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Influence of existing site contamination on sensitivity of *Rhinella fernandezae* (Anura, Bufonidae) tadpoles to Lorsban®48E formulation of chlorpyrifos

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Abstract Effects of the widely employed insecticide Lorsban®48E formulation of chlorpyrifos (CPF) was studied on *Rhinella fernandezae* tadpoles, a native species of Argentina, Brazil, Paraguay and Uruguay, under the hypothesis of a differential response of organisms from ponds of two sites with different degree of anthropogenic disturbance: S1 an unpolluted area, and S2 area with high degree of antropogenic disturbance. To collect a representative sample of the genotypic variability of each population, small portions from six clutches were taken randomly from each site when the period of clutching was finished. Embryos and tadpoles were maintained under controlled laboratory conditions. Toxicity tests were conducted under standardized conditions to study acute and chronic lethal (mortality) and sublethal effects (behavior, growth, and abnormalities), within the range of concentrations of 0.010 to 5 mg/L. Chronic effects were assessed with organisms from one of the demes (S1). CPF showed high toxicity on the tadpoles, inducing lethal and sublethal effects at 96 h exposure within a narrow range of concentrations from 0.066 to 0.887 mg/L. Results indicate that *R. fernandezae* tadpoles are below the 30th percentile in the species sensitivity distribution of existing data. The acute LC50, NOEC, and LOEC values were 0.151, 0.066, and 0.133 mg/L for S1, and 0.293, 0.177, and 0.266 mg/L for S2, respectively. Considering all acute end-points evaluated, the effects of CPF showed no significant differences ($p = 0.3484$) between the studied populations. CPF has more severe effects at higher concentrations than at higher times of exposure. Contaminants in S2 do not seem to induce local adaptation. Sublethal effects data and measured environmental concentrations indicate potential risk for populations inhabiting agroecosystems.

Keywords Chlorpyrifos · *Rhinella fernandezae* · Lethal effects · Sublethal effects

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Introduction

Chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate; CPF) is a widely used, multipurpose organophosphate (OP) insecticide, classified as Class II (moderately toxic) by the U.S. environmental protection agency (USEPA) (CASAFE 2011), and able to bioconcentrate in different groups of aquatic organisms (Varó et al. 2000). Its primary mode of action is the inhibition of cholinesterase activity, an enzyme involved in neurotransmission in muscles and nerve synapses, inducing neurotoxic effects in insects and nontarget organisms by ending transmission of neural impulses (Barron and Woodburn 1995; Krieger 2010). Thus, excessive acetylcholine builds up in the synapses and can initially cause hyperactivity, leading to uncontrolled muscle spasms and, depending on the dose, eventually result in paralysis, respiratory failure, and death (Barron and Woodburn 1995). In the year 2000, the USEPA regulated the restriction of CPF use in order to protect the health and environment (Gaizick et al. 2001). However, in Argentina it is one of the most widely used insecticides, particularly in the production of food commodities. Cappello and Fortunato (2008) classified it as frequently used in the production of leaf and fruit vegetables, and very frequently used in the production of fruits. It has been incorporated as one of the insecticides used in biotech soybean crops and categorized as one of the most widely used insecticides during 2009 and 2010 (Casabé et al. 2007; CASAFE 2011).

Marino and Ronco (2005) found detectable levels of CPF in streams adjacent to soybean fields in the Pampas region of Argentina with an average of 1.7 μg/L (<0.2–10.8), detection associated to spray events and rains after spraying. Concentrations of the insecticide in stream water measured by Jergentz et al. (2005), in the main soybean area of the country, were 0.21 and 0.45 μg/L during a period with two rainfall events of 67 and 13 mm/day, respectively. Concentrations in the suspended particulate matter were 63 and 225.8 μg/kg, for the same rainfall events. Several other studies (Moore et al. 2002; USEPA 2002; Mazanti et al. 2003) of small water bodies adjacent to croplands in North America report concentrations of CPF ranging from 73 to over 700 μg/L.

