Toxicity of the insecticide chlorpyrifos to the South American toad Rhinella arenarum at larval developmental stage

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\textbf{A B S T R A C T}

Chlorpyrifos (CPF) is an insecticide widely used for pest control in the fruit-productive region of North Patagonia, Argentina, where it is found in superficial waters. The aim of this study was to establish the toxic effects of CPF in Rhinella arenarum toad larvae as a potentially exposed species. We determined the 96 h-LC50 (1.46 ± 0.27 mg/L), the LOEC (0.81 mg/L, LC10) and NOEC (0.43 mg/L, LC1) for CPF lethality as endpoint. We also analyzed biochemical biomarkers in larvae exposed to sublethal CPF concentrations. The IC50 for cholinesterase was 0.113 ± 0.026 mg/L, one order of magnitude lower than the LC50. Carboxylesterase activity was inhibited, buffering OP toxicity on cholinesterase. Reduced glutathione increased after 24 h as an antioxidant response, and decreased at 96 h together with catalase activity, due to oxidative stress. These biochemical effects suggest that environmentally relevant CPF concentrations pose a threat to \textit{R. arenarum} larvae progression.

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\textbf{Abbreviations:} CPF, chlorpyrifos; OP, organophosphorus; AChE, acetylcholinesterase; CabE, carboxylesterase; CAT, catalase; GST, glutathione S-transferase; TRAP, total reactive antioxidant potential.

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1. Introduction

Chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate; CPF) is a non-systemic organophosphorus (OP) insecticide frequently used around the world. This pesticide is widely used in Argentina for pest control in crops and orchards. In the valley of Río Negro and Neuquén, North Patagonia, this compound is applied to manage the codling moth Cydia pomonella together with the OP azinphosmethyl. Both OP insecticides have been detected in superficial and shallow ground water in this fruit-producing region (Loewy et al., 2011). Residues of OP and carbamates have been detected in the order of 10–100 μg/L during the productive season, although a worst-case scenario based on the field doses, drift, runoff and repeated treatments suggests that concentrations of the milligram per liter range may be transiently reached (Rosenbaum et al., 2012). Ponds and irrigation channels within this area are the natural habitat of embryos and larvae of the amphibian Rhinella arenarum, the common South American toad. Amphibians are probably the taxonomic group with the highest number of threatened species around the world, with an upper estimate of 56% in 2013 according to the International Union for Conservation of Nature (IUCN). The greatest number of threatened species occurs in Latin American countries and although habitat loss clearly poses the greatest threat to amphibians, the fact that many species are declining for unknown reasons is disturbing (IUCN; http://www.iucnredlist.org/organisations/amphibians/analysis). Chemical pollution of water, including pesticides, may be one of the factors contributing to amphibian population decline; although it is also unknown to which extent it may affect assumed pristine habitats (Fellers et al., 2004). In particular, larval stages of amphibians are highly susceptible to pesticides present in water due to their thin and permeable skin and the continuous water flow through their gills (Wijesinghe et al., 2011). In the case of the insecticide CPF, only 1.7–3.4% of about 7000 entries found in databases (Pesticide Action Network Database, http://www.pesticideinfo.org; ECOTOX Database, http://www.epa.gov/ecotox) correspond to studies developed in amphibians.

Inhibition of acetylcholinesterase (AChE) by OP in the synapses and neuromuscular junctions of the central and peripheral nervous system leads to acetylcholine accumulation and subsequent overstimulation of cholinergic receptors causing symptoms such as hyperexcitability, uncontrolled muscle spasms and paralysis. The traditional view for OP toxicity states that this mechanism of action based on AChE activity depletion leads to mortality. However, a significant number of aquatic species shows no signs of acute toxicity when exposed to OP under laboratory conditions, despite an almost complete inactivation of AChE. This fact does not imply that these species will not be threatened in environmental exposures to such OP concentrations; there are also behavioral responses such as feeding and predator avoidance that may be impaired by AChE inhibition and consequently challenge the survival in field situations (Ferrari et al., 2009b). Severe alterations in motor activity were observed in axolotl juveniles exposed to environmental CPF concentrations (Robles-Mendoza et al., 2011), and other behavioral alterations caused by OP exposure in amphibians were described (Robles-Mendoza et al., 2009; Ruiz de Arcaute et al., 2012). Besides its anticholinesterasic action, CPF exerts diverse toxicological effects at sublethal concentrations, such as the induction of oxidative stress (Ural, 2013). In addition, CPF alters amphibian normal development (Bernabó et al., 2011), behavior (Robles-Mendoza et al., 2009) and polynuclear metabolism (Sotomayor et al., 2012). Biochemical endpoints may be far more sensitive than lethality, reflecting a threat for an organism even at sublethal concentrations and allowing for the selection of suitable biomarkers that could be used in biomonitoring programs.

