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Biochemical changes in certain enzymes of *Lysapsus limellium* (Anura: Hylidae) exposed to chlorpyrifos

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ABSTRACT

Different enzyme biomarkers (AChE: acetylcholinesterase, CbE: carboxylesterase, GST: glutathione-S-transferase, CAT: catalase) were measured in digestive tissues of *Lysapsus limellum* frogs collected from a rice field (RF: chlorpyriphos sprayed by aircraft) and a non-contaminated area (RS: reference site), immediately (24 h) and 168 h after aerial spraying with chlorpyrifos (CPF). CPF degradation was also searched in water samples collected from RF and RS, and found that insecticide concentration was reduced to ≈ 6.78% of the original concentration in RF at 168 h. A significant reduction of AChE and CbE activities was detected in *L. limellum* from RF in stomach and liver at 24 and 168 h, and in intestine only at 24 h, with respect to RS individuals. CAT activity decreased in intestine of *L. limellum* from RF 24 h and 168 h after exposure to CPF, whereas GST decreased in that tissue only at 24 h. In stomach and liver, a decrease was observed only at 168 h in both CAT and GST. The use of biomarkers (AChE, CbE, GST, and CAT) provides different lines of evidences for ecotoxicological risk assessment of wild frog populations at sites contaminated with pesticides.

1. Introduction

The expansion of rice fields is one of the anthropogenic activities associated with the disappearance of wetlands and lentic environments throughout the world (Machado and Maltchik, 2010). Despite landscape simplification, rice fields present abundance of certain aquatic species that can adjust their biological and ecological traits to this homogeneous environment, a fact that was especially demonstrated for anuran amphibians (Bambaradeniya et al., 2004; Duré et al., 2008).

In Argentina, rice is a highly valuable crop cultivated under submerged conditions at the expense of considerable freshwater resources and pesticides (Tilman et al., 2002). Organophosphorus pesticides (OP) are an important group of agrochemicals widely used in this type of agroecosystem. OP are rapidly metabolized in vertebrate and invertebrate organisms (Racke, 1992) and have a relatively short persistence in the environment, in the order of hours to few days (Lacorte et al., 1995), with hydrolysis, photolysis and microbial degradation being the main destruction mechanisms of these ephemeral molecules.

Several biomarkers are increasingly included in environmental management to assess the health of organisms in complex ecosystems (Galloway et al., 2002). Biomarkers have the advantage of detecting early adverse effects resulting from exposure to pesticides and altered physical–chemical parameters (oxygen, pH, and nutrients). Consequently, a suite of biomarkers, such as measurable endpoints at molecular, cellular and physiological levels, may be necessary to successfully determine the health status of populations, and once selected for a particular case, they can be adapted to different ecological scenarios (Sánchez–Hernández, 2007; Almeida et al., 2010). OP pesticides exert acute toxicity by

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inhibiting B-esterase enzymes such as acetylcholinesterase (AChE, EC: 3.1.1.7) in the neuromuscular function; in cases of severe intoxication, OP provoke seizures and respiratory failure, causing the death of biota (Fukuto, 1990). However, OP detoxification is mediated primarily by carboxylesterases (CbEs, EC 3.1.1.1; Sogorb and Vilanova, 2002; Wheelock et al., 2008). Thus, B-esterase inhibition by OP in tissues may persist for days to weeks (Morgan et al., 1990; Wijeyaratne and Pathiratne, 2006), even if they no longer contain detectable traces of pesticides.

Glutathione S-transferases (GST EC 2.5.1.18) are another group of biotransformation enzymes that has been widely used as environmental biomarker. These enzymes catalyze the conjugation of xenobiotics and their metabolites with endogenous glutathione to facilitate their elimination from the organism (Greulich and Pflugmacher, 2004). Another key cellular defense mechanism against hydroperoxides that may be the target of pesticide action is the enzyme catalase (E.C. 1.1.1.6, CAT). CAT activity has been analyzed in different organisms exposed to diverse pollutants, including pesticides (Greulich and Pflugmacher, 2004; Ferrari et al., 2011; Peltzer et al., 2013).

In the present study, differences in levels of enzymatic activity of AChE, CbE (using substrates; a-naphthyl acetate), GST, and CAT of *Lysapsus limellum* frogs present in a rice field were studied and compared with similar data from individuals sampled from a reference site (pristine forest) located in Mid-eastern Argentina. In addition, we investigated chlorpyrifos (CPF) degradation in water collected from each sampling sited. Enzymatic biomarkers are usually used to detect biological effects of exposure to pesticides on amphibian larvae and adults (Robles-Mendoza et al., 2011; Peltzer et al., 2013; Ilizaliturri–Hernández et al., 2013; Attademo et al., 2014a). These endpoints provide important lines of evidences in ecotoxicological risk assessments (den Besten et al., 2003). Therefore, this study may be useful for ecotoxicological risk assessment of anurans present in rice agroecosystems.

2. Material and methods

2.1. Study area

The study was conducted in Santa Fe province, located in Mid-eastern Argentina. Rice productions extending on the floodplains of Paraná River dominate this area (Alvisio, 1998). The area presents a rainy season that extends from October to March and a dry season from April to September. Rice fields in this region are surrounded by few and dispersed forest fragments characterized by native vegetation of the Deltas and Islands of Paraná River and Espinal Ecoregions (Burkart et al., 1999; Peltzer et al., 2004).

2.2. Fields survey

The effects of realistic CPF exposure on amphibians were compared between two sites, intensively managed rice field (RF) and a reference site (RS) (Fig. 1). The former (RS) was located within riparian forests along Paraná River in Garay department, Santa Fe province, Argentina (31°10′21.10″S; 60°15′31.73″W). RF was a rice (*Oryza sativa*) cropland located in San Javier department (30° 05′ 13.56″ S-59° 53′ 19.98″ W). On 30 January 2013, the insecticide formulation (commercial grade; 48% a.i.) of CPF (Terminator, AGROS Soluciones, Argentina) was applied by aircraft at a proportion of 500 cm^3/ha. After that CPF application, mortality of *L. limellum* individuals was detected, reaching approximately 3-4 dead frogs per m^2. Likewise, dead invertebrates (ants, lobsters, and worms) and different species of fishes were also observed. Mortality incidents involving *L. limellum* and other anurans (*Pseudis paradoxa*, *Leptodactylus chaquensis*, *Dendropsophus nanus*) were previously recorded in rice crops (Lajmanovich et al., 2012).

