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Sensitivity of *Boana pulchella* (Anura: Hylidae) Tadpoles to Environmentally Relevant Concentrations of Chlorpyrifos: Effects at the Individual and Biochemical Levels

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Abstract: We report sublethal effects of environmentally relevant concentrations of chlorpyrifos at the individual (swimming alterations) and biochemical (esterase activities and antioxidant enzymes) levels in the Montevideo tree frog *Boana pulchella* larvae. The 50% lethal concentration at 96 h (LC50-96h) for chlorpyrifos in stage-29 *B. pulchella* tadpoles was 0.98 mg/L, which was close to the 65th percentile of published anuran species sensitivity. In *B. pulchella*, chlorpyrifos disrupted biochemical processes: tadpoles showed a significant inhibition of esterase activity and a significant induction of antioxidant enzymes, indicating a response to an environmental challenge causing oxidative stress. Using principal components analysis, we could associate chlorpyrifos reduction in esterase activity with swimming alterations at 0.5 mg/L of the toxicant. The biochemical biomarkers reported in the present study respond at levels 20 times lower than the LC50-96h and were associated with a biologically important response—swimming behavior. The link of responses across different levels of biological organization was demonstrated. The species is suitable as a model for ecotoxicological studies at different levels, including the individual and biochemical levels, and may be considered a good reference organism in environmental control programs. *Environ Toxicol Chem* 2020;39:834–841. © 2020 SETAC

Keywords: Organophosphorus insecticide; Amphibians; Biomarkers; Biochemical level; Swimming alterations

INTRODUCTION

The use and application of biomarkers in ecotoxicology have extensively been discussed since their proposal as useful predictors of ecologically relevant effects (Shugart et al. 1992; Forbes et al. 2006). In recent years, the classical concept has been extended to include higher biological levels (Handy et al. 2003; Denoël et al. 2012). In herpetological ecotoxicology, lethal and sublethal endpoints like mortality and morphological and behavioral alterations during embryonic and larval development were the first and most amply described biomarkers of exposure (Venturino et al. 2003). However, their lower specificity and sensitivity compared with other biochemical targets such as enzymatic activities, other proteins, or metabolites serving as molecular targets or detoxifying pathways have led to a more generalized use. Therefore, the use of a battery of biomarkers covering different targets or pathways and different levels of biological organization has gained consensus (Venturino et al. 2003; Ferrari et al. 2011; Sotomayor et al. 2012, 2015; Liendro et al. 2015; Pérez-Iglesias et al. 2015; Natale et al. 2018). However, in most studies the classical biomarker response is not tightly and consistently linked to responses at higher levels. The aim of the present study was to assess the sensitivity of the most representative species of anurans of the subtropical area of South America in terms of distribution, *Boana pulchella* (Frost 2020), to a highly frequently used insecticide, chlorpyrifos, using ecotoxicological endpoints at different levels of biological organization and evaluating the link of the responses across those levels.

Chlorpyrifos (O,O-diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate) is an organophosphate insecticide, classified as moderately toxic (class II) to humans by the World Health Organization (2010). Its neurotoxic action is exerted by inactivation of acetylcholinesterase, which causes accumulation of acetylcholine and nervous system hyperexcitation (Barron and Woodburn 1995). It has a persistence of more than 2 mo in water and 6 mo in sediment, being even higher at low temperature, light, and pH conditions (Watts 2012). Previous studies in different parts of the world showed that chlorpyrifos enters aquatic ecosystems with concentrations in water,

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suspended particles, and sediment ranging from 5×10^{-7} to 0.017 mg/L, 0.003 to 0.226 mg/kg, and 0.001 to 2.258 mg/kg, respectively (Barron and Woodburn 1995; Jergentz et al. 2005; Marino and Ronco 2005; Claver et al. 2006; Mugni et al. 2011; De Gerónimo et al. 2014; MacLoughlin et al. 2017; Alvarez et al. 2019). Thus, the entire system may be at risk of exposure, including nontarget organisms. Despite the evidence, it has not yet been banned by several organizations including the US Environmental Protection Agency.