The aim of this study was to assess the acute toxicity (96 h) of CPF in the larval stage of the South American toad R. arenarum and determine biochemical effects at lethal and sublethal concentrations on esterases and the detoxifying/antioxidant system.

2. Materials and methods

2.1. Chemicals

High purity-certified standards of chlorpyrifos (99.5% purity) were purchased from Chem Service (West Chester, Pennsylvania, USA). Acetylthiocholine iodide, reduced glutathione (GSH), bovine serum albumin, 1-chloro-2,4-dinitrobenzene (CDNB), dithio-bis-nitrobenzoate (DTNB), β-nicotinamide adenine dinucleotide phosphate-reduced tetrasodium salt (β-NADPH), were purchased from Sigma Co. (St. Louis, MO, USA). All the reagents used were of analytical grade.

2.2. Toad embryo culture

Rhinella arenarum toads were obtained from a pristine area in Los Barreales Lake, located 60 km upstream from the nearest fruit production area in the valley of Río Negro and Neuquén, and kept in captivity outdoors in a small terrarium. Animals used in this study were treated with regard for the alleviation of pain and distress prescribed by the guide for the care and use of laboratory animals (National Institutes of Health, 1996). Embryos were obtained by in vitro fertilization as previously described (Ferrari et al., 2008). Briefly, a testicular homogenate in amphibian Ringer’s solution (0.65 g/L NaCl; 0.01 g/L KCl; 0.02 g/L CaCl₂) was gently dispersed over the oocytes, and after 15 min the fertilized clutch was completely covered with the same media. Independent in vitro fertilizations were performed using different pairs of parents. The newly-fertilized embryos were developed in glass dishes containing amphibian Ringer’s solution and maintained at 20 ± 2°C in a 12h light-12h dark photoperiod until the beginning of the assay. The development solution was changed every 48 h. The tadpoles were fed freely with boiled lettuce from the open mouth stage until the beginning of the assay.
2.3. **Experimental set #1: acute toxicity assays and estimation of toxicological (lethality) parameters**

Ten days after reaching the complete operculum stage (stage 25, Del Conte and Sirlin, 1952), larvae were randomly collected to perform the assays. Exposures to CPF were carried out during 96 h in glass dishes containing 50 larvae in 50 mL of amphibian Ringer’s solution with 0.3% aceton (final v/v) as a vehicle, according to our standard protocol (Venturino et al., 2001). Exposure solutions were made by first preparing a standard solution of the insecticide in aceton and then diluting it with an appropriate amount of amphibian Ringer’s solution. The exact concentrations of CPF in the standard solutions were checked by gas chromatography with nitrogen phosphorus detection. In the acute toxicity assays performed to determine the 96 h-lethal concentration fifty (96 h-LC50), CPF concentrations of 0-, 0.5-, 1-, 1.5-, 2-, 2.5- and 3-mg/L were tested. Four independent experiments were performed using different clutches, and within each independent experiment, the different concentrations were tested by triplicate. The range of concentrations was chosen after a first trial using 0-, 1-, 2-, 4-, 8- and 16 mg/L CPF. The exposure condition was semi-static, with solution renewal every 48 h. Control groups were run in parallel, using Ringer’s solution plus 0.3% aceton, as this concentration has no effects on larvae survival (Ferrari et al., 2008). Larvae were not fed during the assays. Viability of individuals and malformations were monitored with a stereoscopic microscope and mortality was determined by the lack of heart beat and blood circulation (Ferrari et al., 2009a).

2.3.1. **Model fitting to mortality data and estimation of toxicological parameters**

A logistic model was fitted to mortality data after 96 h-exposure using a non linear regression method (Venturino et al., 1992). Data from the four experiments were used both individually and together for fitting the model equation. The LC50 was directly estimated from the fitted equation as one of the model parameters. To estimate the CPF concentrations causing minimal or no lethal effects, the LOEC and NOEC for lethality as endpoint were assessed by a probabilistic approach. The LC1 and LC10 as endpoints were calculated from the fitted equation to estimate the NOEC and LOEC, respectively (Crane and Newman, 2000). Confidence limits (95%) were established using ±2 standard errors (SE) for the model parameters to calculate the boundary concentrations and mortality percentages (Murado and Prieto, 2013). The “minimum detectable response” (MINDER) concentration was estimated as the effective concentration whose confidence interval (CI) is 100% of its value (Murado and Prieto, 2013), using ±2SE for the parameters in the logistic model equation.