The other site (RS) was a wetland without any agricultural activities in the nearby areas and previous reports determined that this site is free of anticholinesterase chemicals (Lajmanovich et al., 2010; Attademo et al., 2014a,b). RS is dominated by different tree species, such as *Salix humboldtiana, Tessaria integrifolia, Enterolobium contortisiliquum*, and *Acacia caven*. The ephemeral

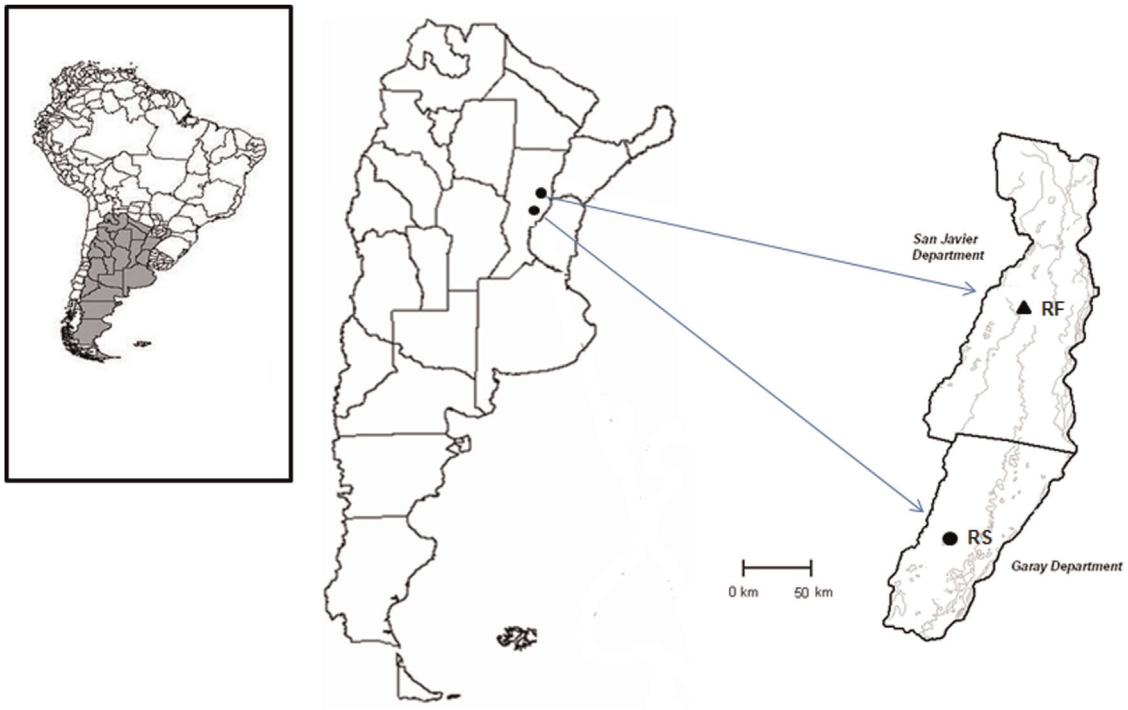

Fig. 1. Location of sampling sites in Mid-eastern Argentina. RS: reference site and RF: rice field subjected to chlorpyrifos application.

ponds and marshes present *Eichhornia crassipes, Pontederia cordata, Saggitaria montevidensis, Typha latifolia, Cortaderia selloana, Cyperus corymbosus, Salvinia biloba*, and *Pistia stratiotes.*

2.3. Study species

L. limellum (Amphibia, Anura, Hylidae) was selected because it is frequently found in modified environments such as rice fields (Duré et al., 2008). It is widely distributed along Paraguay and Paraná rivers ecosystems (Cei, 1980). In Argentina, this frog is categorized as "not threatened" (Vaira et al., 2012) and is distributed in the provinces of Buenos Aires, Formosa, Chaco, Corrientes, Santiago del Estero, Entre Ríos, and Santa Fe. *L. limellum* is a small-sized species, varying from 16 to 21 mm at adult stages, and it is usually associated with aquatic macrophytes (Peltzer et al., 2006; Sánchez et al., 2009).

2.4. Animal sampling

Forty seven adult males of *L. limellum* were collected by hand from the sampling sites on two occasions: immediately (24 h; RS=13 and RF=12; 31 January 2013) and after 168 h (RS=12 and RF=10; 7 February 2013) aerial spraying with CPF. The two observation periods were selected based on previous works (Sánchez et al., 1997; Lajmanovich et al., 2009; Attademo et al., 2011). All the animals captured were males because they are easily identified in the field by their calls and to minimize population impact (ASIH et al., 2001); gravid females were avoided. After capture, animals were rapidly transported to the laboratory in darkened buckets containing water (approximately 2 cm deep) to minimize stress. At the laboratory, frogs were anaesthetized individually by immersion in a buffered solution of 0.1% tricaine methanesulfonate (MS-222), with approval of the animal ethics committee of the Faculty of Biochemistry and Biological Sciences, National University of Littoral, and following the guidelines of ASIH et al. (2001). Each individual was weighed to the nearest 0.0001 g using a digital balance and their snout-vent length (SVL) was measured with a digital caliper. To compare the physiological condition of individuals among populations, condition factor (CF) for each animal was calculated and expressed as 100x[body weight (g)]/[length (cm)]^3 (Bagenal and Tesch, 1978). Each individual was dissected along the mid-ventral line by making a longitudinal incision, and digestive organs (stomach, intestine, and liver) were removed. Digestive organs contribute mainly to the OP detoxification (Attademo et al., 2014b). Tissues were washed in distilled water and placed on filter paper to remove excess fluids. Tissue samples were stored at −80 °C until biochemical analyzes were performed.

2.5. Water parameters

To analyze chlorpyrifos degradation (methyl chlorpyrifos, 99.5% and ethyl chlorpyrifos, 99.5%) in water from RF and RS, samples were collected in triplicate from a sprayed area after 24 and 168 h of pesticide application and simultaneously from the RS. Each water sample vas transported in a glass bottle to the laboratory and either processed immediately or frozen at −18 °C.

In addition, other chemical pesticide screenings (Chem Service, Fluka and Applied Science, USA) were performed in water samples in order to discard the presence of the following chemicals: γ-HCH (lindane) 99%; aldrin 99.9%; dieldrin 97.9%; p,p-DDT, o,p-DDT32 and 65.6% p,p-DDE 99.5%; p,p-DDD 99%; heptaclor 99.2%; heptaclor epoxide 99.5%; α and γ-Chlordane 99%; endosulfan I and II 59.2 and 39.9%, endosulfan sufate 98.5%; endrin 99%; cypermehrin 99%, and permethrin 99.5%. All pesticide residues were identified and quantified by VARIAN 3400 gas chromatograph (GC), with automatic injector, electronic capture detector (ECD), DB-608 Megabore column (30 m × 0.53 mm I.D., 1.5 μm), and DB-5 capillary column (30 m × 0.25 mm i.d.) (J&W Scientific CA, USA), injector temperature 240 °C, detector temperature 320 °C and column temperature 220 °C. For mass spectrometry, a Varian 2000 chromatograph with mass selective detector (MSD) and an HP-5 capillary column (30 m × 0.25 mm ID) was used.

Table 1
Summary of pesticide residue data of water from a reference site (RS) and a rice field (RF) subjected to chlorpyrifos application.

