± 5.40^{fk}	9.45 ± 0.74^{bg}	12.35 ± 1.05^{ce}	102.6 ± 7.88^{cjmn}
	105		**49.77 ± 1.96**	**6.02 ± 0.29**	**6.45 ± 0.38**	**55.69 ± 3.52**

Mean activity (± SEM); column means sharing a common letter indicate statistical difference at the 5% level of probability by Dunn multiple range test.

[a] Average total size (mm) (± SEM) (snout-tail tip).

Table 2
50% butyrylcholinesterase (BChE) inhibition (IC_{50}) (expressed in M) for anuran tadpoles.

Amphibian species	Potential equation	r^2	SEM	IC_{50}
Rhinella fernandezae	$y = 51.569 \times^{-0.1958}$	0.85	16.01	1.17×10^{-6}
Leptodactylus ocellatus	$y = 42.986 \times^{-0.2494}$	0.80	12.23	5.45×10^{-7}
Leptodactylus mystacinus	$y = 34.625 \times^{-0.3108}$	0.81	9.53	3.06×10^{-7}
Physalaemus albonotatus	$y = 46.472 \times^{-0.1243}$	0.86	12.40	5.55×10^{-7}
Physalaemus santafecinus	$y = 4.6694 \times^{-0.2366}$	0.92	17.82	4.44×10^{-11}
Odontophrynus americanus	$y = 28.734 \times^{-0.3383}$	0.90	16.45	1.94×10^{-7}
Hypsiboas pulchellus	$y = 61.274 \times^{-0.1201}$	0.88	17.49	5.43×10^{-6}
Scinax nasicus	$y = 47138 \times^{-0.1548}$	0.88	15.45	7.08×10^{-7}
Scinax squalirostris	$y = 42.465 \times^{-0.1164}$	0.95	11.85	2.45×10^{-7}
Pseudis limellum	$y = 32.046 \times^{-0.1097}$	0.83	15.61	1.73×10^{-8}
Elachistocleis bicolor	$y = 53.918 \times^{-0.1613}$	0.91	10.89	1.59×10^{-6}

SEM: standard error of the *y*-axis.

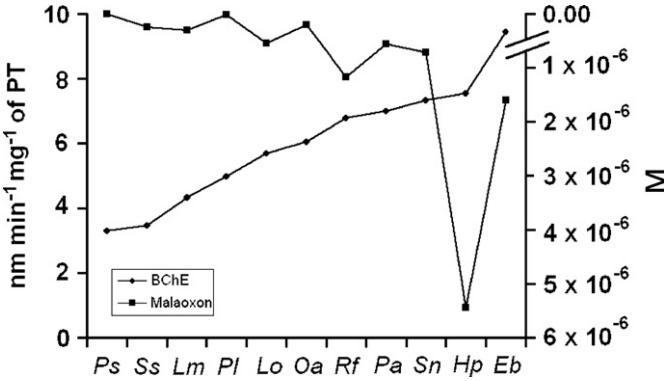

Fig. 1. Relationship between the average butyrylcholinesterase (BChE) activities (left *y*-axis) in 11 larval anurans species. (*Ps*: *P. santafecinus*; *Ss*: *S. squalirostris*; *Lm*: *L. mystacinus*; *Pl*: *P. limellum*; *Lo*: *L. ocellatus*; *Oa*: *O. americanus*; *Rf*: *R. fernandezae*; *Pa*: *P. albonotatus*; *Sn*: *S. nasicus*; *Hp*: *H. pulchellus*; *Eb*: *E. bicolor*) a molar concentration (M) of malaoxon causing 50% of enzyme inhibition (IC_{50}) (right *y*-axis).