2.3.2. **Biochemical determinations**

After the 96 h-exposures, the surviving larvae from controls, 1 and 2 mg/L CPF were collected and processed to assay the enzymatic activities of ChE, CabE, CAT and GST, and the content of GSH as it is indicated below for sublethal experiments.

2.4. **Experimental set #2: sub-lethal exposures to CPF and biochemical biomarkers as endpoints**

Ten days after reaching the complete operculum stage, larvae were randomly collected and exposed to the sublethal concentrations of 0.1 mg/L and 0.5 mg/L CPF up to 96 h. Groups of 200 larvae were exposed in 200 mL of amphibian Ringer’s solution with 0.3% aceton (final v/v) as a vehicle. Larvae were not fed during the assays. Samples of 50 larvae were randomly collected from each dish at 24, 48 and 96 h for the biochemical determinations, and simultaneously 50 mL of media were removed to keep the larvae/volume ratio constant. Three independent experiments were performed using different clutches. Within each independent experiment, the different concentrations were tested by duplicate.

2.4.1. **Sampling and homogenization**

The larvae were immediately cooled on ice, washed with cold Ringer’s solution and homogenized in 2.5 mL of 143 mM potassium phosphate buffer pH 7.5 with 6.3 mM EDTA. One aliquot was separated for the immediate determination of GSH and antioxidant potential; the rest of the homogenate was centrifuged at 10,000 × g for 20 min at 4 °C and the resulting supernatant was aliquoted and kept frozen until performing the enzymatic determinations.

2.4.2. **Enzymatic determinations**

Cholinesterase (ChE) was measured at 25 °C in 1.03 mL of 100 mM phosphate buffer pH 8.0 containing 0.2 mM DTNB, 0.75 mM acetylthiocholine iodide and 25 μL of larvae supernatant. Activity was continuously recorded at 412 nm. Enzymatic activity was corrected for spontaneous hydrolysis of the substrate and was expressed as mIU/mg protein (Ferrari et al., 2004). The concentration value that inhibited fifty percent of control ChE enzymatic activity (IC50) after 96 h of exposure was estimated from the whole range of CPF concentrations, fitting a sigmoidal model to data by nonlinear regression (Ferrari et al., 2004). Carboxylesterase (CabE, EC 3.1.1.1) activity was determined as previously described (Ferrari et al., 2011). Reactions were performed in 1 mL 100 mM phosphate buffer pH 8.0 containing 5% aceton, 1 mM p-nitrophenylbutyrate and 10 μL of larvae supernatant. Activity was continuously recorded at 400 nm. Specific activity was calculated using a molar extinction coefficient of 18.6 mM−1 cm−1 for p-nitrophenol.

Glutathione S-transferase (GST; EC 2.5.1.18) activity was assayed in a final volume of 1.0 mL of 100 mM phosphate buffer pH 6.5 containing 0.5 mM CDNB dissolved in 1% v/v acetonitrile and 2.5 mM GSH as substrates. Baseline (non enzymatic reaction) was continuously recorded at 340 nm, and 10 μL of the supernatant was added. The continuous changes in the absorbance were recorded. Enzymatic activity was corrected for spontaneous hydrolysis of the substrate and was expressed as mIU/mg protein using a molar extinction coefficient of 9.6 mM−1 cm−1 (Ferrari et al., 2008). Catalase (CAT; EC 1.11.1.6) activity was determined by recording the continuous decrease in hydrogen peroxide (H2O2) absorbance at 240 nm. The reaction was performed in 3 mL sodium phosphate buffer 50 mM pH 7.0 containing 25 mM H2O2. The enzymatic activity determination was carried out at the linear range of response.
with respect to substrate concentration. The absorbance of the reaction mixture was strictly controlled to be one unit. Baseline absorbance was controlled to be stable, and $10 \mu$L of supernatant was added to initiate the catalyzed reaction. Specific activity was expressed as IU/mg protein using a molar extinction coefficient of $40 \text{M}^{-1} \text{cm}^{-1}$ (Ferrari et al., 2008).

2.4.3. GSH determination
The crude homogenates were immediately mixed with 10% trichloroacetic acid 1:1 (v/v) and centrifuged at 10,000 × g during 10 min at 4 °C. GSH was measured as acid-soluble thiols in 0.2 mL of supernatants, using 1 mL of 1.5 mM DTNB in 0.25 M sodium phosphate buffer pH 8.0. The mixture was incubated during 20 min and the absorbance at 412 nm was measured. Acid-soluble thiols were quantified using a calibration curve with pure GSH as standard (Venturino et al., 2001).