Environmental contaminants such as agrochemicals are one of the proposed causes of the global amphibian decline phenomenon (Mann et al. 2009; Alroy 2015). When considering available data for chlorpyrifos, effects on anurans from different parts of the world have been evaluated at different levels, including the individual level (responses in terms of survival, development, growth, and morphology; Abbasi and Soni 1991; Cowman and Mazanti 2000; Kerby 2006; Bernabò et al. 2011), the biochemical level (Richards and Kendall 2002; El-Merhibi et al. 2004), and the genotoxic level (Yin et al. 2009). Only few studies have evaluated the effects of chlorpyrifos on native anuran species from Argentina, at both the individual (Ruiz de Arcaute et al. 2012; Salgado Costa et al. 2018) and the biochemical (Sotomayor et al. 2012, 2015; Attademo et al. 2015; Liendro et al. 2015) levels. Those sublethal effects on local species have been mostly reported at environmentally relevant concentrations. Given the diversity in life-history traits, it is important to examine the sensitivities to chlorpyrifos of different native species. In the present study, we examined the sensitivity of a promissory test species in ecotoxicology studies: the Montevideo tree frog, *B. pulchella*, which is also abundant in the pampean region of Argentina (Cei 1980; Vaira et al. 2012), where pesticides like glyphosate and chlorpyrifos are the most widely used products (Cámara Argentina de Fitosanitarios 2016). We integrated the use of well-known biomarkers at the biochemical level, such as esterases, oxidative stress parameters, and antioxidant enzymes, with other frequently used ecotoxicological endpoints in anuran tadpoles at the individual level, such as mortality, swimming alterations, presence of abnormalities, and developmental/growth inhibition.

MATERIALS AND METHODS

Test species

Boana pulchella tadpoles are a suitable in vivo model for assessing lethal and sublethal effects induced by several emerging pollutants, including insecticides (Natale et al. 2018; Sansiñena et al. 2018), herbicides (Peltzer et al. 2013; Pérez-Iglesias et al. 2015; Sansiñena et al. 2018), and heavy metals (Natale et al. 2000, 2006). This species is easy to find in 2 well-defined breeding season periods (winter and spring). It has a discontinuous reproductive pattern, associated with the availability of water and heavy rains. The species lays the eggs in masses attached to submerged stems or leaves of aquatic plants, making them easy to handle and acclimate to laboratory conditions (Natale et al. 2018).

Source of organisms and maintenance

During April 2013, when the period of clutching was finished, we collected eggs of *B. pulchella* from a pond located on the outskirts of La Plata city (Buenos Aires, Argentina) within the flood valley of El Pescado stream (35°01′15,58″S, 57°51′ 21,9″W). The study site contains mainly grasslands and current livestock, with temporary ponds in the topographically lower-lying sectors. Measurements of the quality of water and sediment over the past 20 yr did not reveal the presence of heavy metals or pesticides (Ronco et al. 2001; Sansiñena et al. 2018). We randomly took 10% of 10 different clutches, each clutch having from 300 to 3000 eggs, and transferred them to the laboratory (collection permit 22500-22339/13).

Fertilized eggs were mixed and kept under controlled conditions (25 °C ± 1, 16:8-h photoperiod) with tap filtered water (25 ± 1 °C, pH 7.8–8.2, hardness 96–144 mg $CaCO_3$/L, alkalinity 94–176 mg $CaCO_3$/L, dissolved oxygen 6.3 ± 0.3 mg/L), previously dechlorinated by vigorous bubbling for 48 h, and with continuous aeration. Rearing density was 10 individuals/L. Individuals were fed ad libitum with Tetramin® flakes from stage 25 (first feeding stage) until they reached the desired Gosner stage of development, 29 (for ~8 wk; Gosner 1960), with a weight of 0.094 g (95% confidence interval 0.088–0.101 g) and a snout-to-vent length of 7.68 mm (95% confidence interval 7.07–8.29 mm). The selected larval stage has a stable developmental rate and the minimum mass needed to process samples at the biochemical level.

Experimental design

All tests were performed with tadpoles at stage 29 under controlled laboratory conditions, previously mentioned, and following international recommendations to accomplish with test procedures (ASTM International 2002; US Environmental Protection Agency 2002), with minor modifications for *B. pulchella* (Natale et al. 2018). All procedures for the care and use of laboratory animals agree with local guidelines for vertebrate animal welfare (protocol 023-22-15) as well as with US Public Health Service and/or European Union policy.