4. Discussion

B-esterase activity has been determined in different species of larval anurans by many investigators, but never in more than 4 species simultaneously (e.g. Fulton and Chambers, 1985; Caballero de Castro et al., 1991; Sparling et al., 1997, 2001; Richards and Kendall, 2002; Widder and Bidwell, 2006; Sparling and Fellers, 2007). Likewise, there have been no studies comparing B-esterase activities among different tadpole species in specific assemblages. Additionally, an accurate cross-species comparison from enzymatic measurements reported in different studies is difficult, because such data are not always comparable for the methodologies applied (Chuiko, 2000). For these reasons, the present investigation was carried out with the same assay procedures and simultaneously in the tadpoles of 11 species (all at similar developmental stages range) from six families caught during the same period of the year to compare AChE, BChE, CbE α-NA and CbE 4-NPV activities.

The mean value for tadpole AChE activity here obtained was 49.77 ± 1.96 nmol min^{-1} mg^{-1} PT. Overall, this magnitude of AChE activities was consistent with the results of the other investigations (e.g. 80 nmol $min^{-1}mg^{-1}$ PT in *Xenopus laevis* larvae; Gindi and Knowland, 1979) (89.05 ± 8.78, 96.48 ± 9.29, and 88.14 ± 8.72 nmol min^{-1} mg^{-1} PT in tadpoles of *Lithobates sphenocephala*, *Hyla cinerea*, and *L. catesbeianus*, respectively; Fulton and Chambers, 1985). Similarly, our the average values of BChE and CbE α-NA (6.02 ± 0.29 and 6.45 ± 0.38 nmol $min^{-1}mg^{-1}$ PT, respectively) are also consistent with the results obtained in a sympatric tadpole (*R. arenarum*: 6.31 ± 0.8 and 4.39 ± 0.46 nmol min^{-1} mg^{-1} PT, respectively; Lajmanovich et al., 2010). BChE is considerably more sensitive to ChE-inhibiting pesticides than AChE in most vertebrates (Magnotti et al., 1994;

