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ORIGINAL ARTICLE

Blood biomarkers of common toad *Rhinella arenarum* following chlorpyrifos dermal exposure

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ABSTRACT

Chlorpyrifos (CPF) is a broad spectrum pesticide commonly used for insect control, has great affinity for lipids and is thus a potential for bioaccumulation in aquatic organisms. The aim of this study was to evaluate the toxicity of CPF using the common toad *Rhinella arenarum* via dermal uptake in plastic bucket to simulate their natural exposition in ponds. *R. arenarum* toads were exposed individually to solutions containing a nominal concentration of a commercial formulation of CPF insecticide (5 and 10 mg/L). Different enzyme biomarkers (BChE: butyrylcholinesterase, CbE: carboxylesterase, and CAT: catalase) were measured in blood tissue after exposition. The capacity of pyridine-2-aldoxime methochloride (2-PAM) to reverse OP-inhibited plasma BChE and the ratio of heterophils and lymphocytes (H/L) as hematological indicators of stress were also determined. The normal values of plasma B-sterases (BChE and CbE) were highly inhibited (until ≈ 70%) in toads 48 h after exposure to CPF. The results indicate that 2-PAM produced BChE reactivation as well. The activity of CAT was also inducted for dermal exposure at more than double of that in the control toads (CPF; 5 mg/L). H/L ratios did not reveal a significantly increased stress. The study suggests that CPF via dermal uptake induced neurotoxicity and oxidative stress in the common toad *R. areanum*. Thus, some blood biomarkers employed in our study (i.e. BChE, CbE, 2-PAM, and CAT) might be used as predictors in health and ecological risk assessment of amphibian populations exposed to CPF.

KEY WORDS: *Rhinella arenarum*; blood; chlorpyrifos dermal exposure; B-esterases; catalase

Introduction

Amphibians may be particularly susceptible to anthropogenic chemicals for a multiplicity of reasons. Fundamentally, their complex life cycles (aquatic and terrestrial life stages) expose them to potential chemical contamination in both habitats (Brühl *et al.*, 2013; Lajmanovich *et al.*, 2015; Van Meter *et al.*, 2015). Indeed, terrestrial anurans breathe and absorb water mainly through their hyper-vascularized skin in the ventral pelvic region in adults, and this reason explains the susceptibility to pesticide uptake from contaminated sediments, water, and soil (Sparling *et al.*, 2001). Dermal exposure presents a potentially significant but insufficiently studied route for pesticide uptake in amphibians (Van Meter *et al.*, 2014).

Chlorpyrifos (CPF) is a broad-spectrum organophosphate pesticide widely used around the world in agriculture and domestic use against harmful insects (Lee *et al.*, 2004). Large-scale manufacture and handling of CPF have led to contamination of soil, air, surface and groundwater in many countries (Eaton *et al.*, 2008). Thus different concentrations of CPF have been detected in ground and surface waters worldwide (Turner, 2003). The major route of CPF to aquatic ecosystems is through rainfall runoff and air-drift (Xing *et al.*, 2012). Nowadays, large amounts of CPF are used in Argentine for the agriculture of crops, i.e. an estimate of 6.8 million kilograms per year based on import records, especially on soybean crops, fruits, grains and vegetables for local consumption (SENASA, 2011). Likewise, Marino and Ronco (2005) detected CPF residues in Argentine surface waters at concentrations ranging among 0.2 to 10.8 µg/L. However, in other regions the peak concentration (>10 mg/L) occurred in a drainage ditch; stream residues had declined to undetectable levels within 44 days (NRA 2000). In some cases, the CPF commercial formulations were found to be more toxic than

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the active ingredient, particularly to aquatic organisms (Ali *et al.,* 2009).

The integrated use of a measurable indicator such as cholinesterases (ChEs) may be necessary for biomonitoring programs to assess the risk of pesticide exposure in amphibians living in agroecosystems (Lajmanovich *et al.,* 2008; Mann *et al.,* 2009; Attademo *et al.,* 2011).The use of several biomarkers (i.e. pollutant-induced biological responses at sub-individual level measured by nonlethal methodologies) is one of the first ecotoxicity phases in the risk characterization of pollutants. Therefore, the measurement of blood butyrylcholinesterase (BChE) activity, such as carboxylesterase (CbE) (for neurotoxicity), catalase (CAT) (for oxidative stress) and the ratio of heterophils and lymphocytes (H/L) (for immune response to stress) are used as biomarkers to monitor CPF exposures in amphibian species (Attademo *et al.,* 2015; Lajmanovich el at. 2015). The case of monitoring anti-ChE pesticide exposure in the field using ChEs activity levels has some limitations. Detection of OP-exposed individuals is highly dependent on a well-represented reference group or an intense sampling effort to increase the likelihood of capturing exposed individuals (Rodríguez-Castellanos & Sánchez-Hernández, 2007). As Wilson & Ginsburg (1955) discovered, mono-pyridinium oximes were effective reactivators of OP-inhibited ChE. Several mono-pyridinium and bis-pyridinium oximes have been synthesized and tested (Jun *et al.,* 2008). Since then, the use of oximes such as pyridine-2-aldoxime methochloride (2-PAM) can partially solve the problem of identifying OP-exposed individuals in wild fauna (Sánchez-Hernández, 2007). One option to solve this problem was the use of oximes (Wilson & Ginsburg, 1955). Oximes such as pyridine-2-aldoxime methochloride (2-PAM) revert the OP-inhibited ChE (Jun *et al.,* 2008) and can partially solve the problem of identifying OP-exposed individuals in wild fauna (Sánchez-Hernández, 2007).