2.4.4. Total reactive antioxidant potential (TRAP)
The reaction mixture contained the free radical generating compound 2,2’-azo-bis(2-amidinopropane) 10 mM and 200 μM luminol in 50 mM sodium phosphate buffer, pH 7.4. Aliquots of 10–20 μL of the crude homogenates were added to 3 mL of the reaction mixture to assess their antioxidant potential. The mixture was incubated at room temperature to generate luminescence once the antioxidant potential was overcome. The induction time for chemiluminescence was measured in a liquid scintillation counter Wallac LC1010. Trolox was used as a standard reference for the induction time (Evelson et al., 2001).

2.5. Protein determination
Protein content was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

2.6. Statistical analysis
Differences between treatments were assessed by Factorial ANOVA for each biochemical parameter. A block design was applied as one factor to consider the variability introduced by the clutches, being the other two factors the treatment and the time of exposure. Fisher’s Lowest Significant Differences (LSD) post hoc test was used to assess the statistical differences between factor levels.

3. Results

3.1. Lethal acute toxicity test
Mortality data obtained from the acute toxicity tests are presented in Fig. 1. The non-linear regression analysis (number of replicate assays = 4; total number of data N = 114; concentration levels = 12, 0–16 mg/L CPF) revealed a 96 h-LC50 value of 1.41 ± 0.08 mg/L CPF (mean and standard error, SE) for R. arenarum larvae. The steepness of the mortality curve was high, with a slope of 3.9 ± 0.7. The sigmoidal model was also fitted individually to each experimental data set (results not shown), and the fitted LC50s were compared giving a reasonable range of values and average (range: 0.88–2.33 mg/L; mean 1.46 ± 0.27 mg/L). From the non-linear regression analysis, the LC50 was calculated as an estimation of a NOEC value, which resulted in 0.43 mg/L CPF (95% CI 0.20–0.66 mg/L). This value was close to the experimental concentration of 0.5 mg/L CPF that also caused no significant mortality. In turn, the statistical variation (95% CI) of the 1% mortality was established between 0.1 and 6.6%. An estimation of LOEC, the LC10 was calculated from the fitted equation in 0.80 mg/L CPF (CI 95%: 0.52–1.04 mg/L), while the confidence limits for the 10% mortality were 2.8–24.8%. The fitted LC10 was consistent with the nearest experimental treatment of 1 mg/L CPF, which caused a significant mortality of 15.3 ± 6.8%. The MINDER concentration was also estimated from the fitted logistic equation, resulting in 0.48 mg/L CPF. This concentration corresponded to 1.43% mortality (MINDER). The endpoint criterion MINDER concentration was between the NOEC and LOEC values as expected (Murado and Prieto, 2013).

Larvae surviving the exposure to CPF during 96 h showed a percentage of malformations that increased along with CPF concentration (Table 1). Only larvae exposed to 2 mg/L CPF showed an increase in the percentage of malformations (82.6 ± 17.4%) that was statistically significant compared to control and the other treatments (p = 0.005). The morphological abnormalities most frequently found in

| Table 1 – Percentages of malformations in R. arenarum larvae exposed to CPF. |
|---------------------|---------------------------|-----------------------------|
| CPF concentration (mg/L) | Percentage of malformations (mean ± standard error) |
| 0 (control)       | 2.1 ± 1.4                  |
| 0.5               | 15.6 ± 6.7                 |
| 1.0               | 14.3 ± 2.5                 |
| 1.5               | 24.4 ± 0.0                 |
| 2.0               | 82.6 ± 17.4**               |

Malformations were assessed under stereoscopic microscope on the larvae surviving CPF exposure at 96 h of treatment. Data from triplicate exposures to each concentration and from 4 independent assays were analyzed by ANOVA. There were significant differences between treatments (DF = 4; DF’ error = 9; p = 0.02). Post hoc Fisher’s LSD test was applied (“p = 0.005).
 CPF-exposed larvae were diverse axial malformations such as lateral flexure of the tail, both mild and severe, and necrosis of the caudal fin. Generalized edema was also observed (Fig. 2).