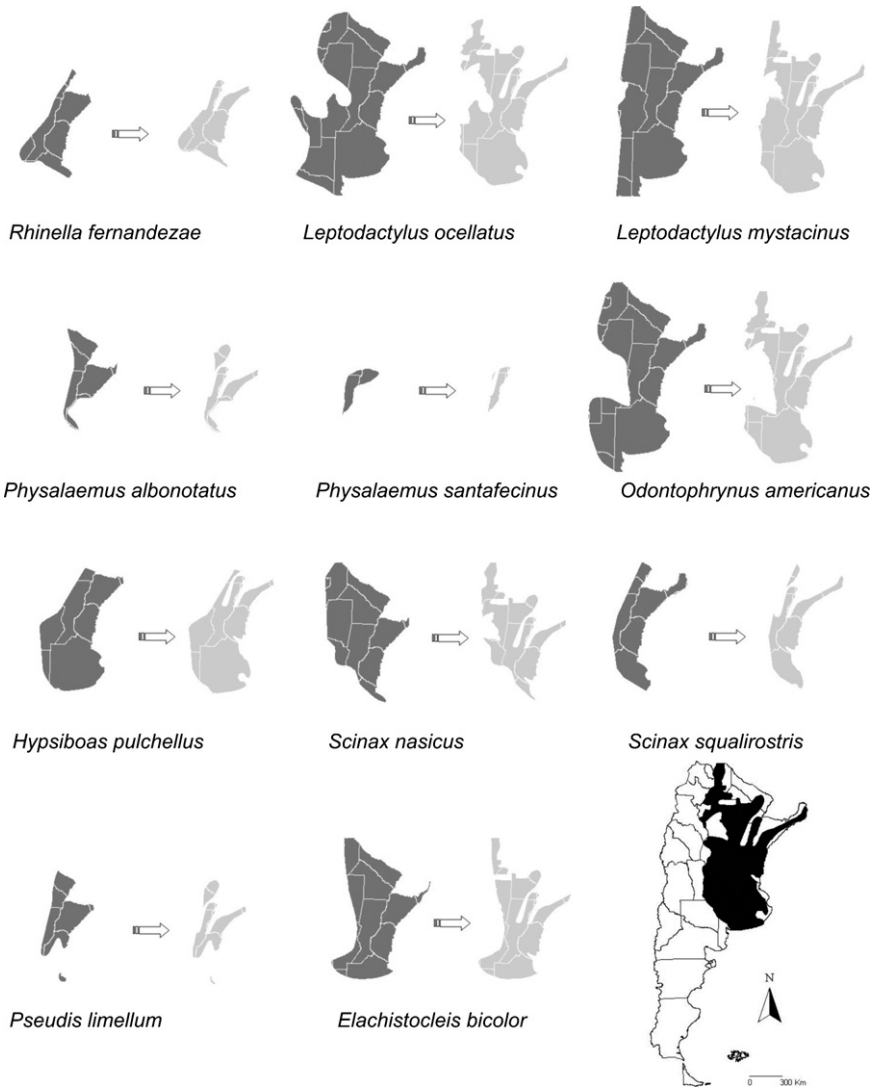

Fig. 2. Maps showing the relationship between geographical anuran distribution and soybean land cover in Argentina. The black area corresponds to soybean land cover, the dark gray to the amphibian species studied, and the light gray shows their overlap.

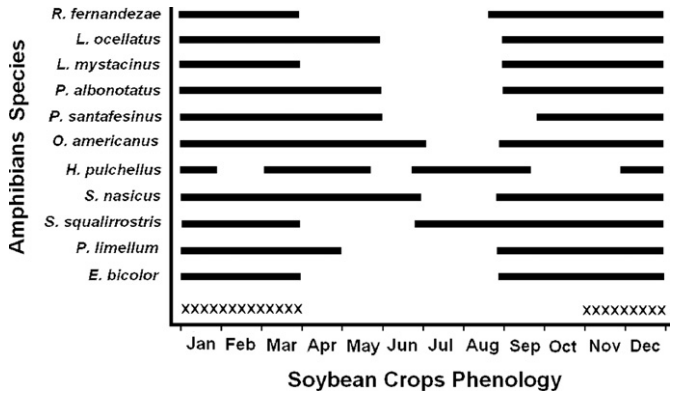

Fig. 3. Relationship between tadpole presence and soybean phenology. The black lines corresponds to the reproductive pattern of anurans (sensu Peltzer and Lajmanovich, 2007), and *x* bar to the soybean growth season (sensu Lorenzatti et al., 2004).

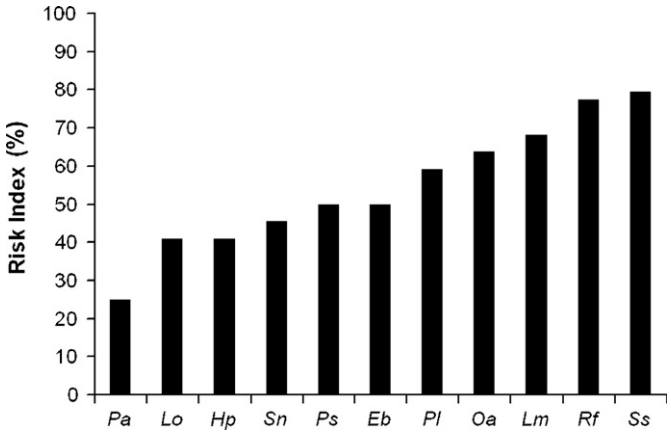

Fig. 4. Relative risk index (RI) that integrates basal buffer and detoxification enzymes (butyrylcholinesterase, BChE; carboxylesterases, CbEs) and soybean land cover amphibian overlap (SLCAO), soybean phenology overlap (SPO) of 11 species larval anuran. (*Pa*: *P. albonotatus*; *Lo*: *L. ocellatus*; *Hp*: *H. pulchellus*; *Sn*: *S. nasicus*; *Ps*: *P. santafecinus*; *Eb*: *E. bicolor; Pl*: *P. limellum*; *Oa*: *O. americanus*; *Lm*: *L. mystacinus*; *Rf: R. fernandezae*; *Ss: S. squalirostris*). Risk scale: 0 (lowest)–100% (highest).

Sparling et al., 1997; Sturm et al., 2000; Sánchez-Hernández, 2007), because the BChE protects brain AChE from inhibition by OP, CB, and PY.