Figure 1. Design of the experimental semi-transparent plastic water bucket used for *Rhinella arenarum* toad dermal absorption.

Several studies have documented an apparent connection between the presence of CPF residues and reductions in amphibian populations, at both local (Fellers *et al.,* 2004) and landscape scales (Davidson *et al.,* 2001). Considering the world average application rate of CPF formulations and that the main part of ecotoxicological research for amphibians was done on aquatic life stages, it is important to emphasize the need of ecotoxicological risk assessment on adult terrestrial non-target amphibians. Following these assumptions and exposing *Rinella arenarum* toads to CPF formulation, we can state that the aims of this study were: (i) to determine the anticholinesterase responses (BChE and CbE activities as well as the ability of 2-PAM to reverse OP-inhibited plasma BChE), and (ii) to determine the oxidative stress response (CAT activities) and immune response (H/L ratio).

Materials and methods

Animals studied

Sixteen adult male of *R. arenarum* were collected by hand from temporary ponds (31°39'52.90"S - 60°42'50.20"W, South Park Lake, Santa Fe, Santa Fe province, Argentina). These sites had not been treated with chemical pesticides as determined by the laws to protect human and wildlife health. *R. arenarum* is used as a good model in ecotoxicology for several blood parameters (Cabagna *et al.,* 2005; Lajmanovich *et al.,* 2008). This toad is frequently found in forests, wetlands, agricultural land and urban territories (Peltzer *et al.,* 2006; Bionda *et al.,* 2015) and it has an extensive Neotropical distribution (IUCN, 2015). It feeds mainly on a variety of arthropods and thus it plays an important role as biological control, particularly in soybean crops (Attademo *et al.,* 2005).

After capture, the toads were quickly transported to the laboratory in darkened buckets containing water to minimize stress. Snout-vent length (SVL) (mm) and body weight (g) were recorded with digital calliper (precision 0.01 mm). The toads were acclimated for 24 h before experiment initiation (individually in semi-transparent plastic water buckets; size: Ø 23 cm × 28 cm) under laboratory conditions with a photoperiod 12-12 h (light 07:00–19:00 h), humidity (65±10%) and temperature 24±2 °C.

Experimental design

The commercial formulation of CPF (48 % active ingredient [a.i.], O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, log K_{OW}=4.7), NUFARM®, Nufarm S.A. (Argentina), was used in this experimental study. The pesticide was tested in this form to mimic the way in which it is applied to cultivated fields and introduced into the environment (Sparling *et al.,* 2010). Likewise, Brühl *et al.* (2013) exposed terrestrial amphibians to pesticide formulations that contain additives.

The animals were randomly distributed into three experimental groups. A control group (CO, n=4) with 500 mL of dechlorinated tap water (DTW; pH 7.4±0.05;
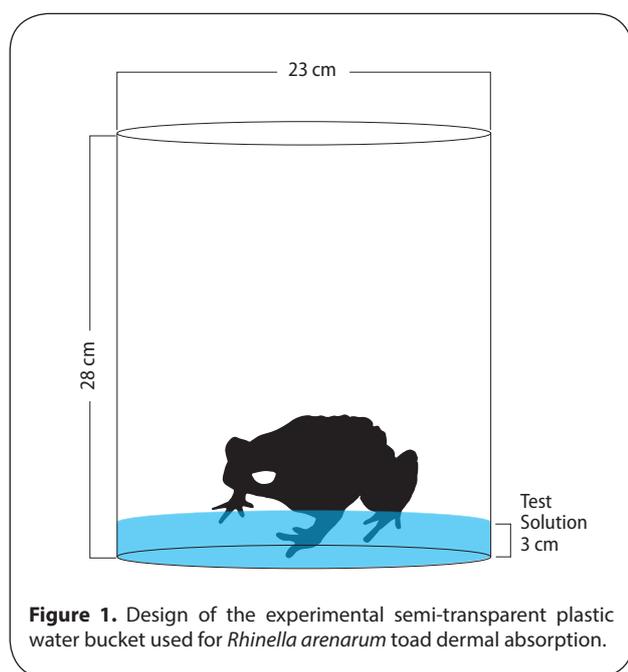
conductivity 165±12.5 µmhos/cm; dissolved oxygen concentration 6.5±1.5 mg/L; and hardness 50.6 mg/L $CaCO_3$), and two groups treated with CPF pesticide formulations containing nominal concentration of 5 and 10 mg/L (n=6). Doses were chosen in ranges usually lacking overt toxicity, mimicking a scenario with no altering clinical signals that may erroneously lead to the assumption of absence of danger (Muller *et al.,* 2014; Lajmanovich *et al.,* 2015). Toads were randomly placed individually into a sterile bucket with 500 mL test solutions (DTW or pesticides) equivalent to 3 cm deep for 48 h, and under the same laboratory conditions as described above (Figure 1).

