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Environmental Toxicology and Chemistry, Vol. 31, No. 9, pp. 2052–2058, 2012

Printed in the USA
DOI: 10.1002/etc.1921

DEVELOPMENTAL AND POLYAMINE METABOLISM ALTERATIONS IN *RHINELLA ARENARUM* EMBRYOS EXPOSED TO THE ORGANOPHOSPHATE CHLORPYRIFOS

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(*Submitted* 29 *March* 2012; *Returned for Revision* 23 *April* 2012; *Accepted* 9 *May* 2012)

Abstract—Organophosphorus pesticides (OPs) are widely applied in the Alto Valle of Río Negro and Neuquén, Argentina, due to intensive fruit growing. Amphibians are particularly sensitive to environmental pollution, and OPs may transiently accumulate in ponds and channels of the region during their reproductive season. Organophosphorus pesticide exposure may alter amphibian embryonic development and the reproductive success of autochthonous species. In the present study, embryos of the common toad *Rhinella arenarum* were employed to assess developmental alterations and to study polyamine metabolism, which is essential to normal growth, as a possible target underlying the effects of the OP chlorpyrifos. As the duration of chlorpyrifos exposure increased and embryonic development progressed, the median lethal concentration (LC50) values decreased, and the percentage of malformed embryos increased. Developmental arrest was also observed and several morphological alterations were recorded, such as incomplete and abnormal closure of the neural tube, dorsal curvature of the caudal fin, reduction of body size and caudal fin length, atrophy, and edema. An early decrease in ornithine decarboxylase (ODC) activity and polyamine levels was also observed in embryos exposed to chlorpyrifos. The decrease in polyamine contents in tail bud embryos might be a consequence of the reduction in ODC activity. The alteration of polyamine metabolism occurred before embryonic growth was interrupted and embryonic malformations were observed and may be useful as a biomarker in environmental studies. Environ. Toxicol. Chem. 2012;31:2052–2058. © 2012 SETAC

Keywords—Amphibian Pesticides Biomarkers Polyamines Ornithine decarboxylase

INTRODUCTION

Organophosphorus pesticides (OPs) have been shown to produce severe effects in diverse organisms, mainly through the inhibition of acetylcholinesterase (AChE), which is considered the most sensitive biomarker of OP and carbamate insecticides exposure [1]. Chlorpyrifos is one of the most widely used OPs, applied in agriculture, veterinary practices, and domestic residences [2]. The inhibition of AChE is not the only way chlorpyrifos elicits its toxicity. In fact, its effects can be seen before the cholinergic system is innervated, suggesting multiple mechanisms of toxicity, including developmental processes [3].

Concern has been expressed in the Alto Valle of Río Negro and Neuquén about undesirable pesticide effects in biota as a result of their intense use in fruit production. Organophosphorus pesticides have been detected in superficial and groundwater in the valley of Río Negro and Neuquén, Patagonia, Argentina [4], an area where more than 35,000 hectares are subjected to intensive agriculture. Organophosphorus pesticide and carbamate concentrations of 1 to 100 μg/L have been detected in superficial water of this region during the growing season, exceeding the criteria for protection of aquatic life. Moreover, higher concentrations of these pesticides may be transiently present in ponds and irrigation channels of orchards because of aerial drift and runoff, considering that insecticide applications can reach a biweekly frequency in concentrations of approximately 100 g/L per hectare [5]. The exceedence of environmental concentrations of OPs in superficial water in this region might range from one to two orders of magnitude, as suggested by biomarker studies performed with amphibian larvae exposed in situ in irrigation channels of the region [6]; although the maximum concentration of the OP azinphos methyl detected in water was 22.5 μg/L, larvae showed altered biomarker responses compatible with 2 mg/L, as suggested by laboratory studies.

Amphibians are good bioindicators of aquatic pollution, and their embryos are easy to manipulate in the laboratory. They are particularly sensitive to numerous environmental contaminants during their embryonic stages because of their position in the food chain, the permeability of their skin to toxic substances, and the vulnerability that metamorphosis causes in larval development [7]. Different xenobiotics affect the normal development of toads during their aquatic life stages and impact adult specimens mostly at the reproductive level [8]. *Rhinella arenarum*, the common South American toad, is widely distributed in Argentina, Brazil, Chile, and Uruguay and may be threatened by exposure to agrochemicals. The effects and mechanisms of action of several OPs and chlorinated pesticides during the embryonic and larval ontogenesis of *R. arenarum* have been reported, evaluating acute and chronic effects as well as primary and secondary molecular targets [9,10].