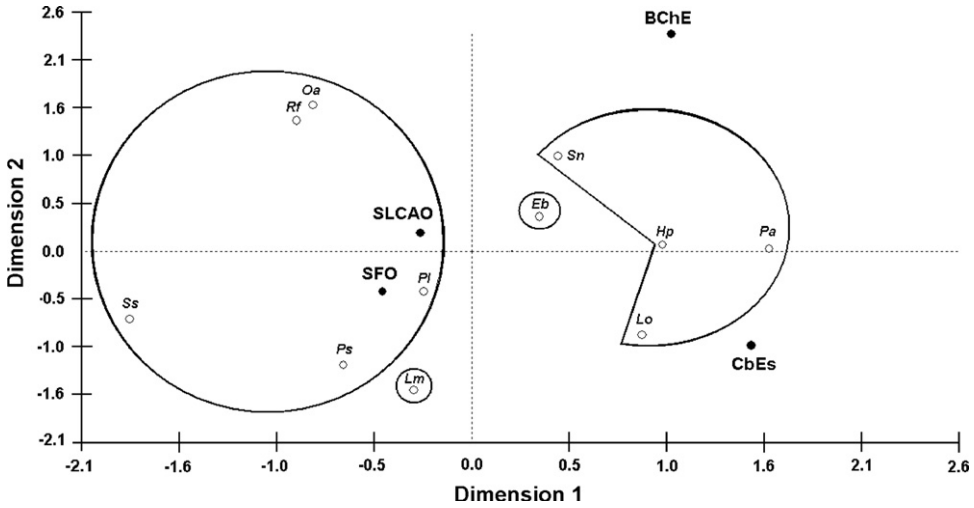

Fig. 5. Plot of the first two dimensions from correspondence analysis of anuran species-risk index (RI) variables (butyrylcholinesterase, BChE and total carboxylesterase CbEs values, soybean land cover amphibian overlap, SLCAO and soybean phenology overlap, SFO) included (*Rf: R. fernandezae*; *Lo*: *L. ocellatus*; *Lm*: *L. mystacinus*; *Pa*: *P. albonotatus*; *Ps*: *P. santafecinus*; *Oa*: *O. americanus*; *Hp*: *H. pulchellus*; *Sn*: *S. nasicus*; *Ss: S. squalirostris; Pl*: *P. limellum*; *Eb*: *E. bicolor*). The first dimension was positively related to enzymes level and negatively with temporal and spatial anuran–soybean overlaps.

Kinetic parameters of tadpole CbE activity differed depending on the substrate used for quantification. Those differences could be interpreted by the presence of multiple isozymes in the crude extract (Laguerre et al., 2009). Multiple CbE isozymes occur also in mammal tissues (Satoh and Hosokawa, 1998) and other non-mammalian species (e.g. earthworm and snail; Sánchez-Hernández and Wheelock, 2008; Laguerre et al., 2009, respectively). Multiple CbE isozymes were found in the crude homogenate of 11 tadpole species, with the highest CbE activities with the 4-NPV substrate. However, the role of the differences among substrates in either biological or toxicological mechanisms has not been completely established (Sánchez-Hernández and Wheelock, 2008). Frequently in small mammals, fishes and birds, ChE activity is greater in younger, smaller organisms than in older, larger ones (e.g. Fishwick et al., 1996; Chuiko et al., 1997; Roy et al., 2005; Sogorb et al., 2007). These relationships were found among BChE and CbE activities and body sizes in adults of 3 species of South American toads (Lajmanovich et al., 2008). However, in the 11 tadpole species studied here, no relationship was found between body size and enzyme activity. Similar results have been determined in other vertebrates (Grue and Hunter, 1984; Gard and Hooper, 1993; Atterberry et al., 1997). Interspecies variation in ChE activity has been reported for birds, mammals (Walker and Thompson, 1991), fish species (Chuiko et al., 2003), and among fish populations (Gibson et al., 1969). Anuran tadpole ChE can fluctuate during the early development (Gindi and Knowland, 1979) and seasons (Venturino et al., 2003). Remarkably, a recent study has found that AChE and CbE activities in a neotropical tadpole (*S. fuscovarius*) are independent of development stage dependence (Leite et al., 2010). Differences among tadpole species apparently have yet to be characterized (Henry, 2000); however, differences in ChE activities have been reported among species of anuran adults (Lajmanovich et al., 2008). Although the B-esterase enzymes studied here presented enclosed ranges among the species, there are clear interspecific differences. While these differences could potentially be attributed to an innate biochemical or physiological basis, more research is necessary to test this hypothesis.