Blood samples (500 µl approximately) were collected by a minimal cardiac puncture using a heparinized syringe after 48 h of dermal exposure. Toads were maintained in the laboratory of Ecotoxicology in the Faculty of Biochemistry and Biological Sciences – National University of Litoral during a certain period of time for recovery and after a general revision of body condition, they were released to the same sites where they had been captured. To do this, we had the approval of the animal ethic committee of the same institution and followed the guidelines of ASIH *et al.,* (2004).

B-esterases

Plasma BChE activity was determined colorimetrically following the method proposed by Ellman *et al.* (1961). The reaction medium included 930 µl of 25 mM Tris-HCl, 1 mM $CaCl_2$ (pH=7.6), 50 µl of 5,5′-dithiobis-2-nitrobenzoic acid (3×10^{-4} M, final concentration), 10 µl of butyrylthiocholine iodide (2×10^{-3} M, final concentration) and 10 µl of plasma. The optical density variation was recorded at 410 nm for 1 min at 25 °C using a Jenway 6405 UV-VIS spectrophotometer. Kinetic experiments were carried out in duplicate. Plasma BChE activity was expressed as µmol of substrate hydrolyzed $min^{-1}ml^{-1}$ of plasma using a molar extinction coefficient of $13.6\times10^{-3}\,M^{-1}cm^{-1}$. We did not determine plasma acetylcholinesterase (AChE) activity because BChE is the enzyme that primarily contributes to total plasma cholinesterase activity in many vertebrate species (Sánchez-Hernández & Moreno-Sánchez, 2002).

Plasma CbE activity was measured by the Gomori method (1953) as adapted by Bunyan *et al.,* (1968). The assay was carried out with 25 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$ (pH=7.6) and 10 µL of plasma at 25 °C. The reaction was initiated by adding 50 µL of α-naphthyl acetate (1.04 mg/mL in acetone – α-NA) as substrate, and stopped after 10 min by addition of 500 µL of 2.5% SDS and subsequently 500 µL of 0.1% Fast Red ITR in 2.5% Triton X-100 in water (freshly prepared). Samples were left in darkness for 30 min and the complex absorbance was read at 530 nm. Hydrolysis of α-NA was expressed as nmol of substrate hydrolyzed $min^{-1}ml^{-1}$ of plasma using a molar extinction coefficient of $33.225\times10^3\,M^{-1}cm^{-1}$.

Chemical reactivation of BChE

Two aliquots of each plasma sample were used for assaying reactivation of BChE activity in the presence of 2-PAM (dilution factor 1/5). An aliquot was spiked with 1.7×10^{-3} mol/L 2-PAM and the other was diluted with an equal volume of distilled H_2O (control). After incubation for 30 min at 25 °C, BChE activity of both aliquots was measured. The activity of BChE of control samples was compared to BChE activity of CPFs treatments. Inhibition of BChE activity by CPFs formulations was assumed when the increase of esterase activity was higher than 5% compared with the corresponding controls (Laguerre *et al.,* 2009).

CAT activity

Plasma CAT activity was measured using the method described by Aebi (1984), and was expressed as H_2O_2 µmol $min^{-1}mg^{-1}$ plasma using a molar extinction coefficient of $H_2O_2\ 40\times10^{-3}\,M^{-1}cm^{-1}$. The reaction medium was composed of 50 mM phosphate buffer (pH=7.2) and 30 mM H_2O_2, and the absorbance was read on the spectrophotometer at a wavelength of 240 nm at 25 °C (quartz cuvette).

Hematological indicators of stress

Two blood smears for each toad were prepared on clean slides, fixed, and stained by the May-Grunwald-Giemsa method (Dacie & Lewis 1984). To determine the counts of heterophil and lymphocyte, 1000 cells per film were examined by light microscopy. All blood counts, including granulocytes (heterophil, basophil and eosinophil) and non-granulocytes (lymphocyte and monocyte) were examined by the same analyst. The results are presented as the percentage of each cell occurring in each film. The heterophil/lymphocyte (H/L) ratio was examined as a response estimator of stress caused by the experimental assay (Davis *et al.,* 2008).

Data analyses

All biomarker data were expressed as mean ± SEM. The influence of pesticide treatments on each variable (B-esterases, CAT and H/L ratio) was analyzed with Kruskal-Wallis and Dunn's tests for post hoc comparisons (Lajmanovich *et al.,* 2013). The differences in the percentage increase of BChE activity after 2-PAM treatments among two CPF exposition doses were calculated using the Mann Whitney-U test for each data set. These statistical analyses were performed using the BioEstat software 5.0 (Ayres *et al.,* 2008). A value of $p<0.05$ was considered to be significant.