Polyamines are ubiquitous polycations essential for cellular division, growth, and differentiation [11–13]. The polyamines putrescine, spermidine, and spermine are widespread among living organisms, and they reach high concentrations in actively

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Published online 19 June 2012 in Wiley Online Library (wileyonlinelibrary.com).

proliferating cells, being involved in a variety of fundamental cellular processes, including transcription, RNA modification, protein synthesis, and the modulation of enzyme activities. Low intracellular levels of polyamines result in the inhibition of cell proliferation and differentiation, and sometimes in cell death [14]. Ornithine decarboxylase (ODC) is the initial and rate-limiting enzyme in the polyamine biosynthetic pathway [15] and may be a target of xenobiotic exposure. Thus, polyamines are key molecules involved in the control of cellular life and death. Polyamines have been proposed as biomarkers of effect in pesticide toxicity [10]. The importance of normal polyamine levels has been assessed during *R. arenarum* development, showing a link between the exposure to the OP malathion and azinphos methyl, oxidative stress, and polyamine metabolism [16].

In the present study, we evaluated the toxicity and teratogenesis caused by continuous chlorpyrifos exposure in *R. arenarum* embryos at the tail bud, heart beat, and complete operculum stages. We further analyzed the effects of chlorpyrifos concentrations well below the median lethal concentration (LC50) value on polyamine levels and ODC activity in embryos at the early tail bud stage, to determine whether polyamine metabolism might precede later developmental effects.

MATERIALS AND METHODS

Chemicals

The insecticide chlorpyrifos (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl phosphorothioate]) (99% purity) was purchased from Chem Service. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, pyridoxal 5-phosphate monohydrate, L-ornithine monohydrochloride, and bovine serum albumin were purchased from Sigma Co. The L-[$^{14}C_1$]-ornithine was purchased from New England Nuclear. Scintillation liquid Optiphase "Hisafe" 3 was purchased from Perkin–Elmer. The Folin-Ciocalteau reagent was purchased from Anedra. All of the other reagents used were of analytical grade.

Fertilization and test conditions

Toad (*R. arenarum*) embryos were obtained from a local supplier and kept in captivity outdoors in a small terrarium containing grass. Female ovulations were induced by an intraperitoneal injection of 2,500 units human chorionic gonadotropin (ELEA laboratory). The oocytes were extracted from the sacs and fertilized with a testicular homogenate in Amphibian Ringer's solution (NaCl 0.65 g/L, KCl 0.01 g/L, $CaCl_2$ 0.02 g/L). The solutions were renewed every 48 h (semi-static conditions), and the embryos were maintained at 20–22°C in a 12-h light, 12-h dark photoperiod without feeding. The conditions were carefully controlled, because chlorpyrifos half-life is 35 d at 25°C and pH 7.0, and the rate of hydrolysis increases with both pH and temperature (http://www.apvma.gov.au/products/review/current/chlorpyrifos_chemistry.php#3_3). Volatilization of chlorpyrifos at 20°C has been found to be negligible (http://www.who.int/whopes/quality/Chlorpyrifos_WHO_specs_eval_Mar_2009.pdf). Toxic effects on development were evaluated at the tail bud (48 h of development), heart beat (120 h), and complete operculum (144 h) stages.

Chlorpyrifos exposure

To determine the LC50 and to evaluate developmental alterations at different embryonic stages, groups of 50 recently fertilized embryos (within 2 h from fertilization, two-cell or four-cell stages) were continuously exposed to 0 to 32 mg/L chlorpyrifos (tail bud stage) or 0 to 16 mg/L chlorpyrifos (heart beat and complete operculum stages) in 50 ml media. Concentrations tested were increased by a 2× factor, but intermediate concentrations were also included when necessary to determine more precisely the LC50 values for each stage assessed. A standard solution of chlorpyrifos was prepared by dissolving the pure chemical in acetone, and the exact concentration of the insecticide in the standard solutions was checked by gas chromatography with a nitrogen-phosphorus detector. The test solutions were prepared by diluting the appropriate volume of standard chlorpyrifos into the required amount of Amphibian Ringer's solution. Acetone in 0.3% final (v/v) concentration was used as a vehicle, because chlorpyrifos is highly soluble in this solvent but not in water (650 g/100 g acetone vs 2 mg/L at 25°C) [17]. For control groups run in parallel, Amphibian Ringer's solution plus 0.3% acetone was used. All assays were performed in triplicate.