3.2. Biochemical responses after 96 h-lethal exposures

The activity of the OP-specific biomarker ChE was significantly affected, as assessed by factorial ANOVA (p = 0.00001; block effects not significant, p = 0.10). After 96 h of exposure to the lethal concentrations of 1 mg/L and 2 mg/L CPF, the specific activity of ChE was significantly diminished by 95 and 97% respectively, when compared to control values (Fig. 3a). Meanwhile, CatE showed a decrease of 70 and 64% due to exposure to 1 and 2 mg/L CPF, respectively (p = 0.000001; Fig. 3b); block (clutch) effects were significant for this enzyme (ANOVA, p = 0.000001, df = 2, df error = 17), but the effects of CPF exposures were similar in magnitude and significant in all the clutches (p = 0.000001, df = 2). The antioxidant enzyme CAT was significantly inhibited by both concentrations of CPF tested (29 and 43%, respectively, p = 0.0005; Fig. 4a); block (clutch) effects were also significant (ANOVA p = 0.00002, df = 2, df error = 14) and the effects of CPF within them were similar. The activity of the detoxifying enzyme GST was not significantly modified by CPF exposure (Fig. 4b), and block (clutch) effects were minimal (ANOVA p = 0.05, df = 1, df error = 12). Conversely, the levels of the antioxidant GSH were significantly increased (67%) after 96 h of exposure to 2 mg/L CPF (p = 0.01; Fig. 4c), while block (clutch) effects were not significant (ANOVA p = 0.12, df = 1, df error = 12).

3.3. Biochemical effects at sublethal CPF concentrations

Biochemical effects of CPF in toad larvae were studied at 0.1 and 0.5 mg/L, considering that both concentrations were respectively below and around the determined NOEC for
The exposure of larvae to sublethal concentrations of CPF caused a significant decrease in esterase activities. The inhibition of ChE activity was dependent on both time of exposure and the concentration tested (Fig. 5a); block (clutch) effect was significant being one set lower than the other two, but within them the same time-concentration dependence for CPF effects was observed (ANOVA p = 0.03, df = 2, df error = 47). After 24 h of exposure, only the maximal sublethal concentration assayed, 0.5 mg/L CPF, caused a significant decrease of ChE specific activity (34% with respect to control values; p = 0.01, df = 2, df error = 15). After 48 h of exposure, both concentrations tested were capable of significantly inhibiting larval ChE activity, causing 27 and 63% of decrease for 0.1 and 0.5 mg/L CPF respectively (p = 0.01, df = 2, df error = 15). After 96 h of exposure, 0.1 and 0.5 mg/L CPF caused 46 and 85% of ChE inhibition, respectively (p = 0.01, df = 2, df error = 15). Using the complete range of CPF concentrations at 96 h, the IC50 for ChE was calculated in 0.113 ± 0.026 mg/L (mean ± SE, N = 25) (Fig. 5c); a concentration of 0.023 mg/L was also estimated as the theoretical limit value to detect differences respect to control (LOEC). A dependence on time of exposure and CPF concentration was also observed for CabE activity in exposed larvae. Carboxylesterase activity levels varied between blocks (clutches) (ANOVA p = 0.03, df = 2, df error = 30, but showed similar effects for CPF exposure. The enzymatic activity was significantly diminished after 24 h of exposure to both concentrations of CPF tested (34 and 60%, respectively; p = 0.03) (Fig. 5b). The decrease in CabE activity was more pronounced after 48 h of exposure reaching 42 and 80% of inhibition for 0.1 and 0.5 mg/L CPF, respectively (p = 0.005). At the end of the assay, the percentage of decrease was similar between both concentrations tested (58 and 64%, respectively, p = 0.000004 versus control).

The antioxidant enzymatic activity of CAT was not significantly affected by the exposure to the sublethal CPF concentrations assayed at 24 or 48 h (Fig. 6a) and showed an inhibition at 96 h that averaged 20%; clutch block affected the average CAT activity (ANOVA p = 0.000001, df = 2, df error = 47). No significant differences between exposed and non-exposed larvae were found for GST activity (Fig. 6b). However, a trend toward a decrease in GST activity could be observed in larvae exposed during 96 h to both CPF concentrations. Clutch block significantly affected average GST activity (ANOVA p = 0.000001, df = 2, df error = 47). The levels of the antioxidant GSH were significantly increased after 24 h of exposure to 0.1 mg/L CPF (43%) and 0.5 mg/L CPF (106%; ANOVA p = 0.001, df = 2, df error = 19) (Fig. 6c). At 48 h, larvae exposed to 0.1 mg/L CPF maintained a significant increase in GSH levels (20%, p = 0.05), while GSH in larvae exposed to 0.5 mg/L CPF returned to control values. At the end of the exposure, GSH content significantly diminished in exposed larvae (44 and 46% with respect to control values; ANOVA p = 0.0002, df = 2, df error = 15). Block (clutch) effects were not significant on the average GSH levels (ANOVA p = 0.10, df = 2, df error = 59). The total antioxidant capacity was significantly diminished in larvae exposed during 24 h to both sublethal CPF concentrations (p = 0.01) (Fig. 6d), returning to control levels at 48–96 h. Block effects were significant on the average values (ANOVA p = 0.000001, df = 1, df error = 41), and in one of the clutches a trend toward TRAP decrease was observed after 24 h of exposure.

