ECOTOX BRASIL
SOCIEDADE BRASILEIRA DE ECOTOXICOLOGIA

Ecotoxicol. Environ. Contam., v. 16, n. 1, 2021, 13-18
doi: 10.5132/eec.2021.01.02

EEC

Swimming ability in tadpoles of *Physalaemus* cf. *cuvieri, Scinax x-signatus and Leptodactylus latrans* (amphibia: anura) exposed to the insecticide chlorpyrifos

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Received June 22, 2021; Accept August 04, 2021

Abstract

We examined the swimming abilities of tadpoles exposed to the organophosphate chlorpyrifos for 24 h (at concentrations of 0, 1, 5 and 10 µg L^{-1}). *Scinax x - signatus, Physalaemus cf cuvieri*, and *Leptodactylus latrans* tadpoles were used as biological models. Our findings evidenced decreased swimming speeds in all tadpole species exposed to chlorpyrifos as compared to the control group, although with significant statistical differences only with *P. cuvieri* and *L. latrans*. Changes in swimming ability may indirectly lead to death or alter important growth and development parameters, as the reduced ability to swim can result in increased vulnerability to predators and impacts on feeding, thus altering their fitness.

Keywords: Amphibians, Chlorpyrifos, Organophosphorus insecticide, Swimming speed, Larvae.

INTRODUCTION

There is no longer any doubt concerning global declines of amphibian populations (Pounds *et al*., 2006; Lajmanovich *et al*., 2012; Daam *et al*., 2019). The causes of those declines are commonly attributed to environmental impacts such as: climate change, habitat destruction and fragmentation, emerging diseases, industrial pollution, and pesticides (Kiesecker *et al*. 2001; Lips *et al*., 2005; Pounds *et al*., 2006; Mann *et al.,* 2009; Lajmanovich *et al*., 2012; Moreira *et al*. 2019).

Most amphibians develop in aquatic environments, which are often contaminated by agricultural pesticides from sprayed fields or derived from leaching, surface runoff, or atmospheric deposition.

Among those agricultural pesticides is chlorpyrifos, a pesticide belonging to the organophosphate family (Pena *et al.*, 2008) that is widely applied to agricultural crops to control various insect pests, although many non-target populations may become exposed to those dangerous contaminants (Boone & Bridges, 2003; Yin *et al*. 2009; Lajmanovich *et al*., 2015).

Organophosphates are inhibitors of cholinesterase enzyme synthesis, which triggers adverse effects on motor activity through the accumulation of the neurotransmitter acetylcholine (ACH). Those accumulations can initially cause hyperactivity, but then uncontrollable muscle spasms or even paralysis, resulting in the death of the animals. (Colombo & Bonfanti, 2005; Rutkoski *et al*., 2020; Silva *et al*., 2020a).

Amphibians are known to be very vulnerable to pesticides that act by inhibiting cholinesterase synthesis (Bridges, 1997; Boone & Bridges, 2003; Boone & Bridges, 2006; Krishnamurthy & Smith, 2010, Salgado Costa *et al*., 2018; Rutkoski *et al*., 2020, Silva *et al*., 2020a, b, c), and may be exposed to various toxic compounds in different habitats during their lifetimes (Boone & Bridges, 2003).

Behavioral studies make it possible to examine the responses of organisms to contamination by a wide variety of chemical compounds (Plaut, 2001), and tests exposing tadpoles to compounds that depress cholinesterase enzyme activity have evidenced significant reductions in their motor activities (Bridges, 1997; Paden *et al*, 2011; Bernabó *et al.*,

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2011; Arcaute *et al.*, 2012; Rutkoski *et al* 2020; Silva *et al*., 2020a) as well as effects on larval muscular integrity (Colombo & Bonfanti, 2005) even at low concentrations, as those compounds act directly on neural transmission (Widder & Bidwell, 2008). According to Silva *et al*. (2020c), the toxic effect of chlorpyrifos on amphibians can be considered species specific.