Assessment of malformations and embryo death

We used a stereomicroscope (Wild M3, Heerbrugg) to follow the development and assess different kinds of malformations. Embryonic stages were established according to Del Conte and Sirlin [18]. Photographs were taken using a Sony digital camera. Data regarding number and type of malformations and number of dead embryos were collected. The malformations were typified according to the *Atlas of Abnormalities* for amphibians [19]. We could also observe and register some alterations in the embryo movements, particularly the swimming activity and body contractions. Death was determined at the tail bud stage as the number of arrested embryos (those not developing to the next stages). At the heart beat and complete operculum stages, the endpoints to establish embryo death were the absence of heart beat and the absence of blood circulation in gills and caudal fin, observed under stereoscopic microscope at 40 ×.

Polyamines and ODC determinations

For polyamine and ODC studies, groups of 50 recently fertilized embryos (two-cell or four-cell stage) were continuously exposed to 50 ml media containing 0 to 16 mg/L chlorpyrifos until the tail bud stage. For polyamine determinations, embryos were gently washed with cold Amphibian Ringer's solution, cooled briefly on ice, and homogenized in 2 ml 143 mM potassium phosphate buffer (pH 7.5) containing 6.3 mM ethylenediaminetetraacetic acid and 0.2 N $HClO_4$. The homogenates were kept in ice for 1 h and centrifuged at 10,000 *g* for 20 min at 4°C; the resulting supernatants were divided into aliquots and kept frozen until performing the analyses. The supernatants were derivatized with 20 mg/ml dansyl chloride, and samples were analyzed for putrescine, spermidine, and spermine content by reverse-phase high-pressure liquid chromatography (HPLC) separation and fluorometric quantitation [20], using a Bondapak C18 column (particle size 10 mm, Waters Corporation). The column was equilibrated with 40% acetonitrile in water before the sample was injected. Polyamines were eluted in a 30-min linear gradient from 40% acetonitrile in water to 100% acetonitrile. The flow rate was 1 ml/min. A Spectra-Physics liquid chromatograph (Spectra-Physics Analytical) with a SpectraSERIES P200 gradient pump and a SpectraSYSTEM FL2000 fluorescence detector (excitation λ 342 nm, emission λ 512 nm) was used. Putrescine, spermidine, and spermine standards were analyzed in parallel, and 1,7-diamine heptane was used as an internal standard for

both samples and calibration standards. Duplicate samples were assayed in two independent experiments.

For ODC determination, control and exposed embryos were homogenized in 1 ml homogenization buffer (H) (N-[2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)], 10 mM, $MgCl_2$ 1.5 mM, KCl 10 mM) (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid, 0.04% Triton X-100, 1 mM dithiothreitol, 0.5 mM pyridoxal-5'-phosphate, and protease inhibitors (0.05 mM phenylmethanesulfonyl fluoride, and 0.001 μg/μl leupeptin, aprotinin, and NaF), and centrifuged at 20,000 *g* for 40 min. Supernatant was collected to perform the assay. The ODC activity was immediately assayed by measuring the release of $^{14}CO_2$ from L-[^{14}C]-ornithine according to Sánchez et al. [21], with slight modifications. The standard reaction mixture consisted of buffer H plus L-ornithine and L-[^{14}C]-ornithine (1 mM, 0.2 μCi) in a final volume of 50 μl. Enzyme reaction was initiated by the addition of 15 μl sample supernatants. Blank controls were run in parallel, using difluoromethylornithine–inactivated samples. A hermetic device was employed to avoid $^{14}CO_2$ loss. The reaction was performed for 1 h at 30°C in agitation, and $^{14}CO_2$ was trapped on a 2 × 2-cm piece of filter paper soaked with 2 N KOH. The reaction was stopped by the addition of 50 μl 0.25 N $HClO_4$ and maintained in the same conditions for 1 h. Filter papers were then transferred to scintillation vials, and 0.5 ml 1% Triton X-100 was added along with 5 ml scintillation liquid Optiphase "Hisafe" 3. Radioactive CO_2 was measured in a liquid scintillation counter (Wallac Winspectral 1414).

Protein determination

Protein content was determined according to Lowry et al. [22], using Folin-Ciocalteau reagent, and bovine serum albumin as standard.

Data analysis

Probit analysis was used to calculate LC50 and LC90 using the PriProbit 1.63 program designed by Masayuki Sakuma (Kyoto University, Kyoto, Japan, http://bru.gmprc.ksu.edu/proj/priprobit/index.asp). The observed mortality of control animals was less than 10%, making Abbot's correction unnecessary. No-observed-effect concentration (NOEC) for lethality was determined as the maximum concentration at which mortality was not significantly different from control values, tested by analysis of variance (ANOVA)–Dunnett's test.