A pesticide stock solution was prepared from 100 mg of chlorpyrifos 95.1% (determined by Gleba; Chemical Abstracts Service no. 2921-88-2) dissolved in 100 mL of absolute ethanol. Dilutions were made with filtered and dechlorinated water (= test water). Two tests were performed simultaneously: one (T1) to determine lethal (mortality) and sublethal endpoints at the individual level up to 96 h (swimming alterations, morphological abnormalities, and development and growth inhibition), the other (T2) to determine sublethal endpoints at the biochemical level up to 96 h. Tadpoles at stage 29 were exposed in groups of 5 individuals (= experimental unit) to 500 mL of chlorpyrifos solutions in glass chambers of 1000 mL (10.5 × 13.0 cm) in quadruplicate. Two control groups were used: a water control and an ethanol solvent control. All solutions were fully renewed every 24 h, and dead individuals were stored in 70% ethanol. Larvae were not fed during exposures.

Mortality, swimming alterations, and morphological abnormalities were determined as the number of affected individuals per test chamber (experimental unit). Developmental inhibition was registered as the mean state of development and growth inhibition as the mean snout-to-vent length, both per test chamber. For biochemical determinations, all individuals of each test chamber ($n = 5$) were homogenized.

Test 1 at the individual level: Lethal and sublethal effects

Thirteen dilutions, each with 4 replicates, were prepared in the following concentration ranges, which were selected considering published data: 0.005, 0.0075, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 0.7, 0.75, 0.8, 0.9, and 1 mg chlorpyrifos/L. Solvent control contained 0.1% absolute ethanol, which is the maximum concentration of ethanol used in dilutions. Mortality was determined every 24 h by visual observation of immobility, absence of cardiac activity, and rapid decomposition of the body. Swimming alterations were recorded every 24 h after gently swirling the media 5 times with a glass rod and observing the swimming activity of each individual tadpole for 1 min. Alterations were noted as irregular swimming, defined as erratic swimming, body twisting, and convulsions according to descriptions made by Brunelli et al. (2009). At the end of the bioassay (96 h), all individuals were anesthetized (250 mg/L benzocaine), fixed in 10% (v/v) aqueous formaldehyde, and then stored for later examination of abnormalities and developmental/growth inhibition. Morphological abnormalities and inhibition of development were determined under a Wild Heerbrugg M8 binocular stereoscope according to the categories proposed by Bantle et al. (1996) and Gosner (1960), respectively. Growth inhibition was assessed by measuring snout-to-vent length with a digital caliper to the nearest 0.01 mm.

Test 2 at the biochemical level: Sublethal effects

Tadpoles were exposed to 0.05, 0.1, and 0.5 mg chlorpyrifos/L in quadruplicate. Solvent control contained 0.05% absolute ethanol, which is the maximum concentration of ethanol used in dilutions. Tadpoles were collected at 48 and 96 h to perform biochemical determinations. Each experimental unit consisting of 5 tadpoles per chamber was independently homogenized in 3 mL of 143 mM cold potassium phosphate buffer (pH 7.5) with 6.3 mM ethylenediaminetetraacetic acid and stored at –20 °C until processing. The following biomarkers were selected considering frequently used biochemical markers in anurans: 1) esterase activity (Venturino et al. 2001) in supernatants of 10 000 *g*: a) acetylcholinesterase (AChE) activity, b) carboxylesterase (CabE) activity; 2) oxidative stress markers in crude homogenates: a) glutathione (GSH) levels, including reduced acid-soluble thiols (Venturino et al. 2001), b) lipid peroxide levels by thiobarbituric acid test (Venturino et al. 2001); and 3) antioxidant enzymes kinetically measured in postmitochondrial supernatants at 10 000 *g*: a) catalase (CAT) activity (Beers and Sizer 1952; Ferrari et al. 2008), b) glutathione S-transferase (GST) activity (Habig et al. 1974; Anguiano et al. 2001).

Analysis of chlorpyrifos concentrations

Before (0 h) and after (24 h) renewal of the media, samples of chlorpyrifos solutions ($n = 12$) were taken and stored in glass bottles at –18 °C. Samples were filtered (0.45 µm), and chlorpyrifos concentrations were determined by liquid chromatography–mass spectrometry with a detection limit of 0.001 mg/L and a quantification limit of 0.003 mg/L. A liquid chromatograph, model Agilent 1100, coupled to an Agilent mass spectrometer, model VL, was used. Chromatographic separation was run in isocratic condition of acetonitrile (high-performance liquid chromatography grade; J.T. Baker): formic acid 0.1% in water (Merck) at an 80:20 ratio and a flow of 0.5 mL/mL on a C18 X-SELECT™ column (75 mm × 4.6 mm and 3 mm pore size; Waters). An electrospray source was used in positive mode with selective ions $m/z = 350$, 352, and 198 for the ionization. Analytical quality and molecular identity criteria followed those proposed by SANTE 11945/2015 (European Commission 2015).