Results

Mean (±SEM) length and body mass of toads were 94.18±2.16 mm and 98.10±7.95 g, respectively. No signs of general behavioral disorders (hyperactivity, loss of coordination in hindlimbs and forelimbs, erratic swimming) were observed in toads exposed to CPF as a response to a possible severe acute neurotoxicity. The mean value of BChE activity in the non-pesticide exposure toads was 6.49±1.01 µmol/min/mL plasma at 48 h. BChE activity
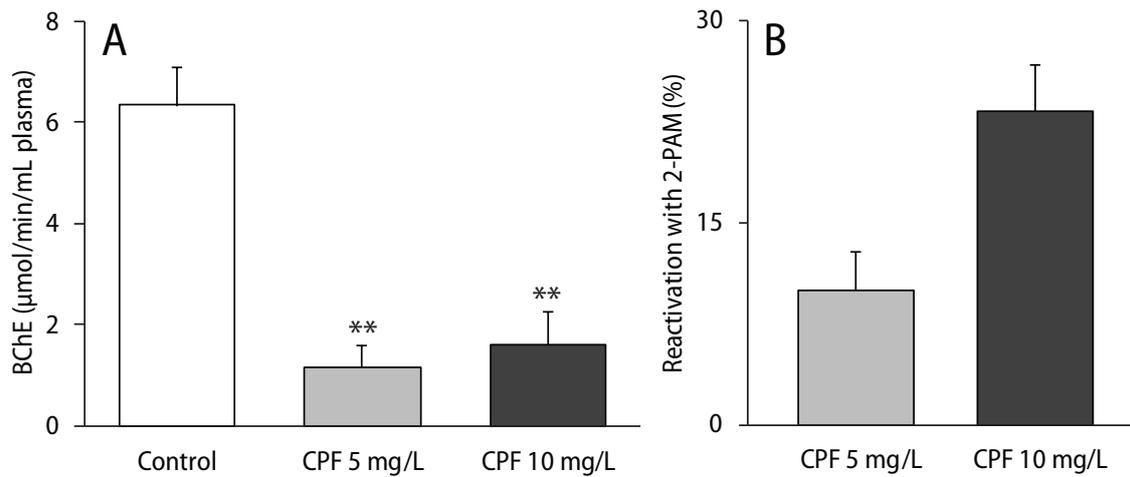
Figure 2. Effects of commercial pesticides exposure (48-h) on butyrylcholinesterase (BChE) activity in *Rhinella arenarum* toads; significantly different form control (** *p*<0.01; Kruskal-Wallis test followed Dunn's post-test) (**a**), and percentage of reactivations after addition of 2-PAM (*p*> 0.05; Mann-Whitney test) (**b**). CO: control (*N* = 4), CPF: chlorpyrifos (*N* = 6). Data are expressed as mean ± SEM.
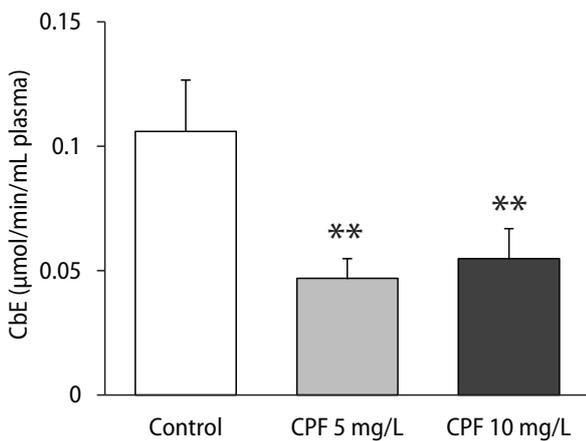
Figure 3. Effects of commercial pesticide exposure (48-h) on carboxylesterase (CbE) activity in *Rhinella arenarum* toads; significantly different from control (** *p*<0.01; Kruskal-Wallis test followed Dunn's post-test). CO: control (n=4), CPF: chlorpyrifos (n=6). Data are expressed as mean ± SEM.
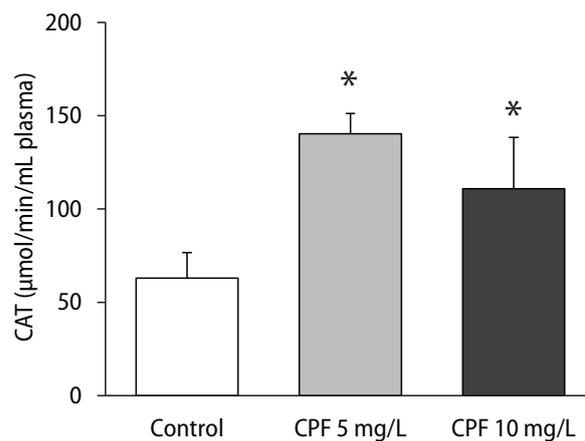
Figure 4. Effects of commercial pesticides exposure (48-h) on the catalase (CAT) activity in *Rhinella arenarum* toads; significantly different from control (* *p*<0.05; Kruskal-Wallis test fallowed Dunn's post-test). CO: control (n=4), CPF: chlorpyrifos (n=6). Data are expressed as mean ± SEM.

varied among groups exposed to CPF formulation (percentage of inhibition of 82.58% [5 mg/L] and 72.72% [10 mg/L]) with respect to the control group (*p*<0.01) (Figure 2a). Oxime-induced reactivation of plasma BChE activity was observed in all toads exposed to CPF formulations. The mean percentage increase of plasma BChE activity in samples containing 5 mg/L of CPF was 11.93% (±2.39), whereas for the samples with 10 mg/L of CPF it was 21.23% (±2.65). The differences between the two groups of samples were not significant (U=12; *p*=0.39) (Figure 2 b).