Mean ± standard error of the mean of enzyme activity and polyamine levels were calculated from two independent experiments with duplicate samples, using the average measurements. Statistical differences between treatments were assessed by ANOVA and Fisher's lowest significant differences post hoc test for polyamines and ODC data. Percentage of malformation data were transformed by the arcsin of the square root of the probability value previous to the analysis. Statistical differences between stages were assessed by ANOVA and Fisher's lowest significant differences post hoc test. The median inhibitory concentration (IC50) for chlorpyrifos on ODC activity was calculated by nonlinear regression.

RESULTS

Toxicological effects

We analyzed the effects of continuous exposure to chlorpyrifos in developing toad embryos. Results obtained from the probit analysis are shown in Table 1. The LC50 value for the tail bud stage (23.3 mg/L) was significantly different from those in the heart beat stage (14.3 mg/L) and the complete operculum stage (13.5 mg/L) ($p = 0.05$). The slope of probit fitting diminished as the embryos developed, being significantly higher at the tail bud stage with respect to the slope obtained for the complete operculum stage ($p = 0.012$). The LC90 values were 33.4 mg/L for embryos at the tail bud stage, 23 mg/L for heart beat, and 23.5 mg/L for complete operculum, but no significant differences were observed among them. The NOEC value for embryos at the tail bud stage was 8 mg/L of chlorpyrifos ($p > 0.05$ vs controls).

Table 1. Toxicological results obtained from the probit analysis in the tail bud, heartbeat, and complete operculum stages of *Rhinella arenarum* embryos[a]

	Stage of development		
	Tail bud	Heart beat	Complete operculum
Exposure time (h)	48	120	144
LC50 (mg/L)	23.3A	14.3B	13.5B
CI 95% (mg/L)	(21.0,25,7)	(13.3,16,2)	(11.0,15.4)
LC90 (mg/L)	33.4A	23.0A	23.5A
CI 95% (mg/L)	(29.5,43.0)	(19.1,37.0)	(19.5,40.0)
$\chi 2$	12.9	1.9	3.2
Slope	8.2 ± 1.7A	6.2 ± 1.7A,B	5.3 ± 1.4B

[a] Recently fertilized embryos exposed for 48, 120, and 144 h up to 32 mg/L of chlorpyrifos. The LC50 (50% lethal concentration) and LC90 (90% lethal concentration) were calculated for the different stages by probit analysis. Different letters mean significant differences ($p \leq 0.05$).

Morphological malformations

Chlorpyrifos induced morphological malformations that became more frequent as its concentration was increased and the embryonic development progressed (Fig. 1). Embryos at the tail bud stage did not show a significant increase in the number of malformations when exposed up to 14 mg/L chlorpyrifos. At the heart beat stage, no significant differences in the percentage of malformed individuals were observed in embryos exposed to 8, 10, or 12 mg/L chlorpyrifos, when compared with control

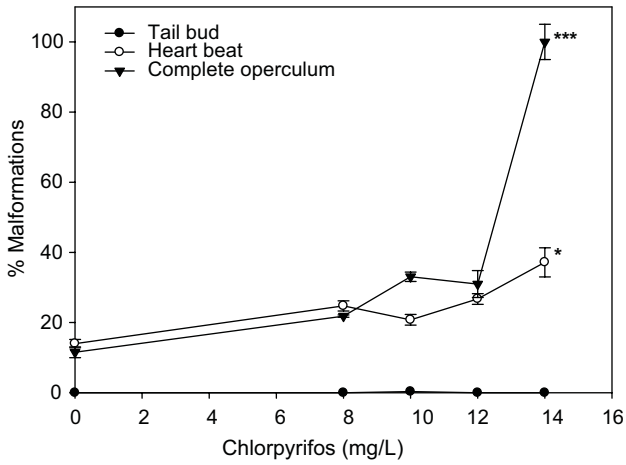

Fig. 1. Stage- and concentration-dependence of the frequency of malformations elicited by chlorpyrifos in *Rhinella arenarum* embryos. The percentages of malformations (%) (mean ± SE) versus increased concentration of chlorpyrifos (mg/L) are shown at different embryonic stages. Significant differences versus controls, at $^{*}p = 0.05$ and $^{***}p = 0.001$, determined by analysis of variance (ANOVA)–Fisher's lowest significant differences tests on arcsin(SQR[p]) transformed data.

values. However, a significant increase in the percentage of malformed individuals was observed in embryos exposed to 14 mg/L chlorpyrifos when compared with control embryos, reaching 30%. When embryos reached the stage of complete operculum, the frequency of morphological alterations abruptly increased to 100% in the individuals exposed to 14 mg/L chlorpyrifos.