**4. Discussion**

In the present study, we assessed the toxicity of the insecticide CPF on larvae of the toad *R. arenarum* under laboratory conditions. The LC50 value showed that CPF was seven times more toxic to *R. arenarum* larvae than the OP azinphos methyl, the most widely applied pesticide in the fruit producing area of Rio Negro Valley, North Patagonia, Argentina, tested under the same laboratory conditions (Ferrari et al., 2004) (Table 2). In fact, CPF was more toxic than other OP and carbamates previously tested on *R. arenarum* larvae, among them the active ingredient parathion that was banned for use two decades ago. Larval stages of this anuran were in turn more sensitive than embryos to OP insecticides based on the LC50 values (Table 2) (Anguiano, 2002; Sotomayor et al., 2012); this increase in sensitivity from embryos to larvae was much more relevant for CPF exposure, being of one order of magnitude. It was previously
Fig. 5 – Esterase specific activity in R. arenarum larvae exposed to sublethal concentrations of chlorpyrifos (CPF) during 24, 48 and 96 h. (A) Cholinesterase (ChE) specific activity. (B) Carboxylesterase (CabE) specific activity. Data (mean ± SEM) from three independent experiments with duplicated treatments are shown. Asterisks indicate significant differences with respect to control values determined by ANOVA and Fisher’s LSD test: **p < 0.01, ***p < 0.001. Different letters indicate significant differences among controls along time, p < 0.05. (C) Estimation of the Inhibitory Concentration-fifty (IC50) for CPF on ChE activity at 96 h of larvae exposure, using non-linear regression fitting on data; IC50 = 0.113 ± 0.026 mg/L, slope 1.20 ± 0.34 (experimental data: mean ± SEM, N = 25).

Fig. 6 – Antioxidant/detoxifying enzymes activity and glutathione (GSH) levels in R. arenarum larvae exposed to sublethal concentrations of chlorpyrifos (CPF) during 24, 48 and 96 h. (A) Catalase (CAT), (B) glutathione S-transferase (GST), (C) GSH content, (D) total reactive antioxidant potential (TRAP). Data (mean ± SEM) from three independent experiments with duplicated treatments are shown. Asterisks indicate significant differences determined by ANOVA and Fisher’s LSD test: *p < 0.05. Different letters indicate significant differences among controls along time, p < 0.05.
reported that OP toxicity for amphibians is highly dependent on the development stage (Berrill et al., 1995). Richards and Kendall (2002) reported that metamorphic larvae of Xenopus laevis were more sensitive to CPF than pre-metamorphic ones. Similarly, Robles-Mendoza et al. (2009) found that embryos of the axolotl Ambystoma mexicanum were less sensitive to an acute exposure to CPF and malathion than axolotl larvae. Besides, larvae of R. arenarum resulted slightly more sensitive to CPF when compared to other amphibian larvae i.e., Rana límnocharis, Rana dalmatina (Ruiz de Arcaute et al., 2012), and Duttaphrynus melanostictus (Wijesinhe et al., 2011). The difference in sensitivity reached one order of magnitude respect to larvae of the reference species Xenopus laevis (Table 2).

In order to analyze the relevance of CPF toxicological data for the survival of common toad larvae in environmental conditions, it is useful to estimate the LOEC and NOEC, which may be obtained from the non-linear regression fitting to mortality-concentration data. The probabilistic approach of LC10 as the LOEC-mortality resulting in 0.80 mg/L CPF proved to be consistent with the experimental data that validated its use as endpoint criterion. We considered the use of the LC1 as an estimation of NOEC-mortality, taking into account that an average 0.8% value in controls represented no significant mortality. The LC1 was also close to the experimental CPF concentration causing no significant mortality. Both elements support our decision to refer the LC1 obtained in this study as the NOEC for CPF in R. arenarum larvae.

These results highlight the probability that R. arenarum may be transiently exposed to toxic concentrations of CPF during the productive season, coincidently with their reproduction and development. Although OP and carbamates were detected in the order of 10–100 μg/L in rivers of the fruit-producing region of the Alto Valle, Argentina (Loewy et al., 2011), small ponds and ditches inside or nearby the orchards may repeatedly receive the concentrated product from applications (Rosenbaum et al., 2012). In particular, CPF has been detected at levels ranging from 0.1 to 1.2 μg/L in rivers and superficial drainage waters, with a frequency of detection 73% in the fruit productive season (Loewy et al., 2011), while in other regions of Argentina like the wetland Pampa, levels of 10 μg/L have been reported (Marino and Ronco, 2005). Data on CPF in soil at orchards in the Alto Valle region reveal very high concentrations reaching up to 14 mg/kg (Loewy et al., 2011), thus sustaining our hypothesis that CPF may transiently reach in the order of mg/L concentrations in stagnant waters located in the aforementioned region.