Pesticide residues ± SE (ng L^{-1})	RS		RF	
	24 h	168 h	24 h	168 h
Chlorpyrifos	ND$^{(10)}$	ND$^{(10)}$	**1388 ± 0.2**	**97.0 ± 5.1**
γ-HCH (Lindane)	ND $^{(1)}$	ND$^{(1)}$	ND$^{(1)}$	ND$^{(1)}$
Aldrin	ND$^{(2)}$	ND$^{(2)}$	ND$^{(2)}$	ND$^{(2)}$
Dieldrin	ND$^{(8)}$	ND$^{(8)}$	ND$^{(8)}$	ND$^{(8)}$
p,p-DDT y o,p-DDT	ND$^{(10)}$	ND$^{(10)}$	ND$^{(10)}$	ND$^{(10)}$
p,p-DDE y p,p-DDD	ND$^{(8)}$	ND$^{(8)}$	ND$^{(8)}$	ND$^{(8)}$
Heptachlor epoxide	ND$^{(2)}$	ND$^{(2)}$	ND$^{(2)}$	ND$^{(2)}$
γ and α-Chlordane	ND$^{(4)}$	ND$^{(4)}$	ND$^{(4)}$	ND$^{(4)}$
Heptachlor	ND$^{(2)}$	ND$^{(2)}$	ND$^{(2)}$	ND$^{(2)}$
Endosulfan I and II	ND$^{(4)}$	ND$^{(4)}$	ND$^{(4)}$	ND$^{(4)}$
Endosulfan sulfate	ND$^{(4)}$	ND$^{(4)}$	ND$^{(4)}$	ND$^{(4)}$
Endrin	ND$^{(5)}$	ND$^{(5)}$	ND$^{(5)}$	ND$^{(5)}$
Cypermethrin	ND$^{(16)}$	ND$^{(16)}$	ND$^{(16)}$	ND$^{(16)}$
Permethrin	ND$^{(28)}$	ND$^{(28)}$	ND$^{(28)}$	ND$^{(28)}$

ND: non-detected at the detection limit mentioned; superscript value indicates the detection limit (ng L^{-1}).

2.6. Enzymatic assays

Whole tissue samples were homogenized on ice in 20% (w/v) buffer containing 0.1% t-octylphenox- y polyethoxy ethanol (tritonX-100) in25mMtris (hydroxymethyl) amino methane hydrochloride (pH 8.0) using a polytron. The homogenates were centrifuged at 10,000 rpm at 4 °C for15 min and the supernatant was collected. AChE activity was determined colorimetrically following Ellman et al. (1961) procedure. The reaction mixture (final volume [F.V.=930 μl]) consisted of 25 mM Tris–HCl containing 1 mM $CaCl_2$ (pH=7.6), 10 μl 20 mM acetylthiocholine iodide (AcSCh), and 50 μl DTNB (3×10^{-4} M, final concentration). Variation in optical density was measured in duplicate at 410 nm at 25 °C for 1 min using a Jenway 6405 UV–vis spectrophotometer. Total Protein concentrations in the supernatants were determined using the Biuret method (Kingbley, 1942). AChE activity was expressed in nmol of hydrolyzed substrate per minute per milligram of protein using a molar extinction coefficient of 13.6×10^3 M^{-1} cm^{-1}. Carboxylesterase was determined using α-naphthyl acetate (α-NA) substrate and specific enzyme activity was expressed as nmol min^{-1} mg^{-1} protein. The hydrolysis of α-NA by CbE was measured as described by Gomori (1953) and adapted by Bunyan and Jennings (1968). The reaction medium (F.V.=1950 μl) contained 25 mM Tris–HCl, 1 mM $CaCl_2$ (pH=7.6) and sample. The reaction was initiated by adding 50 μl α-naphthyl acetate (1.04 mg ml^{-1} in acetone) after a preincubation period of 5 min at 25 °C. The formation of naphthol was stopped after 10 min by adding 500 μl 2.5% sodium dodecyl sulfate and subsequently 0.1% of Fast Red ITR in 2.5% Triton X-100 in deionizer water (prepared immediately before use). The samples were allowed to develop in the dark for 30 min, 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}).

GST activity was determined spectrophotometrically using the method described by Habig et al. (1974) and adapted by Habdous et al. (2002) for mammal serum GST activity. The enzyme assay was performed at 340 nm in 100 mM Na–phosphate buffer (pH