We determined which morphological alterations were more frequent in chlorpyrifos-treated embryos. In early stages, chlorpyrifos mostly caused dorsal curvature, desquamated cellular debris, accumulation of the cells inside the fertilization membrane, and alteration in the consistency of the jelly coat. The axial shortening (reduced growth), axial curvature, and caudal fin curvature were notable at the heart beat stage (Fig. 2A, B). Caudal fin curvature, reduced body and head size, and disparity in growth were the alterations most commonly found among those induced by chlorpyrifos in embryos at the complete operculum stage (Fig. 2C, D). Embryonic exposure to concentrations above 14 to 16 mg/L provoked head malformation, abdominal hydropsy, and bubbling in the body and caudal fin. All of the embryos at the heart beat–complete operculum stages presented some of the above-mentioned malformations when exposed to 16 mg/L chlorpyrifos. We also noticed that embryos at the heart beat and complete operculum stages exposed to 2 mg/L chlorpyrifos already showed reduced swimming activity and a decrease in swimming movements, as well as sustained muscular contractions.

Polyamine levels and ODC specific activity

We assessed polyamine levels at the tail bud stage after exposure to different concentrations of chlorpyrifos. In control animals, putrescine levels were fourfold higher than spermidine levels (12.75 nmol/mg protein vs 3.14 nmol/mg protein, respectively) (Fig. 3). Spermine levels were under the limit of quantitation and were hardly detected. Embryos exposed to 8 mg/L chlorpyrifos showed diminished putrescine levels (33% of decrease, $p = 0.05$) relative to control embryos; however, 16 mg/L did not provoke further decrease. Spermidine levels were affected in a similar way and showed a 42% reduction in embryos exposed to 8 mg/L chlorpyrifos. The exposure of tail bud embryos to chlorpyrifos caused a significant decrease of ODC specific activity in a concentration-dependent manner (Fig. 3). The inhibition of ODC activity ranged from 17% in embryos exposed to 2 mg/L chlorpyrifos to 79% in embryos exposed to 8 mg/L chlorpyrifos. No further decrease in ODC activity was observed when embryos were exposed to higher chlorpyrifos concentrations. The IC50 value for chlorpyrifos on ODC activity, determined by nonlinear regression, was 4.6 ± 0.8 mg/L chlorpyrifos.

We analyzed the correlation between ODC specific activity and the percentage of arrested embryos in the tail bud stage (Fig. 4). The regression line showed a highly significant negative correlation, with an r value of -0.837 ($p = 0.0001$). As ODC activity diminished, the percentage of embryos arrested in the tail bud stage increased, reaching a value of 18%. Because of the downward trend observed in the levels of polyamines and ODC activity with increasing chlorpyrifos concentrations, we performed a correlation analysis between these parameters (Fig. 5). A strong correlation was found between ODC and putrescine ($r = 0.905$; $p = 0.034$) and spermidine levels ($r = 0.986$; $p = 0.002$). The difference in the slopes between both lines implies that putrescine levels (slope = 26.229) were most affected by changes in ODC activity than the levels of spermidine (slope = 7.048).