Amphibians have certain characteristics that make them useful indicator species for measuring the effects of local changes of the environment (Stebbins and Cohen 1995). However, in recent decades, amphibians have been studied extensively because of extinctions, with decreases in numbers of populations being reported on a global scale (Pechman and Wake 1997; Houlahan et al. 2000; Sparling et al. 2001, 2003; Blaunstein and Kiesecker 2002; Stuart et al. 2004). Different factors have been proposed as possible causes of this problem, agrochemical environmental pollution being one of them (Gaizick et al. 2001; Beebee and Griffiths 2005; Mann et al. 2009; Relyea 2009). It has been observed that local selection pressure can lead to a genetic adaptation process or local adaptation (Kawecki and Ebert 2004; Marquis and Miaud 2008; Egea-Serrano et al. 2009; Marquis et al. 2009). Local adaptations can appear in only a few years, even in vertebrates (Carroll et al. 2007; Miaud et al. 2011). This could explain the variations in response to stressors between metapopulations without high levels of gene flow and with similar habitats but different local conditions.

Despite being one of the most used insecticides, information regarding the toxic effects of CPF on amphibians is scarce (Moulton 1996; Calumpang et al. 1997). It is well known that OP insecticides cause severe malformations, including abnormal bending of the tail and notochord abnormalities, in several species of frog embryos (Snawder and Chambers 1989, 1990, 1993; Richards and Kendall 2002, 2003; Bonfanti et al. 2004). Although OP insecticides like phosmet, 2-ethylhexyldiphenyl phosphate, isodecyldiphenyl phosphate, and tri-isobutylphosphate have not been classified as teratogenic compounds in mice and rats (Bleyl 1980; Robinson et al. 1986; Ruckman et al. 1999), there are growing data showing that they are teratogenic in non-mammal developing embryos, such as birds (Meiniel 1981) and amphibians. For example, *Xenopus laevis* tadpoles exposed to CPF displayed abnormal tail flexure with impairment of motility (Vismara et al. 1996; Richards and Kendall 2002).

Taking into account the extensive use of CPF in the Pampas region, and the lack of information on the effects on local anuran species, the aim of the present investigation was (a) to study *Rhinella fernandezae* (Anura: Bufonidae) tadpoles sensitivity (lethal and sublethal effects) to chlorpyrifos in its Lorsban®48E commercial formulation under acute exposure; (b) to test the hypothesis that two tadpole populations from sites with dissimilar degree of anthropogenic disturbance show differences in their acute response to CPF; (c) to assess lethal and sublethal chronic effects on organisms from an unpolluted site (S1), estimating incipiency values.

Materials and methods

Study sites

Two temporary ponds from two areas with different degrees of anthropogenic disturbance were selected. There is a physical barrier between them since approximately 80 years due to a large urbanization (Buenos Aires city) (Vapnarsky 2000), and different degree of contamination. One (S1) is a temporary unpolluted pond (Natale 2006) in a rural area scarcely populated and with low farming activities located near La Plata city (35°01′S, 57°51′W). The other (S2) is located in Tigre (34°25′13.55″S, 58°36′36.15″W), in an area with a high degree of urbanization and disturbance, including multiple sources of contamination such as metals (As, Cd, Cr, Cu, Hg, Pb and Zn) and, organochlorine (α, β and γ HCH; heptachlor, heptachlor epoxide; aldrin; endrin; dieldrin; *op*′ and *pp*′ DDT; *op*′ and *pp*″ DDE; α and γ chlordane and endosulphan II) and organophosphorus (ethyl and methyl parathion; CPF and fenitrothion) pesticides; with levels of metals ranging 4 and 40,000, and pesticides varying between 40- and 400-fold above guidelines established for the protection of aquatic life of surface fresh water (Decree 831/92, 1993 of the Argentine Hazardous Wastes Law 24051/93; Villar et al. 1998; Marbán et al. 1999; Rovedatti et al. 2001; Salibián 2006; Ronco et al. 2008a).

Test species

Rhinella fernandezae is distributed throughout the Littoral Mesopotamian regions from Corrientes to the north and center of Buenos Aires (Argentina), Uruguay, south of Paraguay and south of Brazil (Cei 1980). It inhabits grasslands and highly modified sites such as agroecosystems and urban areas (Narvaes et al. 2004). Individuals of this species are of medium size (56–80 mm), have fossorial habits, and their breeding season is in spring and summer. They are explosive breeders that lay more than 10,000 eggs in long spiral chains, which are attached to submerged stems of aquatic plants and have high growth and development rates, with tadpoles metamorphosing 1 month later (Natale 2006). This species is part of *granulosus* group, composed of 16 forms with very similar biology and ubiquitous distribution.