The study of behavioral endpoints can link varied physiological disorders with ecological processes (Tu *et al*. 2010). Late biomarkers are commonly used in ecotoxicological studies to examine: the effects of different pollutants concentrations on animal behavior, mortality, reproduction, and their tissues, as well as correlating the degree of exposure to their responses, thus making it possible to identify environmental problems (Venturino *et al*., 2003).

It is important to mention that swimming performance can also influence an animal's ability to obtain food resources, thus interfering with their fitness and making them less apt to successfully reproduce and more susceptible to predation (Relyea & Hoverman, 2006).

From that perspective, we sought to verify the effects of sublethal exposures to chlorpyrifos on the swimming speeds of *Scinax x – signatus* Spix, 1824, *Physalaemus* cf. *cuvieri* Fitzinger, 1826, and *Leptodactylus latrans* Steffen, 1815 (Amphibia: Anura) tadpoles. The areas where those species occur are subject to intense agricultural activity, resulting in constant exposure to that product – although the effects of organophosphates on northern South America species remain largely unknown.

MATERIALS AND METHODS

Test organisms

S. x-signatus (developmental phases 39-41), *P. cuvieri* (developmental phases 27-39), and *L. latrans* (developmental phases 27-40) tadpoles were captured in different localities in the municipality of Mucugê, and agro-industrial pole in Bahia State, Brazil, and transported to the laboratory in containers with dechlorinated water.

S. x-signatus is distributed throughout tropical savannas in South America, inhabiting forests edges and open areas, and always close to permanent or temporary bodies of water (Andrade *et al*., 2010).

L. latrans is commonly found in urban areas and has shown high resistance to anthropic environmental impacts. It is widely distributed in tropical regions, and can be found in reservoirs, ponds, or other flooded environments (Ferreira & Tonini, 2010).

P. cuvieri is widely distributed throughout South America in open environments and can be found along the sides the edge of permanent ponds (or occasionally vernal pools) (Lingnau, 2009).

Before initiating the experiments, the tadpoles were acclimated in aquariums (35 L) with dechlorinated water, following Silva *et al*. (2020a, b), and fed with flake feed for ornamental fish (Alcon Basic ®).

Experimental design

Three treatments were performed with different nominal concentrations of the insecticide chlorpyrifos (Klorpan® 0,0-diethyl -0- (3,5,6- trichloro - pyridyl)- phosphorothioate): 1 µg (C1), 5 µg (C5), and 10 µg (C10) of chlorpyrifos/L of water (µg L^{-1}), using dechlorinated water as the negative control (CN). *L. latrans* was only exposed to C10 due to the limited number of individuals of that species available.

The insecticide concentrations used were defined based on records from aquatic environments in Brazil, especially localities with intense agricultural activities (Da Silva, 2006). We chose to evaluate concentrations below 10 µg L^{-1} in light of the results of Rutkoski *et al* (2020) who exposed *Physalaemus gracilis* tadpoles to chlorpyrifos concentrations of 0, 11, 30 and 90 µg L^{-1}. The chlorpyrifos solutions were prepared immediately before each experiment.

Forty tadpoles of *S. x-signatus* and *P. cuvieri,* and 20 *L. latrans* tadpoles were exposed to each of the pesticide concentrations as well as the water control. All exposures occurred during a 24-hour period when the tadpoles were placed individually in 800 mL polypropylene bottles containing the different chlorpyrifos concentrations. The water was well-oxygenated (dissolved oxygen concentration (DO=8.2 mg L^{-1} of water), with a neutral pH (pH 7.0-7.6).