Statistical analysis

The level of significance was set at 0.05 for all tests. Homogeneity of variances and normality were corroborated with the Levene and the Shapiro-Wilk test, respectively. The 50% lethal concentration (LC50), 50% effective concentration (EC50), EC1, EC10, and their 95% confidence limits were obtained by Probit analysis, Ver 1.5. The highest no-observed-effect concentrations and lowest-observed-effect concentrations were estimated by the EC1 and EC10, respectively. Data on morphological abnormalities and inhibition of development were analyzed by a Kruskal-Wallis test followed by a multiple comparison of mean ranks for all groups to assess differences between water control and treatments. The data on growth inhibition were analyzed by a one-way analysis of variance (ANOVA) followed by Dunnett's test to assess differences between water control and treatments. The biochemical endpoints were analyzed by a 2-factor ANOVA with the treatments (solvent control, 0.05, 0.1, 0.5) and time of exposure (48 and 96 h) as factors, followed by a Tukey's honestly significant difference test. The results of biochemical parameters in water control and solvent control were compared by a 2-tailed independent *t* test. A principal component analysis (PCA) considering all biochemical biomarkers (mean per treatment) was performed; then a biplot graph was done corresponding to the main plane. The levels of treatments were projected on the biplot; also, the percentage of swimming alterations was projected as a supplementary variable to establish the relationship between it and the biochemical biomarkers (Husson et al. 2017). Finally, a logistic regression was performed with swimming alteration data at 96 h based on the

concentrations of chlorpyrifos using a generalized linear model with a logit function of the binomial family:

$$\text{logit}(p_i) = \ln\left(\frac{p_i}{1-p_i}\right) = \beta_0 + \beta_1 x_i$$

where p_i is the probability of presenting an alteration in swimming, x_i is the concentration of chlorpyrifos, and β_0 and β_1 are the estimators of the linear model.

RESULTS

Chemical analysis

Nominal and measured concentrations (Table 1) were compared by a 2-factor ANOVA, taking into account the time and expected/observed values; the test revealed no significant differences ($F_{(1,20)} = 0.300$, $p = 0.590$). Thus, all of the LC50/EC50, EC10, and EC1 values were calculated using the nominal concentrations in the exposure solutions at the initial time of testing (0 h).

Lethal and sublethal effects at the individual level: T1

Both control groups exhibited a total absence of any lethal or sublethal effect. The LC50/EC50, EC1, and EC10 of each evaluated endpoint (mortality, swimming alterations) under acute exposure to chlorpyrifos are summarized in Table 2. The EC50 for swimming alterations was approximately one-half of the LC50 (0.439 vs 0.976 mg/L). The respective EC1 and EC10 were also notably lower for swimming alterations than for mortality. It is worth remarking that even the EC1 for mortality was significantly higher than the EC50 for swimming alterations (100% of swimming alterations were detected at the maximum concentration of chlorpyrifos). The increase in the concentration of chlorpyrifos generated a significant increase in the probability of observing swimming alterations. The 0.1 mg/L increment in chlorpyrifos concentration increased approximately one logit unit, with 0.58 mg/L chlorpyrifos being the EC50 estimated for the logistic regression model (Figure 1).

The Kruskal-Wallis analysis carried out to evaluate differences between each treatment with water control showed no significant differences for morphological abnormalities ($H_{(11,128)} = 14.000$, $p = 0.233$) and inhibition of development ($H_{(11,128)} = 18.267$, $p = 0.076$). Considering growth inhibition, no significant differences ($F_{(11,116)} = 1.001$, $p = 0.445$) were found in the length of water control and treated individuals (global mean of treatments 8.58 mm, 95% confidence interval 8.41–8.75 mm; global mean of controls 8.69 mm, 95% confidence interval 8.12–9.26 mm).

TABLE 1: Nominal and measured concentrations of chlorpyrifos (mg/L) before (0 h) and after (24 h) renewal of the media[a]

Nominal concentration	Measured concentration 0 h	Measured concentration 24 h
0.01	0.0132	0.0071
0.025	0.0268	0.0209
0.025	0.0265	0.0201
0.2	0.2096	0.0581
0.2	0.1947	0.1764
0.4	0.4086	0.2183

[a]The 2-factor analysis of variance performed with the time (0 and 24 h) and expected/observed values revealed no significant differences ($p = 0.590$).