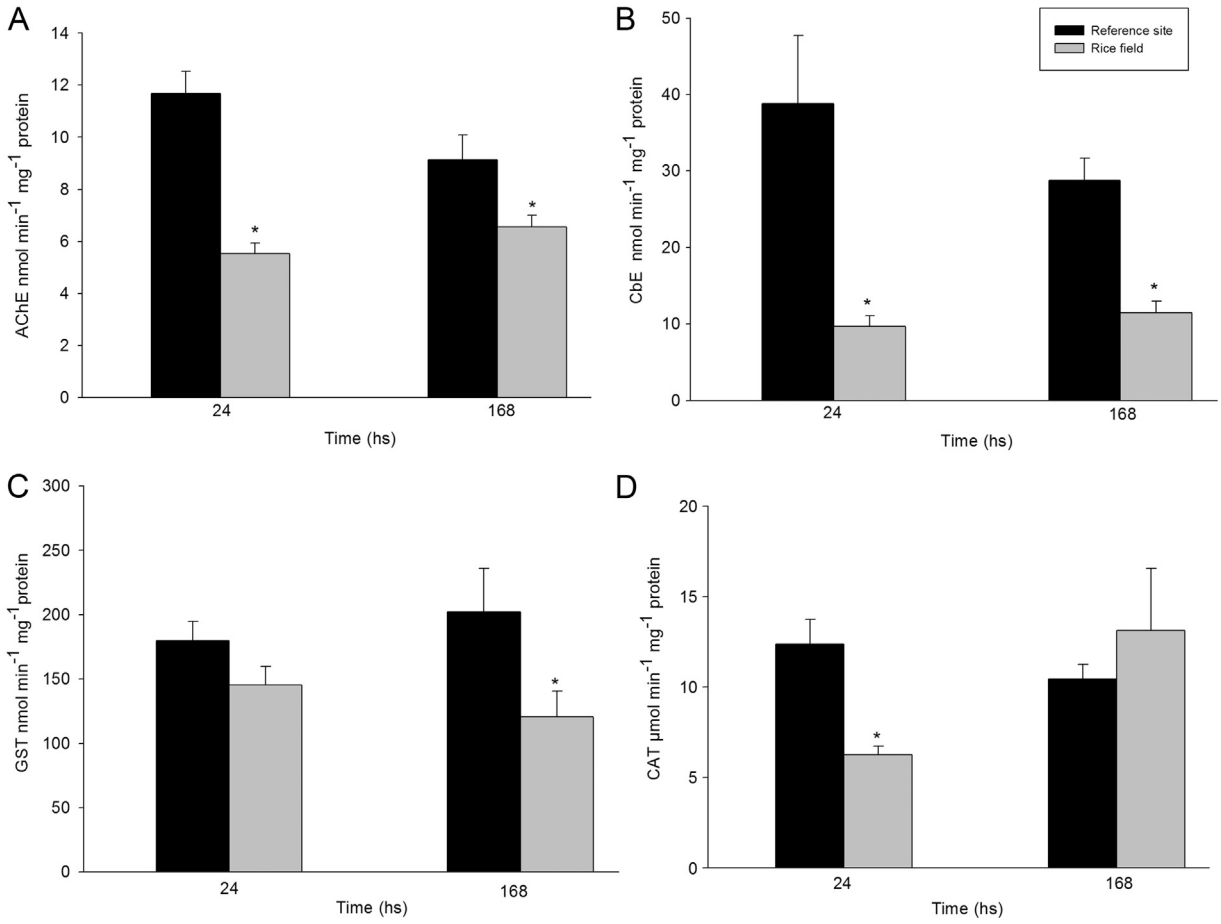

Fig. 2. Comparative values of enzymatic activity in stomach of *L. limellum*. A: acetylcholinesterase (AChE). B: carboxylesterase (CbE). C: glutathione-S-transferase (GST) and D: catalase (CAT). Bars represent the mean ± SEM. ✻P < 0.05 compared with the reference site at 24 and 168 h.

6.5) [F.V.=920 µl]), 20 µl of 0.2 mM 1-chloro-2, 4-dinitrobenzene, 50 µl of 5 mM reduced glutathione, and the sample. Enzyme kinetics assays was performed at 25 °C and whole GST activity was expressed as µmol min^{-1} mg^{-1} protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}

CAT activity was measured using the method described by Aebi (1984), and was expressed as umol H_2O_2 µmol min^{-1} mg^{-1} protein using a molar extinction coefficient of H_2O_2 40×10^{-3} L mol cm^{-1}. The reaction medium was 50 mM phosphate buffer (pH=7.2) and 30 mM H_2O_2 and absorbance was read on the spectrophotometer at a wavelength of 240 nm at 25 °C (quartz cuvette).

2.7. Data analysis

Data of enzymatic activities were expressed as the mean and standard error of the mean ($x \pm$ SEM). The influence of sampling sites (RF and RS) and sampling periods (24 and 168 h) on AChE, CbE, CAT, and GST enzyme activities in tissue was analyzed with the Student *t*-test. The Student *t*-test was also used for comparison of CF. Data was tested for homogeneity and normality of variance (Kolmogorov–Smirnov test and Levene test). Statistical analyzes were performed using INFOSTAT/ P 1.1 for Windows software (Grupo InfoStat Professional, FCA, Universidad Nacional de Córdoba, Argentina). The criterion for significance was $p < 0.05$.

3. Results

3.1. Water parameters

Water concentrations of pesticides were below their respective limits of detection (Table 1). CPF was the only OP detected in water samples from RF, at concentrations of 1388 ng L^{-1} at 24 h and 97 ng L^{-1} at 168 h.

3.2. Condition factor

The mean condition factor (CF) values for *L. limellum* collected from RF was 9.71 ± 0.56 at 24 h and 11.22 ± 0.70 at 168 h; these values were similar to those recorded in frogs from RS: 10.70 ± 0.52 at 24 h and 11.14 ± 0.43 at 168 h (Student $t=1.30$, $p > 0.05$ and $t=1.01$, $p > 0.05$).

3.3. B-esterase and oxidative stress enzymes in digestive tissues

3.3.1. Stomach

B-esterase (AChE and CbE) activity and oxidative stress (GST and CAT) for each site (RF and RS) and two sampling periods (24 and 168 h) are summarized in Figs. 2–4. In frog stomachs, AChE activity was lower for individuals collected from RF than for those from RS at 24 h (Student test $t=6.24$, $p < 0.001$; Fig. 2A) and 168 h ($t=2.71$, $p < 0.05=0.016$; Fig. 2A). Reference CbE activities were 38.83 ± 8.92 nmol min^{-1} mg^{-1} protein TP at 24 h and 29.79 ± 2.86 nmol min^{-1} mg^{-1} protein at 168 h. CbE activity was significantly lower in individuals from RF than in those from RS at