Experimental design

To collect a representative sample of the genotypic variability of each population, small portions from six clutches were taken randomly from each study site (S1 and S2) when the period of clutching was finished and carried to the laboratory for toxicity studies. Embryos and tadpoles were maintained in dechlorinated tap water (pH 7.6–8.3; hardness, 180–250 mg $CaCO_3$/L) with continuous aeration at a temperature of 25 ± 1 °C and a 16:8 h light/dark cycle, until organisms reached stage 25 of Gosner (1960). Toxicity tests were conducted with organisms from each site (S1 and S2). In test T1 corresponding to S1 organisms, acute followed by chronic effects were assessed, and in test T2 corresponding to S2 only acute effects were evaluated.

Acute toxicity tests

Acute exposure was conducted during 96 h. Both tests (T1 and T2) were performed following recommendations proposed by the USEPA (1989) standardized methods, with minor modifications for native species as reported by Natale (2006); Natale et al. (2006) and applied in Vera Candioti et al. (2010) and Agostini et al. (2010). Once the tadpoles reached Gosner stage 25, individuals of each site were mixed, taken randomly, and placed in test chambers according to the experimental design proposed. Preliminary tests allowed us to find a wide concentration range within which to assess lethal and sublethal effects. Toxicity test solutions were prepared from a stock of 1,000 mg/L CPF in Lorsban®48E, the Dow Agroscience commercial formulation. Definitive toxicity tests were performed using ten concentrations per test. T1 was performed using the following concentrations: 0.010, 0.020, 0.075, 0.150, 0.200, 0.500, 0.750, 1, 1.25, and 1.5 mg/L CPF; and T2: 0.010, 0.050, 0.100, 0.200, 0.300, 0.400, 0.500, 1.0, 2.0, and 5.0 mg/L CPF. Testing conditions were the same as those for organism maintenance. Tests were performed in glass chambers with ten individuals and 500 ml of the corresponding test solution. Tests included four replicates (= chambers) per concentration, two negative controls, and a positive control with Cr(VI), according to Natale (2006), with no feeding or aeration, and complete renewal of testing solutions every 24 h after end-point assessment.

Chronic toxicity tests

Chronic exposure was conducted during 30 days only with organisms from S1. Individuals were aerated and fed ad libitum once per week with blenderized lettuce leaves before medium renewal. The test was performed following recommendations proposed by the USEPA (1989) standardized methods, with minor modifications for native species as indicated for acute toxicity tests. Concentrations used are: 0.010, 0.020, 0.075, 0.100, 0.150, 0.200 and 0.500 mg/L CPF. Testing conditions were the same as those for organism maintenance, as explained for acute toxicity tests.

Chemical analysis

The concentrations of CPF in solutions used for toxicity testing (formulation, stock, and test dilutions at initial and final time of testing) were corroborated by chromatographic method using a GC–ECD with an HP5 (15 m and 0.53 mm inner diameter) column and N_2 carrier, with a temperature ramp between 180 and 220 °C, and a limit of detection of 0.02 mg/L (injected sample extract) (Marino and Ronco 2005). Samples of test solutions ($n = 9$) were taken before and after exposure every 24 h to confirm concentrations. Environmental sediment samples ($n = 3$) from each site (S1 and S2) were taken using a corer, and the first 5 cm layers were separated for chemical analysis according to USEPA (1986) and Marino and Ronco (2005). Pretreatment steps for aqueous samples (250 ml) included liquid–liquid extraction with methylene chloride, rotoevaporation (vacuum, 600 mm Hg; bath temperature, 40 °C; taken to dryness with N_2 flow), and resuspension in 1 ml *n*-hexane (Method 3500, USEPA 1986). Sediment samples (25 g) were extracted in a solid–liquid system with 50 ml of methylene chloride and 1 h stirring, separation of organic phase, followed by two steps of extraction by sonication of the solid with 25 ml of the same solvent, filtered, rotoevaporated and taken to dryness with N_2 flow, and finally resuspended in 1 ml *n*-hexane (Method 3550, USEPA 1986). A cleanup procedure with Florisil (60–100 Mesh, activated at 675 °C) was performed for these samples (Method 3620, USEPA 1986). The recovery factor was tested using pure standards on similar matrices. The CPF concentration was analyzed by the same chromatographic methods explained before for test solutions. Solvents and Florisil used for pesticide analysis were from J. T. Baker, and the CPF standard was from SENASA (Argentinean National Service of Agricultural Food Health and Quality).