TABLE 2: Lethal and sublethal endpoints evaluated at 96 h on tadpoles of *Boana pulchella*[a]

Parameter	Mortality	Swimming alterations
LC50	0.976	—
95% CI	(0.973–1.048)	—
EC50	—	0.439
95% CI	—	(0.367–0.511)
EC1	0.761	0.189
95% CI	(0.636–0.817)	(0.102–0.252)
EC10	0.851	0.276
95% CI	(0.778–0.890)	(0.186–0.337)

[a]The corresponding lethal and effective concentrations with the 95% confidence interval are shown, and values are expressed in mg/L.
EC1, EC10, and EC50 = 1, 10, and 50% effective concentrations, respectively; LC50 = 50% lethal concentration.

Sublethal effects at the biochemical level: T2

Both control groups exhibited a total absence of any lethal or sublethal effect (swimming alterations, morphological abnormalities). We found significant differences between control groups only in GST activity (28% of inhibition in solvent control, $t = 3.026$, $v = 3$, $p = 0.023$) and lipid peroxide levels (30% of inhibition in solvent control, $t = 2.677$, $v = 3$, $p = 0.037$) at 96 h.

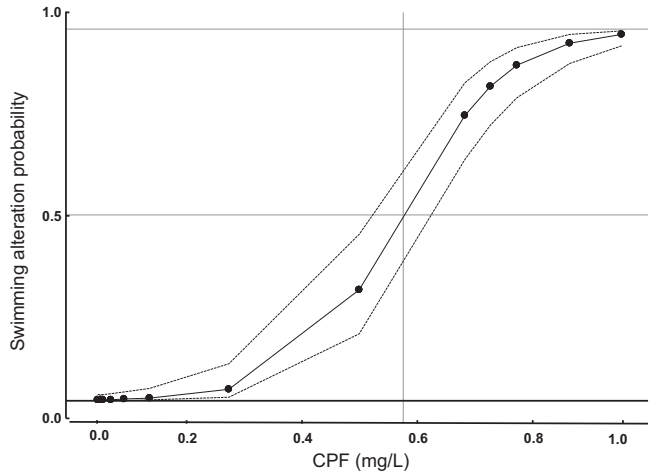

FIGURE 1: Adjusted values and 95% confidence intervals for the logistic model performed with swimming alteration data at 96 h and the concentrations of chlorpyrifos (CPF). Estimated values, standard errors, and probabilities associated with the logistic model performed are as follows: estimated intercept (I) and factor (chlorpyrifos) = –5.98 (I), 10.25 (chlorpyrifos); standard error = 0.93 (I), 1.42 (chlorpyrifos); *z* value = –6.41 (I), 7.19 (chlorpyrifos); probability (*p*) associated with the logistic regression <0.0001 (I/chlorpyrifos).

TABLE 3: Results of the 2-factor analysis of variance performed for each endpoint at the biochemical level considering the concentration of exposure and the time of exposure (48 and 96 h) as factors[a]

Factors	Concentration ($df = 3$)	Time ($df = 1$)	Concentration × time ($df = 3$)
AchE	5.836 ($p = 0.004$)	8.017 ($p = 0.009$)	0.621 ($p = 0.608$)
CabE	33.758 ($p < 0.0001$)	0.228 ($p = 0.637$)	0.804 ($p = 0.504$)
GSH	0.172 ($p = 0.915$)	29.428 ($p < 0.0001$)	1.659 ($p = 0.203$)
LIP	1.304 ($p = 0.296$)	1.227 ($p = 0.279$)	1.432 ($p = 0.258$)
CAT	2.530 ($p = 0.081$)	0.390 ($p = 0.538$)	0.975 ($p = 0.421$)
GST	3.604 ($p = 0.028$)	0.014 ($p = 0.908$)	1.604 ($p = 0.215$)

[a]Given values are F values and probabilities (p values), and the df of the denominator is always the same (= 24).
AChE = acetylcholinesterase; CabE = carboxylesterase; CAT = catalase; GSH = glutathione; GST = glutathione S-transferase; LIP = lipoperoxide.