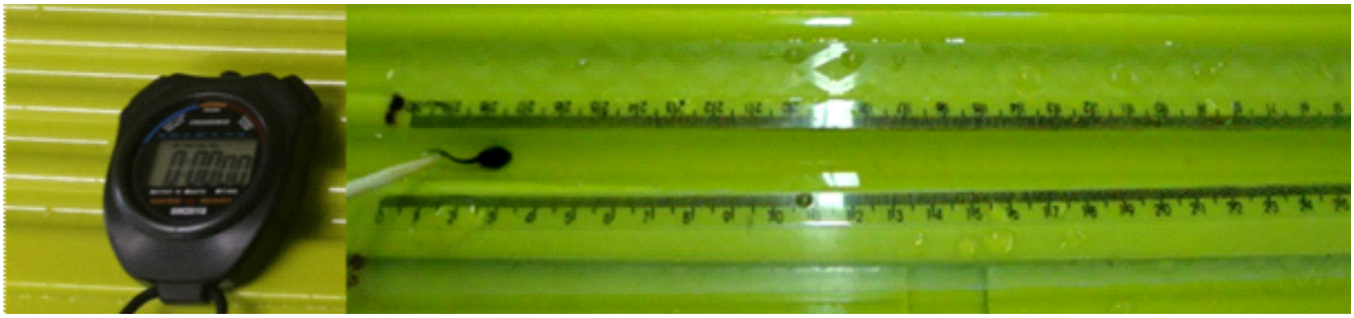

Figure 1 – Material used in the race - polypropylene lane, pipette, rulers and stopwatch.

Swimming ability and morphological abnormalities

Following the methodology was proposed by Bridges (1997) and used by Silva *et al.* (2020a) for tadpoles of *Odontophrynus carvalhoi*: after 24 hours of exposure to chlorpyrifos (or the control) the tadpoles were individually placed in polypropylene lanes (150 cm long x 3 cm wide x 4 cm deep) containing only dechlorinated water (Fig. 1). After an acclimation period of 5 minutes, each tadpole was stimulated at the base of its tail (with a pipette) to trigger a swimming response.

Tadpole swimming ability was assessed by measuring their swimming speed. The time of the swimming behavior (s) and the distance traveled (cm) were determined using a stopwatch and graduated ruler respectively.

After the swimming test, the tadpoles were anesthetized with lidocaine, sacrificed, fixed, and then preserved in 10% formaldehyde. Morphological analyses were subsequently performed, considering the larval stage of each individual.

Statistical analyses

Analysis of variance (ANOVA) was used to determine how the chlorpyrifos treatments affected tadpole swimming speeds, comparing the averages of the speeds registered in each treatment for each species. Normality was confirmed using the modified Shapiro-Wilks test, and homoscedasticity was confirmed using Bartlett's test. Student's t-test was performed with *L. latrans* for the same purpose.

Tukey's multiple comparison test was performed to test for significant differences in the swimming speeds among tadpoles of the species *S. x-signatus* and *P. cuvieri* exposed to chlorpyrifos and the control. All tests were performed using GraphPad Prism 5.00 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

The *P. cuvieri* and *L. latrans* tadpoles exposed to chlorpyrifos were slower when compared with the speed of the control group, showing significant statistical differences.

S. x-signatus tadpoles exposed to different concentrations of chlorpyrifos showed no statistically significant differences in swimming speed values among the different treatments (C1:8.3 ± 3.25 cm s^{-1}; C5: 7.65 ± 3.84 cm s^{-1} and C10: 6.81 ± 2.5 cm s^{-1}) or from the control group (8.65 ± 2.8 cm s^{-1}) ($F = 0.489$; $P = 0.692$) (Fig. 2).

P. cuvieri ($F = 18.97$, $P < 0.0001$) and *L. latrans* ($T = 8.158$, $P < 0.0001$) tadpoles evidenced statistically significant slower swimming speeds as compared to the control group (14.51 ± 8.4 cm s^{-1}; 3.52 ± 1.11 cm s^{-1} respectively) as well as among the tadpole groups exposed to different treatments: C1 (2.66 ± 0.9 cm s^{-1}), C5 (2.24 ± 0.82 cm s^{-1}), and C10 (2.1 ± 0.91 cm s^{-1}) (Fig. 3.); *L. latrans* was tested only at C10 (1.30 ± 0.66 cm s^{-1}) (Fig. 4.).