Acute and chronic lethal end-points

Mortality was evaluated by visual observation every 24 h during acute exposure (T1 and T2) and every 24, 72, 144 and 312 h during chronic exposure (T1). Individuals were considered dead when no movement was detected after gently prodding the tadpoles with a glass rod. After 96 h, all individuals of T2 were identified and fixed in 10 % v/v formaldehyde for further evaluation of growth and abnormalities. Individuals of T1 were kept for 30 days to assess mortality after chronic exposure.

Acute and chronic sublethal end-points

Behavior was registered every 24 h (only during acute exposure of T1 and T2) after gently swirling the water five times with a glass rod and observing for 1 min the swimming activity of each organism; irregular swimming (IS) and immobility (IM) were categorized according to descriptions made by Brunelli et al. (2009). Growth was assessed by measuring body length (snout to tailhead) according to Altig and McDiarmid (1999) with a digital caliper of 0.01 mm after 96 h of T1 and T2, and every 7 days during chronic exposure (T1). Abnormalities were observed under a Wild Heerbrugg M8 binocular stereoscope and determined according to the categories proposed by Bantle et al. (1996) after acute exposure of T1 and T2.

Statistical analysis

The level of significance chosen was 0.050 for all tests. Considering acute exposure, mortality and behavior data were analyzed by the Probit method (Finney 1971) or by linear interpolation method when the Probit was not applicable (USEPA 1989). Concentration–response (C–R) curves at different times (24, 48, 72, 96 h) were estimated with their 95 % confidence limits. Regression (*a* and *b*) and correlation (*r*) coefficients were calculated for each C–R curve. The LC50/EC50 values and the 95 % confidence interval were calculated from estimated C–R curves. The same toxicological parameters and statistical assumptions were corroborated using the probit analysis program, version 1.5 (USEPA 1999). Tests of significance of the regression and correlation were performed following Zar (2010), and were used to compare the C–R curves of both populations.

The proportion of individuals affected per test chamber ($n = 10$) at 96 h was calculated for lethal and sublethal end-points (behavior, growth, abnormalities). Each proportion was angular transformed, and a one-way ANOVA with Dunnett's test (Zar 2010) was performed in order to determine significant differences with the control group. The LOEC and NOEC values were estimated for all acute end-points of T1 and T2. ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and Chi-squared test for normality.

Considering chronic exposure, mortality data were analyzed by the Probit method (Finney 1971). LC50 was calculated at different times (120, 144, 216, 360, 504 and 816 h) with their 95 % confidence limits. Those LC50 values were plotted as a function of time and levels of incipiency were estimated from this plot. The incipient lethal concentration was estimated as the point at which the curve began to run parallel to the x-axis according to Newman and Unger (2003). In order to test chronic survival within T1, regression (*a* and *b*) and correlation (*r*) coefficients were calculated for each time of exposure. Test of significance for the regression and correlation coefficients and comparison of linear regression equations were performed following Zar (2010). With growth data, a one-way ANOVA with Dunnett's test (Zar 2010) was performed in order to determine significant differences with the control group.

Finally, to contrast the hypothesis of the existence of differences between responses of the two populations considering all the acute 96 h assessed end-points (EC/LC50, LOEC and NOEC for mortality, behavior (IM), and abnormalities), a two-tailed paired-sample *t* test was performed according to Zar (2010).

Results

Chemical analysis

The chemical analysis of the stock solution of CPF (nominal concentration, 1,000 mg a.i./L) showed 920 mg/L. All the given data were calculated taking into account the measured concentrations in the tested dilutions at the initial time of testing. An average decay of 23.8 % in the concentrations was detected at the end of exposure. The analysis of sediment samples from S1 ($n = 3$) showed no detectable amounts of CPF (below 0.001 mg/kg dry weight). In the case of samples from S2 ($n = 3$), CPF was detected in two samples obtaining an average value of 2.15 ± 0.35 μg/kg of dry sediment.