The CbE activity (mean ± SEM) in the control group was 0.1095±0.009 µmol/min/mL plasma at 48 h. CPF formulation inhibited CbE enzyme activity significantly (*p*<0.01) with respect to control in toads exposed at 5 mg/L (56.65%) and 10 mg/L (43.23%) (Figure 3).

The mean value of CAT activity in control toads was 60±8.10 µmol/min/mL plasma at 48 h. The induction of CAT enzymatic activity across dermal exposure at the two concentrations tested was significant (*p*<0.05) with respect to the control samples (Figure 4). Our results show that a mean H/L (48-h) ratio in control groups was 0.23±0.04. No differences were found in H/L ratio between toads exposed to both CPF formulations and the control group (KW=2.063; *p*=0.35).

Discussion

Greater than 70% of the worldwide amphibian species present decline (Hayes *et al.*, 2010). These vertebrates are important animal models in toxicology and they often

represent both aquatic and terrestrial forms within the life history of the same species (Helbing 2012). Certainly, frogs and toads have been considered as bio-indicators of aquatic and agricultural ecosystems (Peltzer *et al.,* 2006).

In the present study, BChE and CbE activity was significantly inhibited by dermal exposure to CPF formulations. Previous experiments showed similar results in these B-esterases (Lajmanovich *et al.,* 2015). In the same sense, it is well known that CPF suppresses the activity of ChEs in amphibian aquatic stage (e.g. Widder & Bidwell, 2006; Robles-Mendoza *et al.,* 2011; Liendro *et al.,* 2015). BChE and CbEs are important to reduce OP toxicity (Wheelock *et al.,* 2004; Laguerre *et al.,* 2009) and these isozymes may contribute to pesticide tolerance due to their capability of binding to OPs. Moreover, CbE was reported to comprise a group of isoenzymes that play a role in OP detoxification by acting as an alternative target protecting ChE from inhibition (Wheelock *et al.,* 2008). In the *R. arenarum* individuals used in this study, BChE and CbE may considerably diminish the effective concentration of the pesticide before they reach the blood-brain barrier in order to protect AChE from inhibition in the central nervous system (Walker, 1998).

Oxime-induced reactivation of plasma BChE activity was observed in all toads exposed to CPF formulations. Chemical reactivation of plasma phosphorylated ChEs activity has been used for complementary diagnosis of OP intoxication in birds (McInnes *et al.,* 1996; Parsons *et al.,* 2000; Iko *et al.,* 2003), reptiles (Sánchez-Hernández 2003; Sánchez-Hernández *et al.,* 2004), and amphibians (Attademo *et al.,* 2007; Lajmanovich *et al.,* 2008). In this respect, we performed the 2-PAM reactivation assay, which enabled us to identify organophosphates (OPs) as the agrochemicals responsible for plasma BChE depression in the exposed toads. Plasma BChE reactivation in the presence of 2-PAM appeared to be a more sensitive indicator of exposure to CPF than plasma BChE activity levels.

An antioxidant biomarker, i.e. CAT, was also included in this study, which would help evaluate the impact and risk of sub-lethal concentrations of CPF on *R. arenarum* toads. Indeed, exposure of freshwater gastropod to CPF for 48 h also resulted in a significant increase in CAT activity (Cacciatore *et al.,* 2015). *R. arenarum* larvae exposed to CPF for 96 h also showed changes in CAT activity (Liendro *et al.,* 2015). Oxidative stress may occur if the equilibrium between oxidants and antioxidants is interrupted either by the reduction of antioxidant defences or by the excessive increase of reactive oxygen species (ROS) (Valavanidis *et al.,* 2006). From earlier research it is understood that CPF produces oxidative stress resulting in the accumulation of lipid peroxidation products in different tissues (Verma *et al.,* 2003; 2007). Nevertheless, we cannot conclusively say that similar oxidative stress is caused also in toads. In this sense, four-week exposure of rats to CPF caused noticeable decrease in CAT activity in erythrocytes (Barski *et al.,* 2011).

On the other hand, there is evidence of changes in leukocyte profiles in amphibians under stress (Davis *et al.,* 2008). Severe stress may decrease heterophils

(heteropenia) and increase lymphocytes in the periphery (lymphocytosis), resulting in a low H/L-ratio (Müller *et al.,* 2011). However, we did not find significant relations between leukocyte profiles and exposure to CPF. The lack of differences between toads exposed to CPF and control groups can be related to the fact that CPF in water degrades quickly in the first 24 hours (Wu *et al.,* 2003), or the time lag associated with the leukocyte response to stress may be the longest in ectothermic animals (Pough, 1980).

In conclusion, CPF formulations uptake in toad blood at 48 h produced BChE and CbE inhibition (BChE reactivated by oximes) and oxidative stress. Possibly the multiple effects observed were not restricted to CPF active ingredients and could be influenced by the commercial formulation adjuvants. The experiment proposed of *in vivo* dermal toxicity is a good method to characterize the risk exposures of native adult amphibians exposed to OP insecticides.

Acknowledgments

REFERENCES

Aebi H. (1984). Catalase in vitro. *Met Enzymol* **105**: 121–126.