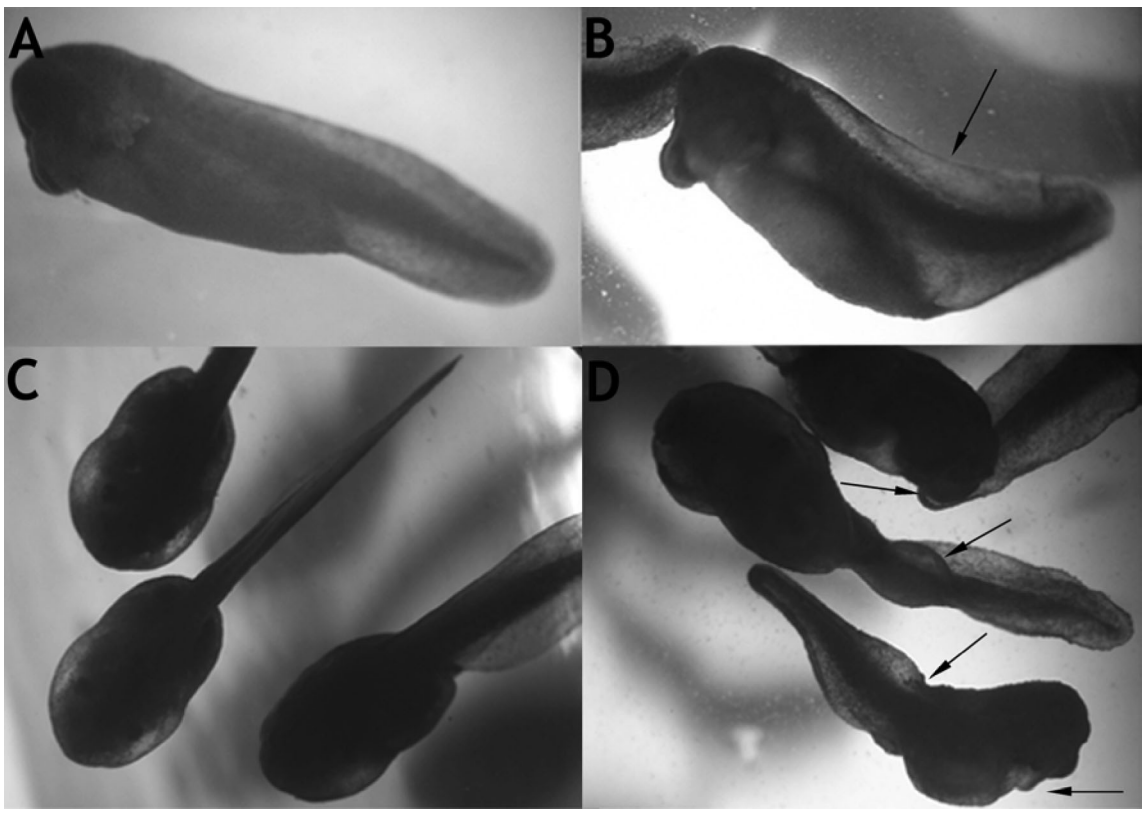

Fig. 2. Morphological alterations in *Rhinella arenarum* embryos exposed to chlorpyrifos. (**A**) Control embryos at the heart beat stage; (**B**) axial and caudal fin curvature (arrow) in embryos at the heart beat stage exposed to 8 mg/L chlorpyrifos; (**C**) control embryos at the complete operculum stage; and (**D**) abdominal hydropsy and bubbling in the body and caudal fin curvature (arrows) in embryos at the complete operculum stage exposed to 8 mg/L chlorpyrifos.

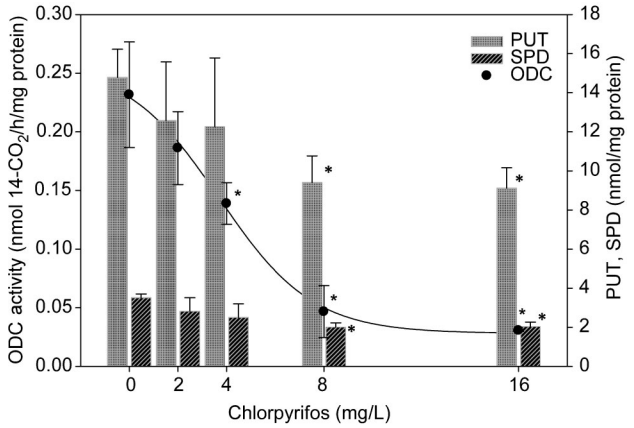

Fig. 3. Effect of chlorpyrifos on polyamine levels and ornithine decarboxylase (ODC) activity in embryos at the tail bud stage. Data from two independent experiments with duplicate samples and duplicate measurements (mean ± SE). The line shows sigmoidal model fitting to data by nonlinear regression; control ODC activity = 0.230 ± 0.021 (nmol CO_2/h/mg protein), and median inhibitory concentration (IC50) = 4.6 ± 0.8 mg/L. *Significant differences versus controls at $p = 0.05$, determined by analysis of variance (ANOVA)-Fisher's lowest significant differences test. PUT = putrescine; SPD = spermidine.