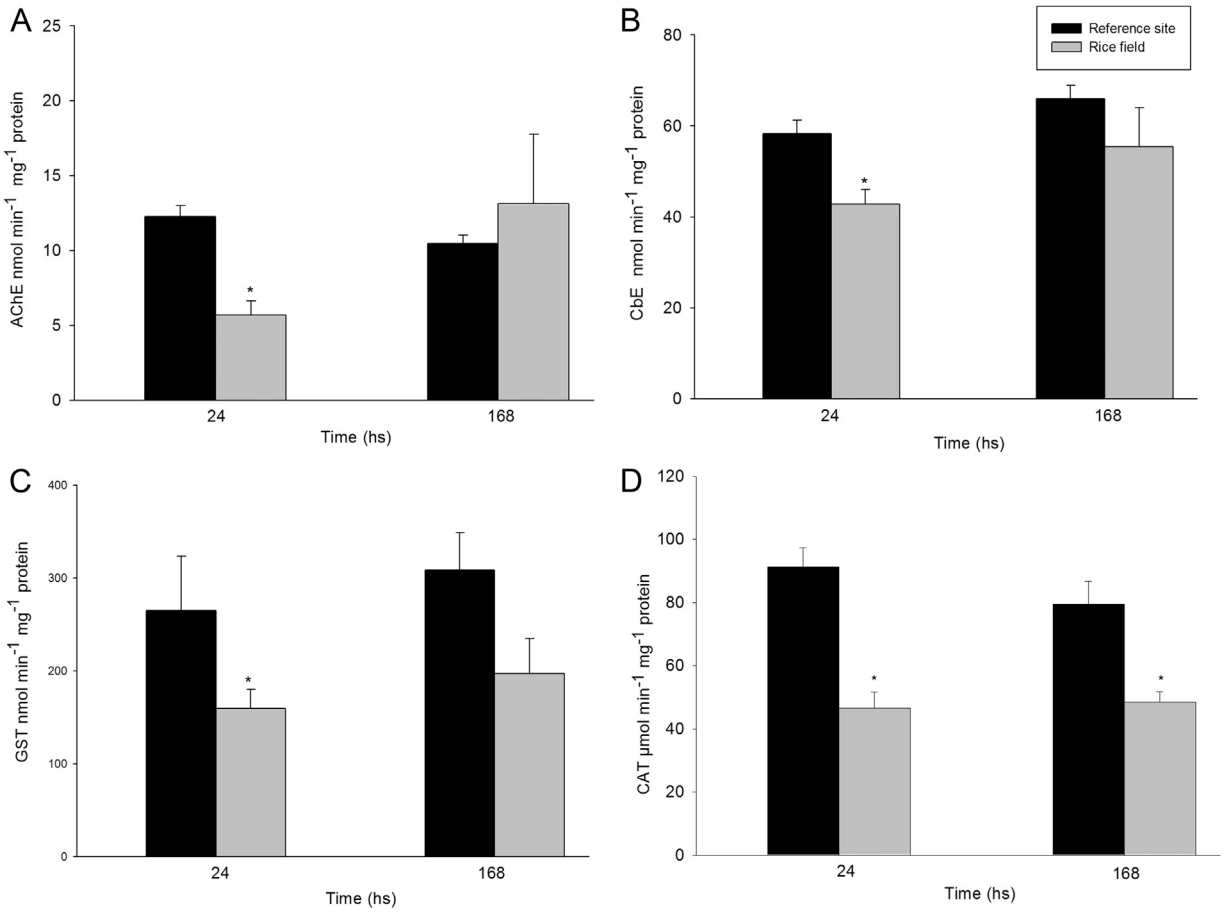

Fig. 3. Comparative values of enzymatic activity in intestine of *L. limellum*. A: acetylcholinesterase (AChE). B: carboxylesterase (CbE). C: glutathione-S-transferase (GST) and D: catalase (CAT). Bars represent the mean ± SEM. ∗$P < 0.05$ compared with the reference site at 24 and 168 h.

24 ($t=2.43$, $p<0.05$; Fig. 2B) and 168 h ($t=2.45$, $p<0.05$; Fig. 2B). GST activity in stomach tissue of RS individuals was 179.82 ± 14.87 nmol min^{-1} mg^{-1} protein at 24 h and 202.17 ± 33.74 nmol min^{-1} mg^{-1} protein at 168 h. GST activity exhibited significantly lower values in individuals collected from RF than in those from RS at 168 h ($t=2.21$, $p<0.05$; Fig. 2C). Similarly, CAT activity was significantly lower in individuals collected from RF at 24 h ($t=9.14$, $p<0.001$; Fig. 2D) than in RS individuals.

3.3.2. Intestine

Mean values of the AChE activity in intestine were 12.26 ± 0.70 nmol min^{-1} mg^{-1} protein at 24 h and 10.47. ± 0.54 nmol min^{-1} mg^{-1} protein at 168 h. AChE activity was significantly lower in individuals collected from RF at 24 h than in RS individuals ($t=5.43$, $p<0.001$; Fig. 3A). Control CbE activity in RS individuals was 58.27 ± 3.00 nmol min^{-1} mg^{-1} protein at 24 h and 65.98 ± 3.01 nmol min^{-1} mg^{-1} protein at 168 h. CbE activity was different between RS and RF at 24 h ($t=2.12$, $p<0.05$; Fig. 3B). GST and CAT activity in the intestine of individuals from RF was 264.77 ± 59.08 nmol min^{-1} mg^{-1} protein and 91.35 ± 6.01 nmol min^{-1} mg^{-1} protein at 24 h; and 308.70 ± 40.18 nmol min^{-1} mg^{-1} protein and 79.45 ± 7.31 nmol min^{-1} mg^{-1} protein at 168 h. GST and CAT activities were significantly lower in individuals collected from RF than in those from RS at 24 h ($t=2.39$, $p<0.05$ and $t=6.72$, $p<0.001$; respectively), but only CAT was significantly lower in RF individuals than in RS individuals at 168 h ($t=3.82$, $p<0.001$; Fig. 3C and D).

3.3.3. Liver

Mean values of the AChE activity were 16.05 ± 1.20 nmol min^{-1} mg^{-1} protein at 24 h and 13.93 ± 0.84 nmol min^{-1} mg^{-1} protein at 168 h in RS. AChE activity was significantly lower in individuals collected from RF at 24 h ($t=4.16$, $p<0.001$) and 168 h ($t=2.48$, $p=0.026$; Fig. 4A) than in RS individuals. Control CbE activity in RS was 127.48 ± 9.92 nmol min^{-1} mg^{-1} protein at 24 h and 107.58 ± 9.71 nmol min^{-1} mg^{-1} protein at 168 h. CbE activity was different between individuals from RS and RF at 24 h ($t=3.98$, $p<0.001$) and 168 h ($t=4.92$, $p<0.001$; Fig. 4B). GST and CAT activity in liver of individuals from RF was 470.78 ± 40.51 nmol min^{-1} mg^{-1} protein and 199.24 ± 9.55 nmol min^{-1} mg^{-1} protein at 24 h; and 515.77 ± 32.21 nmol min^{-1} mg^{-1} protein and 202.93 ± 5.12 nmol min^{-1} mg^{-1} protein at 168 h. GST and CAT activities were significantly lower in individuals collected from RF than in those from RS at 168 h ($t=9.64$, $p<0.001$ and $t=3.94$, $p<0.001$; respectively Fig. 4C and D).

4. Discussion

Chlorpyrifos (CPF) was applied to control numerous pests in rice agroecosystems during the evaluated crop season. A notable degradation (> 12-fold) of CPF over seven days was found in water of the RF. Similar concentrations were reported for Central California Coast (Hunt et al., 2003). Two separate studies agreed that transformation of CPF is rapid during the first few days following application, with this transformation being generally attributed to potential volatilization and degradation (Racke, 1992).

A significant reduction of AChE and CbE activities was detected in *L. limellum* from RF in stomach and liver at 24 and 168 h, and in intestine only at 24 h with respect to RS individuals.