No morphological abnormalities were recorded.

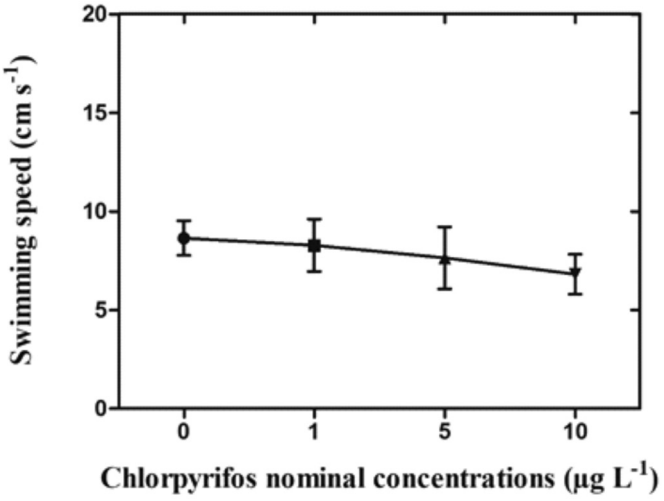

Figure 2 - Swimming speed of *Scinax x-signatus* tadpoles exposed to chlorpyrifos (0, 1, 5 and 10 µg L^{-1}).

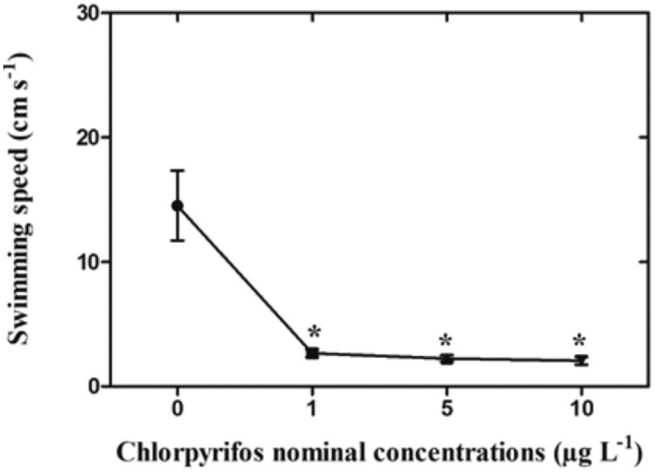

Figure 3 - Swimming speed of *Physalaemus* cf. *cuvieri* tadpoles exposed to chlorpyrifos (0, 1, 5 and 10 µg L^{-1}). *Asterisk indicates a significant difference compared with the control group.

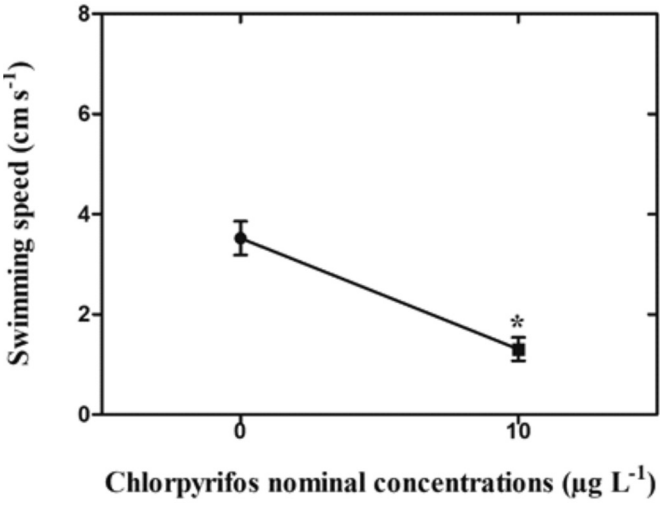

Figure 4 - Swimming speed of *Leptodactylus latrans* tadpoles exposed to chlorpyrifos (0 and 10 µg L^{-1}). *Asterisk indicates a significant difference compared with the control group.