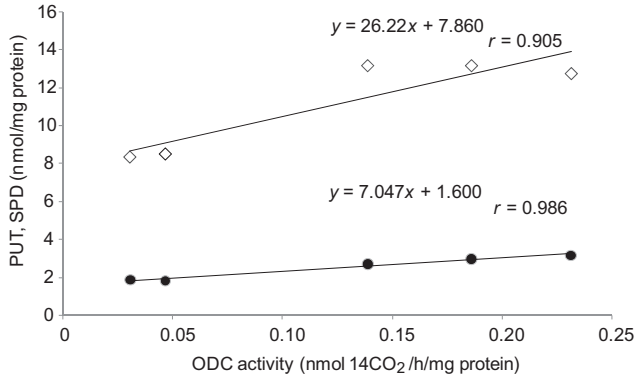

Fig. 5. Correlation analysis between ornithine decarboxylase (ODC) activity and polyamine levels. Putrescine (PUT, ◇) and spermidine (SPD, ●) levels in *Rhinella arenarum* embryos at the tail bud stage exposed to 0, 2, 4, 8, and 16 mg/L of chlorpyrifos were correlated to ODC specific activity. The linear correlation coefficients were $r = 0.905$, $p = 0.034$ for putrescine, and $r = 0.986$, $p = 0.002$ for spermidine.

DISCUSSION

The pesticide chlorpyrifos caused developmental arrest in a stage- and dose-dependent manner in *R. arenarum* embryos. As embryos reached the later embryonic stages, the LC50 value was reduced by approximately 50% (Table 1). Embryos in the tail bud stage are still enclosed in the jelly coat that protects them against contaminants [23,24]. Conversely, at the early stages of development, *R. arenarum* embryos have plenty of yolk platelets. The lipid storages present in the yolk platelets could act as scavengers of OP and thus decrease the bioavailability of these and other lipophilic toxicants at their targets of action [25]. As the embryonic development of *R. arenarum* progresses, the lipid storages in their yolk platelets are continuously mobilized to provide the energy and nutrients that embryos need to hatch from the fertilization membrane and jelly coat and to begin the exchange of dissolved matter through their gills. Finally, although the exposure period to chlorpyrifos increased from the heart beat to complete operculum stages (5 and 7 d of exposure, respectively), the LC50 value was similar in both stages. This suggests that the differential toxicity of chlorpyrifos between early and late embryonic stages of *R. arenarum* is attributable to metabolic and physiological reasons, rather than to a simple increase in the exposure period. At late embryonic stages, the toxic response to chlorpyrifos becomes less homogeneous as suggested by the decrease in the concentration–mortality slopes obtained by probit regression (Table 1). A higher tolerance of early amphibian embryos to OP insecticides has also been observed in *Xenopus laevis* by Bonfanti et al. [26]. Similarly, early *R. arenarum* embryos display a higher tolerance when exposed to OPs other than chlorpyrifos [10]. We also show in the present study that embryos exposed to chlorpyrifos displayed an increase in the percentage of malformations in a time/stage- and dose-dependent manner (Fig. 1). *Xenopus laevis* larvae exposed to chlorpyrifos and malathion displayed an abnormal tail flexure, which impaired their motility [27–29]. *Xenopus laevis* larvae exposed to 3 mg/L chlorpyrifos displayed a reduction in myotome size and disorganized myocytes [26]. In the present study, we also documented the dorsal curvature of the caudal fin as one of the most common malformations observed at the three embryonic stages studied. In a preliminary histological screening in embryos at the tail bud stage, we have also found that myotomes are smaller in embryos exposed to 2 mg/L chlorpyrifos. Embryo motility was also compromised, which has been attributed to the primary action of OPs on AChE inhibition, leading to muscular damage [30]. The altered swimming behavior in individuals exposed to high concentrations of chlorpyrifos correlates with the response observed in *Hyla chrysoscelis* tadpoles exposed to 100 μg/L chlorpyrifos [30]. The abnormal tail flexure observed in *R. arenarum* embryos exposed to chlorpyrifos could be a result of AChE inhibition, leading to axial tail curvature caused by hydrolysis impairment of the neurotransmitter acetylcholine and sustained stimuli before receptor desensitization. However, we have observed AChE inhibition at the tail bud stage only when embryos were exposed to chlorpyrifos concentrations above 8 mg/L [31].