Studies exploring variation of AChE and CbE in tissue samples

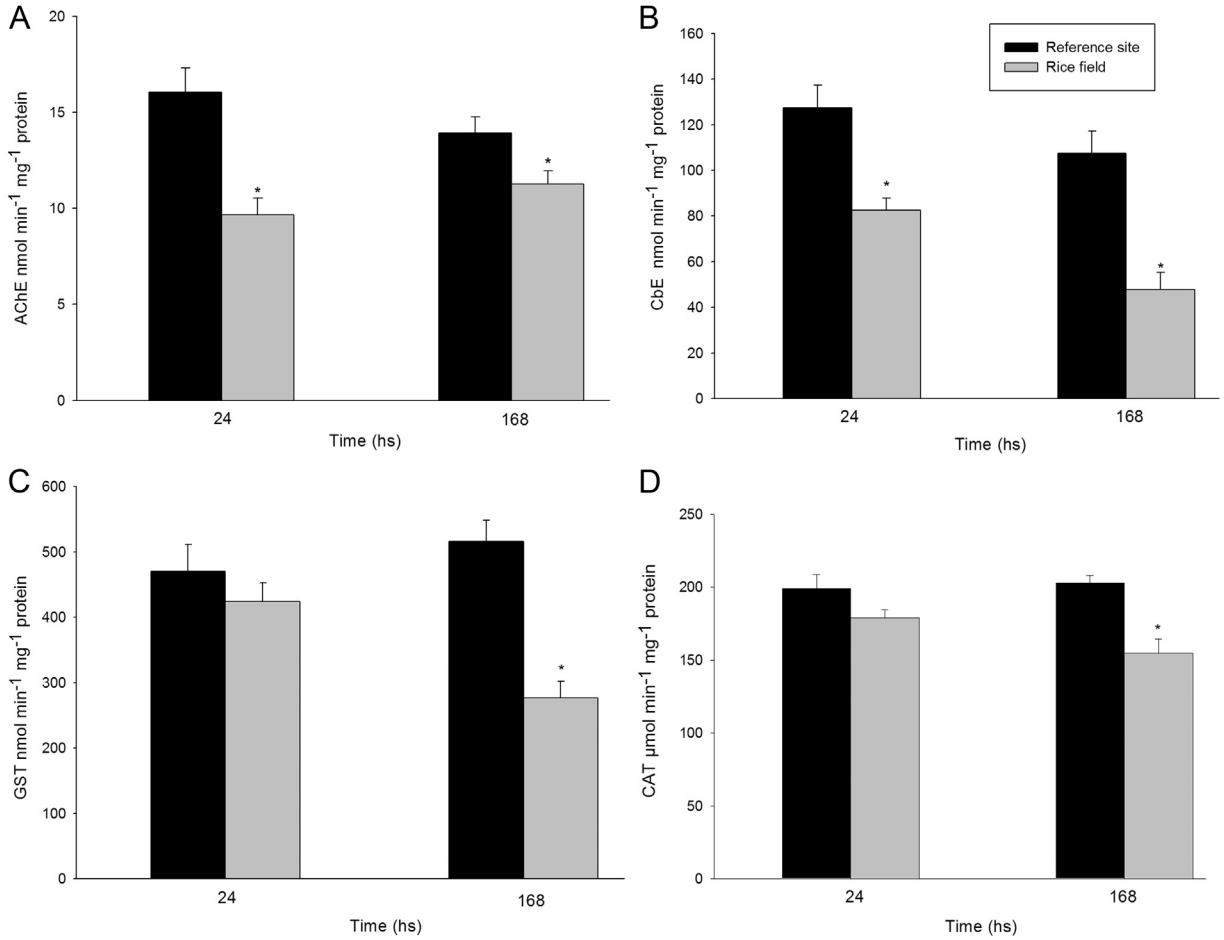

Fig. 4. Comparative values of enzymatic activity in liver of *L. limellum*. A: acetylcholinesterase (AChE). B: carboxylesterase (CbE). C: glutathione-S-transferase (GST) and D: catalase (CAT). Bars represent the mean ± SEM. $*P < 0.05$ compared with the reference site at 24 and 168 h.

of adult frogs are scarce. However, previous experiments in our laboratory showed similar results in another native species (*Hypsiboas pulchellus*) at tadpole stages, as well as in fish (*Synbranchus marmoratus*) exposed to fenitrotion (OP) (Junges et al., 2010). Similarly, Colombo et al. (2005) and Widder and Bidwell (2008) observed a significant reduction of cholinesterase (AChE) activity in anuran tadpoles (*Xenopus laevis, Hyla chrysoscelis*, *Rana sphenocephala*, *Acris crepitans*, and *Gastrophryne olivacea*) exposed to CPF. This enzyme inhibition in tissues by OP may persist for days to weeks (Morgan et al., 1990; Wijeyaratne and Pathiratne, 2006), and is in accordance with the present observation for B-esterases after 168 h of application. Similarly, Strauss and Chambers (1995) found that CPF was a powerful inhibitor of AChE activity in fingerling channel catfish (*Ictalurus punctatus*). After exposure to AChE inhibitors, the length of AChE depression affects the recovery of organisms and their possibility of survival under field conditions (Beauvais et al., 2001), which is consistent with the present results. Wijeyaratne and Pathiratne (2006) observed significant inhibition of brain AChE activity in fish (*Rasbora caverii*) from rice fields, with this inhibited activity persisting even 65 days after insecticide application. Among B-esterases, CbEs are important to reduce OP toxicity (Stenersen, 2004) and these isozymes may contribute to pesticide tolerance due to their capability to bind to OPs, decreasing the effective concentration of the pesticide (Wheelock et al., 2008). Some environmental pesticides found to inhibit AChE activity may also inhibit CbE activity (Küster, 2005); here we found lower CbE activities in all digestive tissues of *L. limellum* individuals from RF.

Moreover, GST activity decreased in intestine of *L. limellum* from the RF at 24 h and stomach and liver at 168 h. This enzyme catalyzes the conjugation of electrophilic xenobiotics to GSH (Gadagbui and James, 2000), plays an important role in protecting tissues from oxidative stress (Ognjanovic´ et al. 2003; Ferrari et al. 2011) and its inhibition could lead to an imbalance of the cellular redox status. Likewise, Frasco and Guilhermino (2002) and Özkol et al. (2012) demonstrated that dimethoate (OP) inhibits GST activity in fish (*Poecilia reticulata*) and in different tissues of frogs (*Rana ridibunda*). Accordingly, a lower level of GST activity has also been reported at 48 and 96 h in congeneric amphibian tadpoles (*Scinax squalirostris*) in rice crops of an agroecosystem (Attademo et al., 2014a).

In addition, CAT activity decreased in stomach and liver of *L. limellum* frogs at 168 h, and only in intestine did it decrease after 24 and 168 h in individuals from RF. A reduction in CAT activity may be caused by the flux of superoxide radicals (O^2-) induced by the CPF (Ahmad et al., 2000). Likewise, Wielgomas and Krechniak (2007) reported the alteration in CAT activity in wistar rat (species) liver after exposure to CPF. Mekail and Sharafaddin (2009) found that CAT activity decreased in brain, liver and kidney of weanling rat treated with OP (diazinon). More recently, Özkol et al. (2012) reported a decrease in CAT activity in tongue of frogs (*R. ridibunda*) exposed to omethoate (OP). The use of GST and CAT enzymes in adult frogs exposed to CPF reinforces a rational use of biomarkers of oxidative stress in biomonitoring of pesticides.

5. Conclusion

Overall, our results demonstrate (1) CFP applications in rice field produces mortality of *L. limellum* adults, (2) inhibition of

AChE and CbE at 24 and 168 h in all digestive tissues in individuals from the rice field, (3) a decrease of GST and CAT activities in stomach and liver of *L. limellum* individuals at 168 h, and in intestine at 24 and 168 h after CPF application. The demonstrated toxicity in *L. limellum* at biochemical level is alarming and a potential negative effect of pesticide exposure on sympatric amphibian species seems likely. The use of enzyme activities as biomarkers are of great importance in understanding the impacts of pesticides on amphibian that occur in rice agroecosystems and in establishing safe limits on the use of these products in these environments. Based upon our results, it can be concluded that biomarker responses in anurans from rice agroecosystems is very useful for providing lines of evidences that can contribute to environmental monitoring programs designed for various aspects of environmental risk assessment (ERA).