Acute and chronic lethal effects

Acute LC50 values estimated through Probit analysis regarding the data collected every 24 h exposure period in both tests (T1 and T2) are detailed in Table 1. ANOVA show significant differences (F = 39.12, df = 9, $p < 0.050$) between the proportions of dead individuals for each concentration under acute exposure. Dunnett's test allowed calculating the acute LOEC and NOEC values in mg/L,

Table 1 Results of acute lethal toxicity to CPF

Time (h)	LC50	95 % CI	*n*
Mortality			
T1			
24	1.215[a]	–	2
48	0.612[a]	–	3
72	0.222[a]	–	3
96	0.151	0.133–0.168	5
T2			
24	0.869	0.505–1.508	3
48	0.550	0.497–0.639	4
72	0.479	0.435–0.532	4
96	0.293	0.248–0.337	6

LC50 values and intervals are given in mg CPF/L

CI 95 % 95 % confidence interval, *n* number of data used in the calculation of LC50 value, *T1 and T2* tests with organisms from S1 and S2, respectively

[a] Calculated by linear interpolation method when the Probit was not applicable

Table 2 Results of chronic lethal toxicity test (T1) to CPF with organisms from S1

Time (h)	LC50	95 % CI	*n*
Mortality			
T1			
120	0.113	0.031–0.389	4
144	0.041	0.038–0.420	4
216	0.016	0.000–0.453	4
360	0.016	0.000–0.436	4
504	0.011	0.000–0.382	4
816	0.005	0.000–0.263	4

LC50 values and intervals are given in mg CPF/L

CI 95 % 95 % confidence interval, *n* number of data used in the calculation of LC50 value

which are 0.133 and 0.066 for T1, and 0.266 and 0.177 for T2, respectively (MS = 0.723, df = 32, $p < 0.050$ for T1, and MS = 1.405, df = 37, $p < 0.050$ for T2).

Chronic LC50 values of T1 estimated through Probit analysis regarding data collected for the interval 120 through 816 h exposure time are detailed in Table 2. The incipiency curve and corresponding LC50 (0.016 mg/L) are shown in Fig. 1. The comparison of the concentration response curves for different exposure times (120, 144, 216, 360, 504 and 816 h) show no significant differences (F = 0.04, df = 5, $p = 0.999$) in the slopes but significant differences (F = 21.93, df = 5, $p < 0.001$) in the elevations. Tukey multiple comparison test show significant differences between LC50 values for the assessed exposure times until 216 h, the time at which the value became constant to the end of the experiment (see Fig. 1).

Acute and chronic sublethal effects

Behavior

Sublethal EC50 values estimated through Probit analysis considering data of effects of CPF on mobility of organisms for all acute exposure times are given in Table 3. ANOVA results showed that IS end-point did not differ significantly (F = 1.93, df = 9, $p = 0.086$ for T1; and F = 1.27, df = 10, $p = 0.282$ for T2, respectively) between concentrations and control group. On the contrary, IM end-point showed significant differences (F = 4.36, df = 9, $p < 0.005$ for T1; and F = 5.51, df = 10, $p < 0.005$ for T2), allowing us to find the acute LOEC and NOEC values (MS = 0.726, df = 30, $p < 0.050$ for T1; and MS = 1.675, df = 35, $p < 0.050$ for T2) for this end-point (Table 4).

Growth

ANOVA results corresponding to the acute exposures showed significant differences (F = 4.65, df = 7, $p < 0.050$) in the body length between treatments from T2 exposed to 0.177 mg/L CPF and the control group, allowing us to find LOEC and NOEC values for growth inhibition (Table 4). There are no significant differences in the body length between treatments from T1 (under acute (F = 2.23, df = 6, $p = 0.097$) and chronic exposure (F = 3.13, df = 5, $p < 0.050$) and the control group.

Abnormalities

Two types of axial abnormalities were detected and classified according to Bantle et al. (1996). One was lateral flexure of the tail, a slight deviation from its normal position, and the other was severe lateral flexure of the tail, which is an almost 90° deviation from the normal position of the tail. Abnormalities were recorded within the concentration range of 0.044–1.774 mg/L in both tests under acute exposure. ANOVA results show significant differences (F = 7.32, df = 10, $p < 0.050$ for T1; F = 6.43, df = 10, $p < 0.050$ for T2) in the presence of abnormalities between treatments and negative controls, allowing us to find the acute LOEC and NOEC values for this end-point (Table 4).