Considering the presence of ethanol as a solvent in the treatment groups, we used the solvent control as a reference in all of the following analyses according to published methodology (Organisation for Economic Co-operation and Development 2014). Table 3 summarizes the results of the 2-factor ANOVA performed for each biochemical parameter. In summary, the tests showed no significant interactions between concentrations and time of exposure. Thus, independent effects for both factors were assessed. The biochemical effects of chlorpyrifos are shown in Figure 2. We found a significant decrease of AChE activity in tadpoles exposed to 0.1 mg chlorpyrifos/L (21%) and 0.5 mg chlorpyrifos/L (21%). The effect of chlorpyrifos on AChE was also dependent on the time of exposure, with the degree of decrease being significantly higher at 96 than at 48 h. A significant inhibition was observed for CabE activity in tadpoles exposed to 0.05 mg chlorpyrifos/L (31%), 0.1 mg chlorpyrifos/L (33%), and 0.5 mg chlorpyrifos/L (74%). The effect for the higher concentration (0.5 mg/L) was also significantly different from those for the other chlorpyrifos concentrations. Finally, an increased GST activity (28%) was found in larvae exposed to 0.5 mg chlorpyrifos/L. No significant differences were found between control and treatments in GSH levels or lipid peroxide or CAT activity (Figure 2).

PCA

Considering the PCA performed with the mean data of both times (48 and 96 h) for each concentration at the biochemical level, the first and second components shown in the main plane explained >74% of the variability in the response of the 6 biochemical biomarkers (Figure 3). The projection of the treatments in the biplot showed a clear segregation of the variability of the