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References

Aebi, H., 1984. Catalase in vitro.Met. Enzymol. 105, 121–126.
Ahmad, N., Gupta, S., Mukhtar, H., 2000. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kappaB in cancer cells versus normal cells. Arch. Biochem. Biophys. 376, 338–346.
Almeida, J., Oliveira, C., Gravato, C., Guilhermino, L., 2010. Linking behavioural alterations with biomarkers responses in the European seabass *Dicentrarchus labrax L.* exposed to the organophosphate pesticide fenitrothion. Ecotoxicology 19, 1369–1381.
Alvisio, A., 1998. Arroz. Modelos zonales de producción en el movimiento CREA: Región Litoral Norte. Cuad. Act. Tec. 61, 141–145.
Attademo, A.M., Peltzer, P.M., Lajmanovich, R.C., Cabagna-Zenklusen, M.C., Junges, C.M., Basso, A., 2014a. Biological endpoints, enzyme activities, and blood cell parameters in two anuran tadpole species in rice agroecosystems of mid-eastern Argentina. Environ. Monit. Assess. 186, 635–649.
Attademo, A.M., Peltzer, P.M., Lajmanovich, R.C., Basso, A., Junges, C., 2014b. Tissue-specific variations of esterase activities in the tadpoles and adults of *Pseudis paradoxa* (Anura: Hylidae). Water Air Soil Pollut. 225, 1903.
Attademo, A.M., Cabagna-Zenklusen, M., Lajmanovich, R.C., Peltzer, P.M., Junges, C., Basso, A., 2011. B-esterase activities and blood cell morphology in the frog *Leptodactylus chaquensis* (Amphibia: *Leptodactylidae*) on rice agroecosystems from Santa Fe Province (Argentina). Ecotoxicology 20, 274–282.
ASIH, HL, SSAR, 2001. Guidelines for use of live amphibians and reptiles in field research. ⟨http://www.utexas.edu/depts/asih/herpcoll.htlm⟩. (accessed on 13.06.01).
Bagenal, T.B., Tesch, F.W., 1978. Methods for assessment of a fish production in fresh 376 waters. In: Bagenal, T.B. (Ed.), Age and Growth, vol 377. Blackwell Scientific Publications, Oxford, pp. 101–136.
Bambaradeniya, C.N.B., Edirisinghe, J.P., De Silva, D.N., Gunatilleke, C.V.S., Ranawana, K.B., Wijekoon, S., 2004. Biodiversity associated with an irrigated rice agroecosystem in Sri Lanka. Biodivers. Conserv. 13, 1715–1753.
Bunyan, P.J., Jennings, D.M., 1968. Organophosphorus poisoning; some properties of avian esterase. J. Agric. Food Chem. 16, 326–331.
Beauvais, S.L., Jones, S.B., Parris, J.T., Brewer, S.K., Little, E.E., 2001. Cholinergic and behavioural neurotoxicity of carbaryl and cadmium to larval rainbow trout (*Oncorhynchus mykiss*). Ecotoxicol. Environ. Saf. 49, 84–90.
Burkart, R., Barbaro, N.O., Sánchez, R.O., Gómez, D.A., 1999. Eco-regiones de la Argentina. PRODIA. Buenos Aires, Argentina.
Cei, J.M., 1980. Amphibians of Argentina. Monitore Zoologico ItalianoMonografía 2, p. 609.
Colombo, A., Orsi, F., Bonfanti, P., 2005. Exposure to the organophosphorus pesticide chlorpyrifos inhibits acetylcholinesterase activity and affects muscular integrity in *Xenopus laevis* larvae. Chemosphere 61, 1665–1671.
den Besten, P.J., deDeckere, E., Babut, M.P., Power, B., Del Valls, T.A., Zago, C., Oen, A. M.P., Heise, S., 2003. Biological effects-based sediment quality in ecological risk assessment for European waters. J. Soils Sedim. 3, 144–162.
Duré, M.I., Kehr, A.I., Schaefer, E.F., Marangoni, F., 2008. Diversity of amphibians in rice fields from northeastern Argentina. Interciencia 33, 523–527.
Ellman, G.L., Courtney, K.D., Andreas, V., Featherstone, R.M., 1961. A new and rapid calorimetric determination of cholinesterase activity. Biochem. Pharmacol. 7, 88–95.
Ferrari, A., Lascano, C., Pechen de D'Angelo, A.M., Venturino, A., 2011. Effects of azinphos methyl and carbaryl on *Rhinella arenarum* larvae esterases and antioxidant enzymes. Comp. Biochem. Physiol. 153, 34–39.
Frasco, M.F., Guilhermino, L., 2002. Effects of dimethoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata*. Fish Physiol. Biochem. 26, 149–156.
Fukuto, T.R., 1990. Mechanism of action of organophosphorus and carbamate insecticides. Environ. Health Perspect. 87, 245–254.
Gadagbui, B.K.M., James, M.O., 2000. Activities of affinity-isolated glutathione S-transferase (GST) from channel catfish whole intestine. Aquat. Toxicol. 49, 27–37.
Galloway, T.S., Millward, N., Browne, M.A., Depledge, M.H., 2002. Rapid assessment of organophosphorous/carbamate pesticide exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. Aquat. Toxicol. 61, 169–180.
Greulich, K., Pflugmacher, S., 2004. Uptake and Effects on detoxication enzymes of cypermethrin in embryos and tadpoles of amphibians. Arch. Environ. Contam. Toxicol. 47, 489–495.
Gomori, G., 1953. Human esterases. J. Lab. Clin. Med. 142, 445–453.
Habdous, M., Visvikis, S., Visvikis, S., 2002. Rapid spectrophotometric method for serum glutathione S-transferases activity. Clin. Chem. Acta 326, 131–142.
Habig, W.H., Pabst, M.J., Jakoby, W., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Hunt, J.W., Anderson, B.S., Phillips, B.M., Nicely, P.A., Tjeerdema, R.S., Puckett, H.M., Stephenson, M., Worcester, K., deVlaming, V., 2003. Ambient toxicity due to chlorpyrifos and diazinon in a Central California Watershed. Environ. Monit. Assess. 82, 83–112.
Ilizaliturri-Hernández, C.A., González-Mille, D.J., Mejía-Saavedra, J., Espinosa-Reyes, G., Torres-Dosal, A., Pérez-Maldonado, I., 2013. Blood lead levels, δ-ALAD inhibition, and hemoglobin content in blood of giant toad (*Rhinella marina*) to asses lead exposure in three areas surrounding an industrial complex in Coatzacoalcos, Veracruz, Mexico. Environ. Monit. Assess. 185, 1685–1698.
Junges, C.M., Lajmanovich, R.C., Peltzer, P.M., Attademo, A.M., Bassó, A., 2010. Predator–prey interactions between *Synbranchus marmoratus* (Teleostei: Synbranchidae) and *Hypsiboas pulchellus* tadpoles (Amphibia: *Hylidae*): Importance of lateral line in nocturnal predation and effects of fenitrothion exposure. Chemosphere 81, 1233–1238.
Kingbley, G.R., 1942. The direct biuret method for the determination of serum proteins as applied to photoelectric and visual calorimetry. J. Lab. Clin. Med. 27, 840–845.
Küster, E., 2005. Cholin and carboxylesterase activities in developing zebrafish embryos (*Danio rerio*) and their potential use for insecticide hazard assessment. Aquat. Toxicol. 75, 76–85.
Lacorte, S., Lartiges, S.B., Garrigues, P., Barcelo, D., 1995. Degradation of organophosphorus pesticides and their transformation products in estuarine waters. Environ. Sci. Technol. 29, 431–438.
Lajmanovich, R.C., Attademo, A.M., Peltzer, P.M., Junges, C.M., 2009. Inhibition and recovery of brain and tail cholinesterases of *Odontophrynus americanus* tadpoles (Amphibia: Cycloramphidae) exposed to fenitrothion. J. Environ. Biol. 30, 923–992.
Lajmanovich, R.C., Peltzer, P.M., Junges, C.M., Attademo, A.M., Sanchez, L.C., Basso, A., 2010. 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. Ecotoxicol. Environ. Saf. 73, 1517–1524.
Lajmanovich, R.C., Peltzer, P.M., Attademo, A.M., Cabagna Zenklusen, M., Junges, C. M., 2012. Los agroquímicos y sus impactos en los anfibios: un dilema de difícil solución. Quím. Viva 3, 184–198.
Machado, I.F., Maltchik, L., 2010. Can management practices in rice fields contribute to amphibian conservation in southern Brazilian wetlands? Aquat. Conserv. Mar. Freshw. Ecosyst. 20, 39–46.
Mekail, A., Sharafaddin, Z.A., 2009. A study of the activity of catalase and glutathione S- transferase in wearling and adult rats intoxicated with diazinon, carbaryl and lambda-cyhalothrin. Proc. 2nd Kurdistan Confer. Biol. Sci. 12, 138–145.
Morgan, M.J., Fancey, L.L., Kiceniuk, J.W., 1990. Response and recovery of brain acetylcholinesterase activity in Atlantic salmon (*Salmo salar*) exposed to fenitrothion. Can. J. Fish Aquat. Sci. 47, 1652–1654.
Ognjanovic´, B.I., Pavlovic´, S.Z., Maletic´, S.D., Zikic´, R.V., Stajn, A.S., Radojičic´, R.M., Saičic´, Z.S., Petrovic´, V.M., 2003. Protective Influence of Vitamin E on antioxidant defense system in the blood of rats treated with cadmium. Physiol. Res. 52, 563–570.
Özkol, H., Tuluce, Y., Celik, I., Isık, I., 2012. Omethoate modulates some oxidant/antioxidant parameters in frogs (*Rana ridibunda* Pallas). Toxicol. Ind. Health 28, 320–326.
Peltzer, P.M., Lajmanovich, R.C., Attademo, A.M., Junges, C., Cabagna Zenklusen, M., Repetti, R., Sigrist, M., Beldomenico, H., 2013. Effect of exposure to contaminated pond sediments on survival, development, and enzyme and blood biomarkers in veined treefrog (*Trachycephalus typhonius*) tadpoles. Ecotoxicol. Environ. Saf. 98, 142–151.
Peltzer, P.M., Lajmanovich, R.C., Attademo, A.M., Beltzer, A.H., 2006. Anuran diversity across agricultural pond in Argentina. Biodiver. Conserv. 15, 3499–3513.
Peltzer, P.M., Bock, G., Tardivo, R., Lajmanovich, R., 2004. Effects of habitat loss and fragmentation on anurans in Espinal Eco-region, Argentina: a GIS approach. Froglog 63, 3–4.