Ali D, NagpureNS, Kumar S, Kushwaha B, Lakra WS. (2009) Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Food Chem Toxicol* **47**: 650–656. https://doi.org/10.1016/j.fct.2008.12.021

ASIH, HL, SSAR (2004). Guidelines for use of live amphibians and reptiles in field research. Available at: http: //www.utexas.edu/depts/asih/herpcoll.htlm. Accessed 13 June 2014.

Attademo AM, Peltzer PM, Lajmanovich RC. (2005). Amphibians occurring in soybean and implications for biological control in Argentina. *Agric Ecosyst Environ* **106** (1): 389–394. https://doi.org/10.1016/j.agee.2004.08.012

Attademo AM, Peltzer PM, Lajmanovich RC, Cabagna M, Fiorenza G. (2007). Plasma B-esterases and glutathione S-transferase activities in the toad *Chaunus schneideri* (Amphibia, Anura) inhabiting rice agroecosystems of Argentina. *Ecotoxicology* **16**: 533–539. https://doi.org/10.1007/s10646-007-0154-0

Attademo AM, Cabagna-Zenklusen MC, Lajmanovich RC, Peltzer PM, Junges C, Bassó A. (2011). B-esterase activities and blood cell morphology in the frog *Leptodactyluschaquensis* (Amphibia: Leptodactylidae) on rice agroecosystems from Santa Fe Province (Argentina). *Ecotoxicology* **20**: 274–282. https://doi.org/10.1007/s10646-010-0579-8

Attademo AM, Peltzer PM, Lajmanovich RC, Cabagna-Zenklusen MC, Junges CM, Basso A. (2014). 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. https://doi.org/10.1007/s10661-013-3404-z

Attademo AM, Peltzer PM, Lajmanovich RC, Cabagna-Zenklusen M, Junges C, Lorenzatti E, Aró C, Grenón P. (2015). Biochemical changes in certain enzymes of *Lysapsus limellium* (Anura: Hylidae) exposed to chlorpyrifos. *Ecotoxicol Environ Saf* **113**: 287–294

Ayres M Jr, Ayres D, Santos A. (2008). *BioEstat*. Versão5.0. Belém, Pará, Brazil: Sociedade Civil Mamirauá, MCT-CNPq.

Barski D, Spodniewska A, Zasadowski A. (2011). Activity of superoxide dysmutase, catalase and glutathione peroxidase in rats exposed to chlorpyrifos and erofloxacin. *Pol J Vet Sci* **14**: 523–529.

Bionda C de L, Kost S, Salas NE, Lajmanovich R, Sinsch U, Martino A. (2015). Age structure, growth and longevity in the common toad, *Rhinella arenarum*, from Argentina. *Acta Herpetol* **10**: 55–62.

Brühl CA, Schmidt T, Pieper S, Alscher A (2013) Terrestrial pesticide exposure of amphibians: an underestimated cause of global decline? *Sci Rep* **3**: 1135. https://doi.org/10.1038/srep01135

Bunyan PJ, Jennings DM, Taylor A. (1968). Organophosphorus poisoning. Properties of avian esterases. *J Agric Food Chem* **16**: 326–331. https://doi.org/10.1021/jf60156a028

Cabagna-Zenklusen MC, Lajmanovich RC, Stringhini GA, Sanchez-Hernandez J, Peltzer P. (2005). Hematological parameters of health status in common toad Bufo arenarum in agroecosystems of Santa Fe Province, Argentina. *Appl Herpetol* **2**: 373–380.

Cacciatore LC, Nemirovsky SI, Verrengia Guerrero NR, Cochón AC. (2015). Azinphos-methyl and chlorpyrifos, alone or in a binary mixture, produce oxidative stress and lipid peroxidation in the freshwater gastropod *Planorbarius corneus*. *Aquat Toxicol* **167**: 12–129. https://doi.org/10.1016/j.aquatox.2015.07.009. aaaaaa

Dacie JV, Lewis SM. (1984). *Practical hematology*. Churchill Livingstone, New York.

Davidson C, Shaffer HB, Jennings MR. (2001). Declines of the california redlegged frog: climate, uv-b, habitat, and pesticides hypotheses. *Ecol Appl* **11**: 464–479.

Davis AK, Maney DL, Maerz JC. (2008). The use of leukocyte profiles to measure stress in vertebrates: A review for ecologists. *Funct Ecol* **22**: 760–772.

Eaton DL, Daroff RB, Autrup H, Bridges J, Buffler P, Costa LG, Coyle J, McKhann G, Mobley WC, Nadel L, Neubert D, Schulte-Hermann R, Spencer PS. (2008). Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Crit Rev Toxicol* **38** Suppl 2: 1–125. https://doi.org/10.1080/10408440802272158

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**: 88–95.

Fellers GM, McConnell LL, Pratt D, Datta S. (2004). Pesticides in mountain yellow-legged frogs (*Rana muscosa*) from the Sierra Nevada Mountains of California, USA. *Environ Toxicol Chem* **23**: 2170–2177.

Gomori G. (1953). Human Esterases. *J Lab Clin Med* **42**: 445–453.