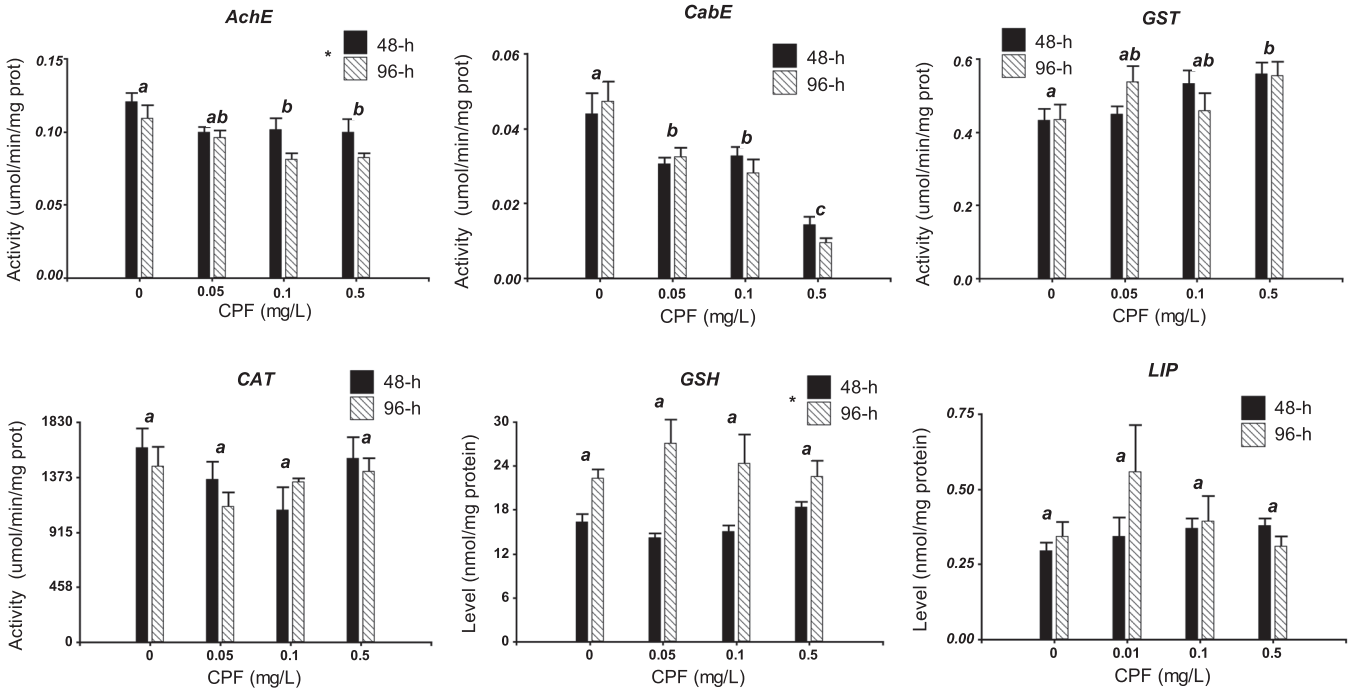

FIGURE 2: Mean ± standard error of biochemical parameters measured in *Boana pulchella* larvae exposed to chlorpyrifos (CPF). Bifactorial analysis of variance was performed with chlorpyrifos concentrations (0.05, 0.1, 0.5 mg/L) and times of exposure (48 and 96 h) as factors. Different letters indicate significant differences between treatments ($p < 0.05$); *significant differences between times of exposure. AchE = acetylcholinesterase; CabE = carboxylesterase activity; CAT = catalase; GSH = glutathione; GST = glutathione S-transferase; LIP = lipid peroxide; 0 = solvent control (0.05% absolute ethanol).

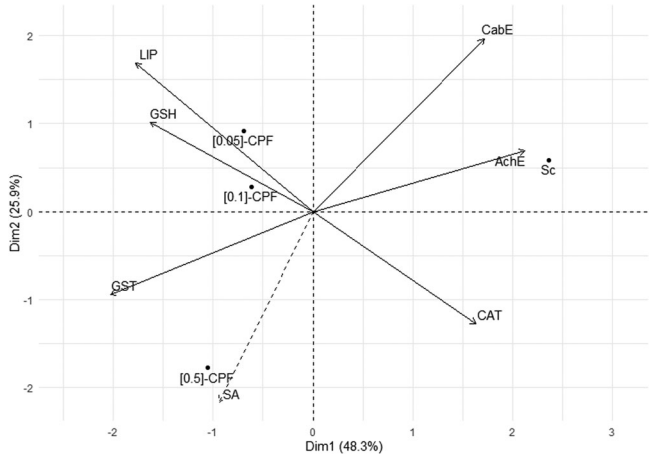

FIGURE 3: Principal component analysis for chlorpyrifos effects on biochemical variables. The 6 biochemical markers are shown as active variables (continuous arrows), and the proportions of individuals with swimming alterations are projected as a supplementary variable (discontinuous arrow). Points indicate mean per treatment. AchE = acetylcholinesterase; CabE = carboxylesterase; CAT = catalase; CPF = chlorpyrifos; Dim, dimethyladenosine transferase; GSH = glutathione; GST = glutathione *S*-transferase; LIP = lipid peroxide; SA = swimming alterations; Sc = solvent control.

control from the chlorpyrifos treatments by the first component. In this case, chlorpyrifos treatments were associated with diminished values of AChE and CabE activities. In turn, chlorpyrifos treatments were separated by the second component from positive (0.05 and 0.1 mg/L chlorpyrifos) to negative (0.5 mg/L chlorpyrifos) values, being associated with a concentration gradient. This segregation was mainly related to increased GST activity. The PCA allowed analyzing all biomarkers at the biochemical level and integrating them with the individual level of response that showed significant differences (= swimming alterations). The swimming alterations were also related with increased activity of GST and decreased activity of CabE and AChE (note that swimming alterations were located opposite to both esterases). Alterations in swimming were clearly close to the highest chlorpyrifos treatment (0.5 mg/L), which was approximately the EC50 for this individual endpoint (Figure 1).

DISCUSSION

We used a battery of biomarkers covering different levels of biological organization. The efficacy of the biochemical biomarkers reported in the present study is clearly evidenced when their effective concentrations are compared with the acute toxicity effects for chlorpyrifos. In fact, the lowest chlorpyrifos concentration tested (0.05 mg/L) at the biochemical level was 20 times lower than the LC50-96 h for *B. pulchella* (0.976 mg/L). Also, responses at the biochemical level could be related to those at the individual level. There was an association of swimming alterations with the decrease in AChE activity as a neurotoxicity biomarker, the decrease in the buffer enzyme CabE, and the increase in the detoxifying enzyme GST, evidenced at 0.5 mg chlorpyrifos/L. This association shows that chlorpyrifos disrupts key mechanisms in *B. pulchella* tadpoles, consequently challenging survival and playing an important role in population dynamics. In this regard, esterase inhibition has been reported to affect relevant behavior of different aquatic organisms not only in amphibians (Liendro et al. 2015) but also in fishes (Ferrari et al. 2009). Although it is difficult to establish connections between different levels of biological organization, we integrated the use of biomarkers at the biochemical and individual levels.