Racke, K.D., 1992. Degradation of organophosphorus insecticides in environmental matrices. In: Chambers, J.E., Levy, P.Z. (Eds.), Organophosphates: Chemistry, Fate, and Effects. Academic Press, San Diego, USA, pp. 48–78.
Robles-Mendoza, C., Zúñiga-Lagunes, S.R., Ponce de León-Hill, C.A., Hernández-Soto, J., Vanegas-Pérez, C., 2011. Esterases activity in the axolotl *Ambystoma mexicanum* exposed to chlorpyrifos and its implication to motor activity. Aquat. Toxicol. 105, 728–734.
Sánchez, J.C., Fossi, M.C., Focardi, S., 1997. Serum B esterases as an nondestructive biomarker for monitoring the exposure of reptiles to organophosphorus insecticides. Ecotoxicol. Environ. Saf. 38, 45–52.
Sánchez-Hernández, J.C., 2007. Ecotoxicological perspectives of B-esterases in the assessment of pesticide contamination. In: Plattenberg, R.H. (Ed.), Environmental Pollution. New Research Nova Science Publishers, New York, pp. 1–45.
Sánchez, L.C., Peltzer, P.M., Lajmanovich, R.C., 2009. Structure of wetland-breeding anuran assemblages from the southern section of Paraná river, Argentina. Herpetol. J. 19, 173–184.
Sogorb, M.A., Vilanova, E., 2002. Enzymes involved in the detoxification of organophosphorus, carbamatos and pyrethroid insecticides through hydrolysis. Toxicol. Lett. 128, 215–228.
Stenersen, J., 2004. Chemical pesticides: mode of action and toxicology. CRC Press LLC, Boca Raton, FL.
Strauss, D.L., Chambers, J.E., 1995. Inhibition of acetylcholinesterase and aliesterases of fingerling channel catfish by chlorpyrifos, parathion, and S,S,S tributyl phosphorotrithioate (DEF). Aquat. Toxicol. 33, 311–324.
Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., Polasky, S., 2002. Agricultural sustainability and intensive production practices. Nature 418, 671–677.
Vaira, M., Akmentins, M., Attademo, A., Baldo, D., Barrasso, D., et al., 2012. Categorización del estado de conservación de los Anfibios de la República Argentina. Cuad. Herpetol. 26, 131–159.
Wheelock, C.E., Phillips, B.M., Anderson, B.S., Miller, J.L., Miller, M.J., Hammock, B.D., 2008. Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). Rev. Environ. Contam. Toxicol. 195, 117–178.
Widder, P.D., Bidwell, J.R., 2008. Tadpole size, cholinesterase activity and swim speed in four frog species after exposure to sub-lethal concentrations of chlorpyrifos. Aquat. Toxicol. 88, 9–18.
Wielgomas, B., Krechniak, J., 2007. Toxicokinetic interactions of cypermethrin and chlorpyrifos in rats. Polish J. Environ. Stud. 16, 267–274.
Wijeyaratne, W., Pathiratne, A., 2006. Acetylcholinesterase inhibition and gill lesions in *Rasbora caverii*, an indigenous fish inhabiting rice associated waterbodies in Sri Lanka. Ecotoxicol 15, 609–619.