Hayes TB, Falso P, Gallipeau S, Stice M. (2010). The cause of global amphibian declines: a developmental endocrinologist's perspective. *J Exp Biol* **213**: 921–933. https://doi.org/10.1242/jeb.040865.

Helbing CC (2012). The metamorphosis of amphibian toxicogenomics. *Front Genet* **3**: 37. https://doi.org/10.3389/fgene.2012.00037.

Iko WM, Archuleta AS, Knopf FL. (2003). Plasma cholinesterase levels of mountain plovers (*Charadriusmontanus*) wintering in central California, USA. *Environ Toxicol Chem* **22**: 119–1125.

IUCN (2015). An Analysis of Amphibians on the 2015 *IUCN Red List of Threatened Species. Version 2015-4*. Available at: www.iucnredlist.org/amphibians. Accessed 11 June 2015.

Jun D, Kuca K, Picha J, Koleckar V, Marek J. (2008). Potency of novel oximes to reactivate sarin inhibited human cholinesterases. *Drug Chem Toxicol* **31**: 1–9. https://doi.org/10.1080/01480540701688238

Laguerre C, Sanchez-Hernandez JC, Köhler HR, Triebskorn R, Capowiez Y, Rault M, Mazzia C. (2009). B-type esterases in the snail *Xeropicta derbentina*: an enzymological analysis to evaluate their use as biomarkers of pesticide exposure. *Environ Pollut* **157**: 199–207. https://doi.org/10.1016/j.envpol.2008.07.003

Lajmanovich RC, Sánchez-Hernández JC, Peltzer PM, Fiorenza G, Cabagna MC, Bassó A. (2008). Levels of plasma B-esterases and glutathione-S-transferase activities in three South American toad species. *Toxicol Environ Chem* **90**: 1145–1161.

Lajmanovich RC, Junges CM, Attademo AM, Peltzer P, Cabagna Zenklusen MC, Bassó A. (2013). Individual and mixture toxicity of commercial formulations containing glyphosate, metsulfuron-methyl, bispyribac-sodium, and picloram on *Rhinella arenarum* tadpoles. *Water Air Soil Pollut* **224**: 1404. https://doi.org/10.1007/s11270-012-1404-1

Lajmanovich RC, Attademo AM, Simoniello MF, Poletta G, Junges CM, Peltzer P, Grenón P, Cabagna Zenklusen MC. (2015). Harmful effects of the dermal intake of commercial formulations containing chlorpyrifos, 2,4-D, and glyphosate on the common toad *Rhinella arenarum* (Anura: Bufonidae). *Water Air Soil Pollut* **226**: 427. https://doi.org/10.1007/s11270-015-2695-9

Lee WJ, Blair A, Hoppin JA, Sandler DP, Dosemeci M, Alavanja MC. (2004). Cancer incidence among pesticide applicators exposed to chlorpyrifos in the Agricultural Health Study. *J Natl Cancer Inst* **96**: 1781–9. https://doi.org/10.1093/jnci/djh324

Liendro N, Ferrari A, Mardirosian M, Lascano CI, Venturino A. (2015). Toxicity of the insecticide chlorpyrifos to the South American toad *Rhinella arenarum* at larval developmental stage. *Environ Toxicol Pharmacol* **39**: 525–35. https://doi.org/10.1016/j.etap.2014.12.022

Mann RM, Hyne R V, Choung CB, Wilson SP. (2009). Amphibians and agricultural chemicals: Review of the risks in a complex environment. *Environ Pollut* **157**: 2903–2927. https://doi.org/10.1016/j.envpol.2009.05.015

Marino D, Ronco A. (2005). Cypermethrin and chlorpyrifos concentration levels in surface water bodies of the Pampa Ondulada, Argentina. *Bull Environ Contam Toxicol* **75**: 820–6. https://doi.org/10.1007/s00128-005-0824-7

McInnes PF, Andersen DE, Hoff DJ, Hooper MJ, Kinkel LL. (1996). Monitoring exposure of nestling songbirds to agricultural application of an organophosphorus insecticide using cholinesterase activity. *Environ Toxicol Chem* **15**: 544–552. https://doi.org/10.1002/etc.5620150420

Müller C, Jenni-Eiermann S, Jenni L (2011). Heterophils/Lymphocytes-ratio and circulating corticosterone do not indicate the same stress imposed on Eurasian kestrel nestlings. *Funct Ecol* **25**: 566–576. https://doi.org/10.1111/j.1365-2435.2010.01816.x

Muller M, Hess L, Tardivo A, Lajmanovich R, Attademo A, Poletta G, Simoniello MF, Yodice A, Lavarello S, Chialvo D, Scremin O. (2014). Neurologic dysfunction and genotoxicity induced by low levels of chlorpyrifos. *Neurotoxicology* **45**: 22–30. https://doi.org/10.1016/j.neuro.2014.08.012

NRA (2000). The NRA Review of Chlorpyrifos. Volume 1. National Registration Authority for Agricultural and Veterinary Medicines, Canberra. http://www.apvma.gov.au/products/review/docs/chlorpyrifos_summary.pdf. Accessed 13 June 2017.