Cholinesterase activity decreases in *B. pulchella* tadpoles exposed to chlorpyrifos, indicating the classical cholinergic inhibition at early tadpole development. Although both AChE and CabE displayed significant inhibition when comparing activities with the control group, the trends associated with a dose–response curve (monotonic trend) were only observed with CabE. Comparatively, the decrease in CabE activity was more pronounced than the decrease in AChE activity. This is in line with the protective role classically attributed to CabEs on AChE activity, having more affinity for organophosphate binding and acting as a suicide molecule that avoids cholinesterase inactivation (Jokanović 2001; Ferrari et al. 2011). Although we did not find oxidative stress induction as reported by other authors (Liendro et al. 2015; Sotomayor et al. 2015), we report an increase in GST activity as a detoxifying enzyme (Venturino et al. 2001). In turn, GST induction might be associated with an antioxidant response that could prevent oxidative stress signs (Sotomayor et al. 2015). Note that the solvent generated a slight inhibition in GST and lipid peroxide after 96 h of exposure, which may be related to liver detoxification. However, from the outcome of our investigation it is not possible to draw any conclusions about this. Further study of the issue would be of interest.

The effects of chlorpyrifos reported at the individual and biochemical levels on *B. pulchella* larvae are within the range of chlorpyrifos levels measured in local environments. Specifically, the highest concentrations measured in suspended particles were 0.226 mg chlorpyrifos/kg (Jergentz et al. 2005; MacLoughlin et al. 2017). These values are within the 95% confidence interval of the EC1 and the EC10 that induce swimming alterations at 96 h in *B. pulchella* tadpoles and are 4.5 times higher than the concentrations that induce effects at the biochemical level. Irregular swimming compromises the larvae's ability to orient correctly and is an ecologically relevant parameter that may act as an indicator of physiological stress (Semlitsch et al. 1995; Natale et al. 2018). Therefore, if this scenario occurs in nature, it would make larvae more susceptible to predation. In the present study, behavioral effects occurred before any other individual effect and in association with biochemical effects, indicating a neurotoxic action (Semlitsch et al. 1995). This is in concordance with other studies that proposed that amphibians are particularly susceptible to pollutants and thus represent good bioindicators or sentinels of local conditions (DeGarady and Halbrook 2006; Sansiñena et al. 2018). Regarding registered abnormalities, it is not possible to confirm that they were type-specific and caused by chlorpyrifos because they are similar to some nonspecific responses as postulated by Ruiz de Arcaute et al. (2012).

Regarding anurans, the use of toxicity tests as an ecotoxicological tool has spread as a water quality validation tool. In this sense, ecotoxicology provides tools to assess the effects of pollutants and to inform decision-making for environmental protection and management by toxicity tests (bioassays) to evaluate effects at the individual and subindividual levels (McCarthy and Shugart 1990; Forbes et al. 2006). Another interesting approach is to consider anuran sensitivities to a particular contaminant. Salgado Costa et al. (2018) considered all the LC50-96 h data available for chlorpyrifos and stage-25 anuran tadpoles, to calculate a species sensitivity distribution. In the present study, we determined an LC50-96 h of 0.976 mg chlorpyrifos/L for *B. pulchella*. When comparing this value with the mentioned distribution, *B. pulchella* is close to the 65th percentile of probably affected anuran species (Abbasi and Soni 1991; Cowman and Mazanti 2000; Richards and Kendall 2002; El-Merhibi et al. 2004; Kerby 2006; Yin et al. 2009; Bernabò et al. 2011; Ruiz de Arcaute et al. 2012; Salgado Costa et al. 2018). The study species, *B. pulchella*, is more sensitive than the 50% of the nonnative species considered in that distribution and more sensitive than the only species from Argentina proposed as a model organism in ecotoxicology. Taking into account the responses both at the individual and biochemical levels, this species would be suitable to determine biomarker responses in environmental impact evaluations. In addition, the multiple endpoint assessment allowed better understanding of the multilevel effects occurring from exposure to chlorpyrifos and the interaction between the stress and the organism. We recommend deepening this type of integration to perform holistic studies, with the final objective being to assess environmentally relevant concentrations that induce simultaneous effects at all levels of biological organization. The widespread distribution of *B. pulchella* and its conspicuous presence in the habitats, high sensitivity to environmental pollutants (= sentinel species), larvae size, duration of larval stage, plasticity, and easiness to breed under controlled laboratory conditions make this species a good candidate for ecotoxicological studies and a good biomonitor in environmental control programs in the region.

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Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (gnatale@quimica.unlp.edu.ar).

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