In view of the probable environmental episodes of pulse exposures, it is worth analyzing the biochemical effects of CPF in R. arenarum larvae at sublethal concentrations. The most relevant effect observed in this study was esterase inhibition. Cholinesterase activity was significantly inhibited in a concentration-dependent manner in the whole range of the tested concentrations, with an estimated IC50 value of 0.113 mg/L CPF. The estimated LOEC (EC10) value for ChE inhibition of about 0.02 mg/L CPF was in the order of the environmental freshwater concentrations reported in the Alto Valle region (Loewy et al., 2011). The IC50 value is clearly below the NOEC for lethality and is one order of magnitude lower than the estimated LC50. This result suggests that a significant degree of ChE inhibition may be relatively well tolerated by R. arenarum larvae, making it a useful biomarker in amphibians (Richards and Kendall, 2002; Widder and Bidwell, 2008). According to Venturino and Pechen de D’Angelo (2005), low levels of ChE inhibition (below 60%) generally do not affect viability or the normal progression of amphibian embryonic development, while more than 90% of inhibition is associated with lethality. However, other aquatic organisms such as certain fish species are able to survive very high levels of

Table 2 – Comparative toxicity of CPF in anuran species and other anticholinesterasic pesticides in R. arenarum.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>Exposure time</th>
<th>LC50 (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haplobatrachus tigerinus</td>
<td>Tadpole</td>
<td>96 h</td>
<td>0.019</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Pseudacris regilla</td>
<td>Tadpole</td>
<td>96 h</td>
<td>0.121</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Rana boylii</td>
<td>Tadpole</td>
<td>96 h</td>
<td>0.205</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Rhinella femaritae</td>
<td>Tadpole</td>
<td>48 h</td>
<td>0.612</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Bufo bufo</td>
<td>Tadpole</td>
<td>96 h</td>
<td>0.800</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Ambystoma mexicanum</td>
<td>Larva</td>
<td>96 h</td>
<td>1.36</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Rhinella arenarum</td>
<td>Embryo</td>
<td>96 h</td>
<td>14.3</td>
<td>Sotomayor et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>Larva</td>
<td>96 h</td>
<td>1.46</td>
<td>This study</td>
</tr>
<tr>
<td>Rana límnocharis</td>
<td>Tadpole</td>
<td>48 h</td>
<td>2.40</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Duttaphrynus melanostictus</td>
<td>Larva</td>
<td>7 days</td>
<td>3.00</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Rana dalmatina</td>
<td>Tadpole</td>
<td>96 h</td>
<td>5.17</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td>Xenopus laevis</td>
<td>Embryo</td>
<td>96 h</td>
<td>2.41</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td></td>
<td>Larva</td>
<td>96 h</td>
<td>14.6</td>
<td>U.S. EPAa</td>
</tr>
<tr>
<td></td>
<td>Metamorphosis</td>
<td>96 h</td>
<td>0.560</td>
<td>U.S. EPAa</td>
</tr>
</tbody>
</table>

Other pesticides in R. arenarum

<table>
<thead>
<tr>
<th>Compound</th>
<th>Stage</th>
<th>Exposure time</th>
<th>LC50 (mg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azinphosmethyl</td>
<td>Embryo</td>
<td>144 h</td>
<td>15.6</td>
<td>Anguiano (2002)</td>
</tr>
<tr>
<td>Parathion</td>
<td>Embryo</td>
<td>120 h</td>
<td>19.8</td>
<td>Anguiano et al. (1994)</td>
</tr>
<tr>
<td>Malathion</td>
<td>Embryo</td>
<td>96 h</td>
<td>4.50</td>
<td>Anguiano et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Larva</td>
<td>96 h</td>
<td>4.40</td>
<td>Rosenbaum et al. (1999) and Lascano et al. (2011)</td>
</tr>
<tr>
<td>Carbyl</td>
<td>Larva</td>
<td>96 h</td>
<td>24.6</td>
<td>Ferrari et al. (2004)</td>
</tr>
</tbody>
</table>

a ECOTOX Database http://www.epa.gov/ecotox.
b Approximate value estimated from both references.
ChE inhibition due to OP exposure in the laboratory (Ferrari et al., 2009b). Esterases inhibition has been reported to affect relevant behavior of different aquatic organisms, related to prey capture and avoidance of predators. Most of the studies that link esterase inhibition and behavioral responses have been performed in fish species (Ferrari et al., 2009b), but some authors have evaluated those responses in amphibians (Robles-Mendoza et al., 2009, 2011; Ruiz de Arcaute et al., 2012). Studies performed in fish have established significant correlations between brain AChE inhibition, ranging from 20 to 55%, and behavioral alterations in feeding, locomotion and swimming (Rao et al., 2005; Tierney et al., 2007). Similarly, significant behavioral alterations were reported in frog tadpoles exposed to sublethal herbicide concentrations that caused 50% of AChE inhibition (Peltzer et al., 2013).