Parsons KC, Matz AC, Hooper MJ, Pokras MA. (2000). Monitoring wading bird exposure to agricultural chemicals using serum cholinesterase activity. *Environ Toxicol Chem* **19**: 1317–1323. https://doi.org/10.1002/etc.5620190514

Peltzer PM, Lajmanovich RC, Attademo AM, Beltzer AH. (2006). Diversity of anurans across agricultural ponds in Argentina. *Biodivers Conserv* **15**: 3499–3513.

Pough FH. (1980). Advantages of ectothermy for tetrapods. *Amer Nat* **115**: 92–112.

Robles-Mendoza C, Zúñiga-Lagunes SR, Ponce de León-Hill CA, 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.

Rodríguez-Castellanos L, Sanchez-Hernandez JC. (2007). Chemical reactivation and aging kinetics of organophosphorus-inhibited cholinesterases from two earthworm species. *Environ Toxicol Chem* **26**: 1992–2000. https://doi.org/10.1897/06-625R1.1

Sanchez-Hernandez JC, Moreno-Sanchez B (2002) Lizard cholinesterases as biomarkers of pesticide exposure: enzymological characterization. Environ Toxicol Chem **21**: 2319–2325.

Sánchez-Hernandez JC (2003). Evaluating reptile exposure to cholinesterase-inhibiting agrochemicals by serum butyrylcholinesterase activity. *Environ Toxicol Chem* **22**: 296–301.

Sánchez-Hernández JC, Carbonell R, Henríquez Pérez A, Montealegre M, Gómez L. (2004). Inhibition of plasma butyrylcholinesterase activity in the lizard *Gallotia galloti* palmae by pesticides: a field study. *Environ Pollut* **132**: 479–88. https://doi.org/10.1016/j.envpol.2004.05.008

Sánchez-Hernandez JC (2007). Ecotoxicological perspectives of B-esterases in the assessment of pesticide contamination. In: Plattenberg RH (ed) Environmental Pollution: New Research. *Nova Science Publishers*, pp 1–45

SENASA. Importacón Agroquimicos. (2011). http://www.senasa.gov.ar/conteni- do.php?to=n&in=524&ino=524&io=7331.

Sparling DW, Fellers GM, McConnell LL. (2001). Pesticides and amphibian population declines in California, USA. *Environ Toxicol Chem* 20: 1591–1595.

Sparling DW, Linder G, Bishop CA, Krest S. (2010). *Ecotoxicology of Amphibians and Reptiles*, Second Edi. CRC Press

Turner L. (2003). *Chlorpyrifos: Analysis of Risks to Endangered and Threatened Salmonand Steelhead.* U.S. Environmental Protection Agency (EPA) Office of Pesticides (OPP)

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol Environ Saf* **64**: 178–89. https://doi.org/10.1016/j.ecoenv.2005.03.013

Van Meter RJ, Glinski DA, Hong T, Cyterski M, Henderson WM, Purucker ST. (2014). Estimating terrestrial amphibian pesticide body burden through dermal exposure. *Environ Pollut* **193**: 262–268.

Van Meter RJ, Glinski DA, Henderson WM, Garrison AW, Cyterski M, Purucker ST. (2015). Pesticide uptake across the amphibian dermis through soil and overspray exposures. *Arch Environ Contam Toxicol* **69**: 545–56. https://doi.org/10.1007/s00244-015-0183-2

Verma RS, Srivastava N. (2003). Effect of chlorpyrifos on thiobarbituric acid reactive substances, scavenging enzymes and glutathione in rat tissues. *Indian J Biochem Biophys* **40**: 423–428.

Verma RS, Mehta A, Srivastava N. (2007). In vivo chlorpyrifos induced oxidative stress: Attenuation by antioxidant vitamins. *Pestic Biochem Physiol* **88**: 191–196. https://doi.org/10.1016/j.pestbp.2006.11.002

Walker C. (1998). The use of biomarkers to measure the interactive effects of chemicals. *Ecotoxicol Environ Saf* **40**: 65–70.

Wheelock CE, Miller JL, Miller MJ, Gee SJ, Shan G, Hammock BD. (2004). Development of toxicity identification evaluation procedures for pyrethroid detection using esterase activity. *Environ Toxicol Chem* **23**: 2699–2708.

Wheelock CE, Phillips BM, Anderson BS, Miller JL, Miller MJ, Hammock BD. (2008). Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). *Rev Environ Contam Toxicol* **195**: 117–178.

Widder PD, Bidwell JR. (2006). Cholinesterase activity and behavior in chlorpyrifos-exposed *Rana sphenocephala* tadpoles. *Environ Toxicol Chem* **25**: 2446–2454.

Wilson IB, Ginsburg S. (1955). A powerful reactivator of alkylphosphate-inhibited acetylcholinesterase. *Biochim Biophys Acta* **18**: 168–170. https://doi.org/10.1016/0006-3002(55)90040-8

Wu J, Laird DA. (2003). Abiotic transformation of chlorpyrifos to chlorpyrifos oxon in chlorinated water. *Environ Toxicol Chem* **22**: 261–264.

Xing H, Li S, Wang Z, Gao X, Xu S, Wang X. (2012). Histopathological changes and antioxidant response in brain and kidney of common carp exposed to atrazine and chlorpyrifos. *Chemosphere* **88**: 377–383. https://doi.org/10.1016/j.chemosphere.2012.02.049