Although some investigators (Mortensen et al., 1998) have not found correlation between acute toxicity (measured by LC_{50}) and baseline enzyme activities (measured by IC_{50}), recent studies reinforced the opposite pattern (e.g. Kramer et al., 2009). As explained by Mortensen et al. (1998), IC_{50s} obtained from incubation of crude homogenates may not indicate the inherent sensitivity of the enzyme to the anticholinesterase pesticide, but they are an indication of the capacity of the tissue or blood to sequester the circulating OP. In vitro IC_{50} values could be useful in determining tissue differences in buffering capacity, and indirectly to establish what amphibian species are most vulnerable in "real-life" situations (Lajmanovich et al., 2008). Taking into account this assumption, *P. santafesinus* could have the lowest tolerance capacity to malaoxon because of its low IC_{50} value. However, further work is still necessary to assess if the IC_{50} outcomes are reproduced in field situations in terms of sensitivity of the tadpoles to OP toxicity. Likewise, Ferrari et al. (2004) compared OP and CB susceptibility of two aquatic vertebrates (including *Rhinella* tadpole), and observed that the IC_{50} was related to LC_{50}. Furthermore, more recently studies by Relyea and Jones (2009) and Jones et al. (2009) determined that 9 species of larval anurans had differential sensitivity to pesticides among the species or families, and suggested some phylogenetic patterns to this susceptibility. Indeed, in the last study the LC_{50} values among species differed by two orders of magnitude. Our results can help to elucidate this question of variability in sensitivities to pesticides, because different species of amphibians show interspecific variation in sensitivity, taking into account variations in such enzymes that are responsible for buffering and detoxification of pesticides.

Standard methodologies for assessing the risk of chemicals in the environment are based on a framework of hazard identification, exposure and toxicity assessments and conclude with risk characterization (USEPA, 1996). However, new approaches have emerged to deal with the difficult issue of assessing the environmental impact of contaminants in its broadest sense, i.e., at the ecosystem level. The procedures presented provide a tool for identifying amphibian species at risk in regions dominated by soybean agriculture, determining the temporal distribution of risk, exploring landscape management and considering some biochemical parameters. This procedure results in a spatially and ecologically based risk assessment that can incorporate multiple sources of uncertainty. Besides incorporating anuran spatial and temporal overlap with soybean cultivation, it would be interesting to incorporate ecological attributes or autoecological parameters (e.g. spatial guild, food strategy, reproductive mode) of amphibian species to characterize level of risk. Moreover, additional investigation may include experiments conducted in

the laboratory or with manipulative field experiments, which may identify the most effective means of risk reduction for a particular species or stressor.

Our results suggest that we can categorize *S. squalirostris* and *R. fernandezae* as the most vulnerable species from the community studied because of their low enzyme values and the high overlap in reproductive period and geographical distribution with soybean crop phenology and land cover use, respectively. Moreover, *P. albonotatus* has the lower RI value. At this point, it is important to note that although we performed in vitro approach to reinforce the RI procedure, these determinations together with ecological attributes (such as mentioned before) could explain the variation in RI values obtained for each species. For instance, *P. albonotatus* was recorded in 73.3% of the total ponds sampled surrounded by soybean croplands in Argentina (Peltzer et al., 2006) and it was categorized as an invasive species for its abundance in agricultural systems. One explanation may be that the high level of CbEs activity observed in the present study may allow it to persist at such environments with potential agrochemical contamination.

Considering role of agricultural activities and the insufficiently strict regulations on agrochemicals, particularly in Latin American countries, this study reveals a promising avenue of research of anuran species and their bioecological responses.

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