Finally, the two-tailed paired-sample *t* test to determine differences between the effects of CPF on the individuals of the two populations of *R. fernandezae* tadpoles under acute exposure, considering all the end-points evaluated,

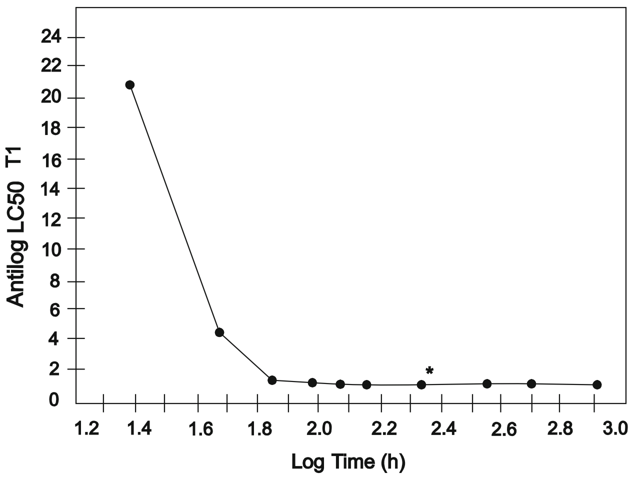

Fig. 1 Antilogarithm (antilog) of CPF LC50 from test 1 (T1) versus log time (duration of chronic test). The incipient LC is estimated as the point at which the curve begins to run parallel to the *x*-axis. *Incipient LC50

showed no significant differences ($t = -1.01$, df $= 6$, $p = 0.3484$).

Discussion

Despite the widespread and extensive use of pesticides, ecotoxicological research examining their effects on amphibians is poorly represented compared to studies on other aquatic organisms (Richards and Kendall 2002; Mann et al. 2009). When comparing the number of contaminant-related scientific publications between 1996 and 2008 involving the vertebrate class ($n = 17{,}375$), only 645 (3.5 %) are about amphibians (Sparling et al. 2010). Ecotoxicological studies on CPF involving anurans are not an exception. The only information that was found was the one compiled by Sparling et al. (2010), and the USEPA (2011), in addition to the mention of Ronco et al. (2008b).

According to PNUMA (1998), CPF is bioaccumulative and toxic, two of the three attributes identifying a substance as hazardous. If we also take into account that CPF is one of the most used insecticides in Argentina (CASAFE 2011), risk to human health and biota from ecosystems in the region are further increased. Moreover, the measured levels of CPF in the environment are within the range of those inducing sublethal effects in the laboratory on *R. fernandezae*. The upper limit of the range of concentration measured in Argentina of CPF in the environment (Jergentz et al. 2005; Marino and Ronco 2005) was 1.2 times greater than the toxicity threshold found in the laboratory that causes sublethal effects on mobility of organisms. However, lethal and sublethal effects in the field could be also due to mixtures of pesticides including CPF, as it was reported by Ronco et al. (2008b) for *Hypsiboas pulchellus* and *R. arenarum* inhabiting environments adjacent to soybean fields in the Pampas region, being this

Table 3 Results of acute sublethal effects to CPF

Time (h)	EC50	95 % CI	*n*
IS			
T1			
24	–	0.062[a]–0.443[a]	3
48	–	0.062[a]–0.443[a]	3
72	–	0.133[a]–0.177[a]	3
96	–	0.062[a]–0.177[a]	2
T2			
24	–	0.089[a]–0.443[a]	3
48	0.301	0.231–0.541	3
72	0.337	0.248–0.647	3
96	–	0.009[a]–0.355[a]	4
IM			
T1			
24	0.124[b]	0.018[a]–0.665[a]	3
48	0.133[b]	0.062[a]–0.443[a]	3
72	0.124[b]	0.009–3.042	3
96	0.168[b]	0.133[a]–0.177[a]	2
T2			
24	0.639[b]	0.009[a]–0.177[a]	3
48	0.195	0.097–0.479	4
72	0.231	0.124–0.541	4
96	0.018[b]	0.009[a]–0.177[a]	3

EC50 values and intervals are given in mg CPF/L

CI 95 % 95 % confidence interval, *n* number of data used in the calculation of EC50 value, *IS* irregular swimming, *IM* immobility, *T1 and T2* tests with organisms from S1 and S2, respectively

[a] The minimum and maximum concentrations in which the effect was observed (rank effect)

[b] Calculated by linear interpolation method when the Probit was not applicable

Table 4 Acute NOEC and LOEC values for behavior, growth inhibition and abnormalities of T1 and T2

Endpoint	T1	T2
LOEC behavior (IM)	0.062	0.089
NOEC behavior (IM)	0.017	0.001
LOEC growth	–	0.177
NOEC growth	–	0.089
LOEC abnormalities	0.020	0.050
NOEC abnormalities	–	0.010