Other biomarkers were included in this study, which would help to evaluate the impact and the risk of sublethal concentrations of CPF on R. arenarum development. Carboxylesterase 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 (Jokanović, 2001). In the present study, CabE was not able to prevent larval ChE inhibition after 96 h of exposure to lethal concentrations of CPF. In turn, exposure to sublethal concentrations resulted in an early inhibition of CabE, which showed a greater sensitivity than ChE. Therefore, at sublethal CPF concentrations and short times of exposure, a protecting role of CabE could be observed, as reported for other anticholinesterasic insecticides (Ferrari et al., 2011).

Oxidative stress biomarkers also indicated the impact of CPF in R. arenarum larvae; CAT activity was diminished in R. arenarum larvae after 96 h of exposure to lethal concentrations of CPF and showed the same effect at sublethal concentrations, suggesting a direct impact of ROS on the enzyme. A similar effect was reported for other anticholinesterasic pesticides and anurans (Ferrari et al., 2011; Kanter and Celik, 2012). Due to the relevant role played by this antioxidant enzyme, its inhibition could lead to an imbalance in the redox status in favor of a pro-oxidant situation. This impact was also observed on GSH levels after 96 h of exposure to sublethal concentrations of CPF, as shown for other OP (Ferrari et al., 2009a). The antioxidant GSH acts as a substrate for GST in conjugation reactions and in redox reactions catalyzed by GST and GSH-peroxidases, being depleted in oxidative stress conditions (Jokanović, 2001). Besides its participation as a substrate for antioxidant enzymes, GSH may also directly react with oxy-radicals and oxidants. Conversely, larval GSH levels were increased after 24 h of exposure to both sublethal concentrations of CPF as an early antioxidant response generated by the oxidative metabolism of the pesticide. This induction has been reported for other OP (Kanter and Celik, 2012) and carbamates (Ferrari et al., 2009a), and for in situ exposed R. arenarum larvae in irrigation channels within an agricultural site potentially receiving pesticide drift (Rosenbaum et al., 2012). The levels of GSH result from the balance of diverse processes of consumption and regeneration. The latter includes GSH de novo synthesis, which is under control of the transcription factor Nrf-2 through its binding to the antioxidant response element (Kensler et al., 2007). In our study, the total antioxidant potential was diminished after 24 h of larvae exposure to sublethal CPF concentrations, indicating an impact of ROS that might be triggering Nrf-2-controlled responses. The increase in GSH levels caused by lethal CPF concentrations may also comprise other effects such as the inactivation of GSH-dependent antioxidant enzymes (i.e. GSH-peroxidases) due to generalized oxidative damage (Ferrari et al., 2009a) paradoxically diminishing GSH use as antioxidant.

ROS increase caused by CPF in breast cancer cells is directly involved in MAPK signaling leading to the inhibition of cell proliferation (Ventura et al., 2015). During embryonic development, the processes affecting oxidative stress, DNA damage and repair and ROS-mediated responses may be determinant for teratogenic alterations (Wells et al., 2005). Thus, malformations caused by CPF during R. arenarum development may be also linked to ROS imbalance. The increasing number of alterations during larvae exposure to CPF may finally result in a loss of viability or the incapacity to progress into metamorphosis. The lateral flexures in caudal fin in R. arenarum larvae may result from ChE inhibition by CPF, which together with caudal fin necrosis lead to altered swimming (Sotomayor et al., 2012). All these effects turn out to be highly relevant to larval survival in field conditions.

In summary, CPF may challenge R. arenarum larvae survival at environmental concentrations transiently reached in ponds and small courses of water during the fruit producing season. Low CPF concentrations may cause esterase inhibition and oxidative stress in R. arenarum larvae, also compromising their normal progression to metamorphosis.

Conflict of interest

N. Liendro and M. Mardirosian report grants from Agencia Nacional de Promoción Científica y Tecnológica during the conduct of the study.

Transparency document

The Transparency document associated with this article can be found in the online version.

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