We further characterized the response of the polyamine pathway to seek any alteration that might precede the effects

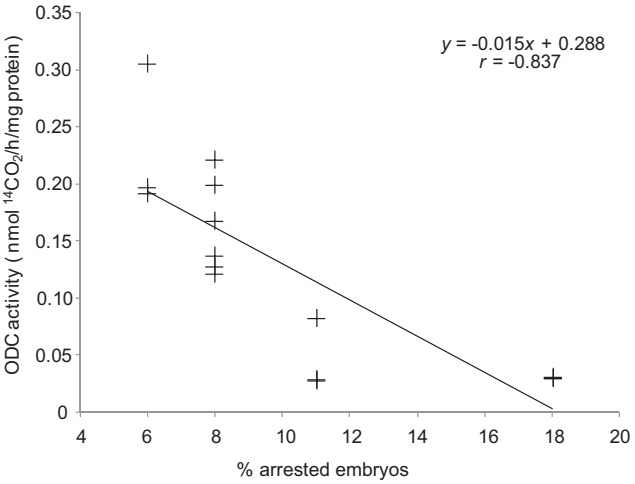

Fig. 4. Correlation analysis of ornithine decarboxylase (ODC) activity and frequency of arrested embryos. Ornithine decarboxylase specific activity expressed as nanomoles of $^{14}CO_2$ released per hour per milligram of protein versus percentage (%) of *Rhinella arenarum* arrested embryos in the tail bud stage exposed to 0, 2, 4, 8, and 16 mg/L chlorpyrifos. The linear correlation coefficient r was -0.837 ($p = 0.0001$).

observed later during *R. arenarum* embryonic development. Polyamines have been proposed as biomarkers of effect in abnormal development triggered by the exposure to OP pesticides [10]. We found that the levels of putrescine and spermidine are diminished approximately 33 and 42%, respectively, in embryos exposed to 8 mg/L chlorpyrifos in comparison with control embryos (Fig. 3). Because ODC is involved in the synthesis of putrescine, it might be possible that putrescine and spermidine levels are diminished because ODC activity is also impaired by chlorpyrifos. Ornithine decarboxylase activity was significantly diminished (40%) because of exposure to 4 mg/L chlorpyrifos. The early response obtained with chlorpyrifos at the tail bud stage during *R. arenarum* development, and at concentrations below those causing any other observable effects, seems to be specific for this OP; other OP insecticides such as malathion and azinphos methyl do not affect polyamine levels and ODC activity at the tail bud stage even at lethal OP concentrations [16]. Chlorpyrifos probably triggers specific mechanisms because of its particular chemical structure. Some of these differential responses, which are not linked to the generic OP structure, are related to cell growth and differentiation, central nervous system cell damage, and disruption of brain development [32–35]. A decrease of polyamine levels may result in the inhibition of cell proliferation and differentiation and cell death [36]. We showed here that ODC activity is directly related to putrescine and spermidine levels and that it is also inversely correlated with the percentage of arrested embryos (Figs. 4 and 5). This supports the hypothesis that polyamine levels are affected by chlorpyrifos because of diminished activity of ODC, thus leading to cellular arrest that will eventually impact on the developmental process of the embryos [14].

From the comparison between the IC50 value for chlorpyrifos on ODC activity (4.6 mg/L), the LC50 (23.3 mg/L), and the estimated NOEC (8 mg/L) at the tail bud stage, we conclude that ODC is a good early biomarker. ODC activity is diminished in exposed embryos at concentrations in which developmental effects are not yet observed (1/12 LC50), and even more, acetylcholine esterase activity is not inhibited [31]. Ornithine-decarboxylase-specific activity is highly valuable for assessing short-term effects in early stages of development in *R. arenarum* embryos.

The relatively high concentrations of chlorpyrifos and other OPs needed to trigger developmental alterations or other toxic manifestations in amphibian embryos and larvae may cast doubt about the real environmental impact of those pesticides. Nevertheless, pesticide exposure is recognized as one of the causes of amphibian population declines [37]. The reported maximum environmental concentrations of OP in water currently range in the micrograms per liter order, as it has been recently published for chlorpyrifos (1.16 μg/L) and azinphos methyl (22.5 μg/L) for superficial and ground water in the Alto Valle de Río Negro y Neuquén [4]. In many cases, these reports do not apply to the real habitat of amphibian aquatic stages, which occur in small ponds and slow-flowing channels and streams. The ponds and channels located in productive orchards are heavily impacted by the 500 tons of formulated pesticides that are applied annually in the region [5]. This is also suggested by biomarker responses in environmental studies on *R. arenarum* larvae exposed in irrigation channels [6]. Then, polyamine metabolism and in particular ODC activity becomes an attractive biomarker for OP exposure, not related to the common cholinergic pathway but giving complementary information to be used in environmental evaluations of pesticide impact.

Acknowledgement—The present study was supported by grants from the Universidad Nacional del Comahue [PI 04-I004], and Fondo Nacional de Ciencia y Técnica PICT Red [2007–214], Argentina.

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