Concentration values are given in mg CPF/L

IM immobility

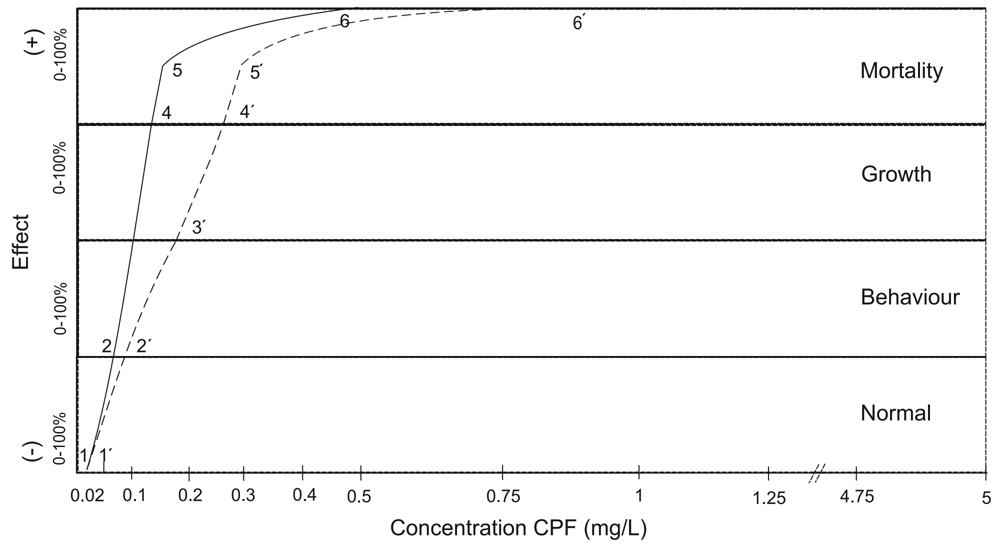

Fig. 2 Category of effects of chlorpyrifos on two populations of *R. fernandezae* tadpoles at different acute exposure concentrations. T1, T2; 1 & 1′: behavior NOEC; 2 & 2′: behavior LOEC; 3′: growth LOEC; 4 & 4′: mortality LOEC; 5 & 5′: mortality 96 h LC50; 6 & 6′: lowest concentration showing 100 % mortality

report the only published data on the effects of the insecticide on anuran native species of Argentina. Greater concern should be also taken into account in the assessment of the risk of using the insecticide, since applications of CPF in the field often coincide with anuran breeding and developmental periods, being eggs and tadpoles the most sensitive stages in the amphibian life cycle (Linder et al. 2003).

In the present study, CPF in the Dow Agroscience commercial formulation Lorsban®48 E, shows high toxicity on *R. fernandezae* tadpoles, from two sites with different degrees of anthropogenic disturbance, inducing lethal and sublethal effects at 96 h exposure within a narrow range of concentrations from 0.066 to 0.887 mg/L with no differences in sensitivity between the two populations. A comparison of the assessed lethal effects (96 h LC50 values in mg/L, T1:0.150, T2: 0.293) with existent information for other species: *Hoplobatrachus tigerinus*, 0.019 (Abbasi and Soni 1991); *Bufo gargarizans*, 0.80 (XiaoHui et al. 2009); *Anaxyrus americanus*, 1 (Cowman and Manzanti 2000); *Lithobates pipiens*, 3 (Gaizick et al. 2001); *X. laevis*, 14.6 (Richards 2000, Richards and Kendall 2002), allowed placing *R. fernandezae* tadpoles below the 30th percentile in the species sensitivity distribution of existing data. This comparison has been assumed valid taking into account that it is known that variations in the sensitivity of a given species to a toxicant could be due to differences in testing methods (Sprague 1995), and that ecotoxicological databases and other ecotoxicological tools (e.g. environmental risk assessment), take those differences as not significant. We also emphasize the need for introducing other variables which are as important like local adaptation and species origin, both of which have not been considered in literature.

Considering the acute effects tested on behavior, we can conclude that CPF induces irregular swimming (IS) and immobility (IM) as reported by other authors for other pesticides (Berrill et al. 1993; Bridges 1997; Ingermann et al. 2002; Rehage et al. 2002; Punzo and Parker 2005; Widder and Bidwell 2008; Brunelli et al. 2009; Agostini et al. 2010; Vera Candioti et al. 2010). Regarding growth, CPF induces a significant growth inhibition in individuals exposed to concentrations above 0.177 mg/L, as reported by other authors for CPF and other organophosphate pesticides (Gurushankara et al. 2007; Widder and Bidwell 2008). Growth inhibition is detected in individuals prior to death, as shown in Fig. 2. Although abnormalities have been registered in the present study, it is not possible to affirm that they were type-specific caused by CPF exposure, since they are similar to some non-specific responses due to different stressors (Bantle et al. 1996; Perkins et al. 2000; Allran and Karasov 2001; Greulich and Pflugmacher 2003; Fort et al. 2004; Sumanadasa et al. 2008; Agostini et al. 2010; Krishnamurthy and Smith 2010, 2011). Figure 2 summarizes the analyzed acute effects and shows the increase in severity of effects, from normal individuals (no effects) to individuals with deficiencies in swimming (behavioral effects), to inhibition of growth, and then to mortality, with increasing concentration of CPF.

Taking into account chronic and acute exposure data we can conclude that CPF exhibits a very rapid effect as seen in Fig. 1. The fig. also shows the incipient value which indicates that far away that value mortalities are not expected (LC50 at 216 h = 0.016 mg/L, 9.5 times lower than LC50 at 96 h). Considering results as a whole, CPF induces severer effects at higher concentrations than at longer times of exposure. This pattern is shown by the decrement in the slopes along the time (Rand 1995). Like other OP insecticides, CPF is a potent toxicant within very short exposure times, reaching faster the incipient value than other toxicants (i.e. chromium (VI), Natale et al. (2006); cypermethrin, Agostini et al. (2010), for anurans).

Considering that the sampled clutches from each site (S1 and S2) were representative of the genetic variability of each population, we can conclude that there are no significant differences in sensitivity between the two populations of *R. fernandezae* tadpoles under acute exposure to the studied CPF formulation. This is despite the different environmental conditions, the pond locations separated from each other by the city of Buenos Aires since almost ten decades, added to exposure to multiple sources of contamination in S2. Taking into account the results as a whole, we reject our original hypothesis and conclude that the two populations of *R. fernandezae* tadpoles do not show differences in their acute responses to CPF.

Williams (1966) and Kawecki and Ebert (2004) proposed the concept of local adaptation that takes into consideration genotypic and phenotypic changes are due to changes in the environments where populations inhabit. Although Ortiz-Santaliestra et al. (2010) and Miaud et al. (2011) incorporated this concept within the field of ecotoxicology, these last authors could not observe population changes in exposed anurans from environments with different type of pollution. The results obtained in the present research are in agreement with the observations from these previous studies (Ortiz-Santaliestra et al. 2010; Miaud et al. 2011). Taking into account the concept of local adaptation, we can consider several interpretations of our results. (1) In the case that the ancestral population of *R. fernandezae* had high genetic variability (a prerequisite in the process of local adaptation), we could not differentiate the populations, (1a) because of an absence of changes in sensitivity to CPF, since it had not acted as a selection factor in S2; (1b) because of the existence of a temporal variation in the selection factor pressure that translates to a lack of specific responses to CPF; (1c) because genotypic variability of both demes has resulted in large variability in responses, due to phenotypic plasticity of the species; or, (1d) regardless of the causes, because the local adaptation processes are occurring at present, differences could not be detected. (2) Assuming the possibility that the ancestral population had low genotypic variability, this process could not take place, since the existence of genetic variability is a prerequisite for the process of local adaptation (Coors et al. 2009; Kawecki and Ebert 2004) and hence demes do not respond differentially to the selection pressure at which they are exposed.

Final remarks

Although local adaptation was not detected, it does not imply that the process of divergent natural selection is not operating, even more if we consider that a major geographical barrier (the city of Buenos Aires) existed for about 80 years. To be able to draw conclusions on the genetic differentiation of populations of *R. fernandezae* and their phenotypic expression in relation to the sensitivity of the species to pollution, other toxicants should be studied to confirm the present results in addition to genetic studies, using quantitative genetics to identify allele variability between populations and their frequency of appearance. This would allow the detection of patterns of interaction between demes and habitats within the context of the existence of gradients of pollution and loss of habitats. Data on environmental chemistry, genetics, and the biology of populations within an ecotoxicological context would be necessary for a holistic interpretation.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The experiments performed in this study complied current ethical standards of Argentina.

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