DISCUSSION

We observed a significant reduction in the swimming speed of *P. cuvieri* and *L. latrans* tadpoles exposed to sublethal chlorpyrifos concentrations. No morphological abnormalities were noted, although their time of exposure to the contaminant (24 hours) was probably too short to evidence morphological alterations.

Although alterations of swimming behaviors were evident in the tadpoles of all three species exposed to chlorpyrifos, only the tadpoles of *P. cuvieri* and *L. latrans* showed statistically significant reductions in their swimming speed when compared to the control group. The tadpoles of *S. x-signatus* presented statistically similar values, although it must be noted that larval development in that species had advanced to phases 39 – 41, when limbs are well developed (Gosner, 1960) and could considerably decrease their swimming ability.

Based on chlorpyrifos toxicity research with amphibians, there are indications that sensitivity to that pesticide is highly species-specific, and influenced by the larval developmental stage of the species (Wildder & Bidwell, 2006). Those chemical compounds may cause subtle changes over time, with indirect effects on their survival, and make them more vulnerable to natural environmental pressures (Silva *et al*., 2020a).

Behavioral changes, such as reduced swimming speeds, suggest a neurotoxic effect. Several behavioral effects are known to be triggered in organisms exposed to organophosphate, as that insecticide inhibits acetylcholinesterase and therefore impacts locomotive performance even at low concentrations (Widder & Bidwell, 2008; Rutkoski *et al*., 2020; Silva *et al*., 2020a).

According to Scott & Sloman (2004) and Relyea & Hoverman (2006), although tadpole exposure to pesticides does not necessarily cause direct mortality, the resulting modifications of their behavior can indirectly affect their survival, as the reduction of their swimming capacity could result in increased vulnerability to predators, alter important growth and development functions, and also impair their ability to feed.

Plaut (2001), in his investigations of larval phases among amphibians, observed that their swimming ability is the main characteristic determining their survival in aquatic environments, and it may also influence the ability of these animals to obtain the food necessary for their development. Watkins (1996) observed in his study with *Pseudacris regilla* tadpoles a correlation between swimming ability and predation risks, with slow swimming tadpoles being more vulnerable to predation by water snakes than faster tadpoles.

Although several studies have reported inactivity in tadpoles exposed to organophosphate class pesticides, Peltzer *et al*. (2013) observed that swimming speed and distance increased at certain concentrations, probably reflecting the fact those levels of acetylcholinesterase inhibition were not sufficient to impact swimming speed. Other researchers reported inactivity and slow tadpole responses when exposed to chlorpyrifos, including in *Acris crepitans*, *Hyla chrysoscelis*, *Gastrophryne olivacea*, *R. sphenocephala* (Widder & Bidwell, 2008), *X. laevis* (Richards & Kendall 2002; Bonfanti *et al*., 2004), *Ceratophrys ornata* (Salgado Costa *et al.,* 2018), *Physalaemus gracilis* (Rutkoski *et al.*, 2020), and *Odontophrynus carvalhoi* (Silva *et al*., 2020a).

In the present study, chlorpyrifos was toxic and had a negative impact on the swimming ability of *P. cuvieri* and *L. latrans* tadpoles at sublethal concentrations. Therefore, chlorpyrifos were evidenced to have negative effects on tadpoles that inhabit agroecosystem pools, making them more susceptible to predation and/or less efficient at foraging.

ACKNOWLEDGMENTS

We thank the Brazilian Ministry of the Environment for the permit to conduct the study (ICMBio permit no. 27305-1) and the Ethics Committee on Animal Use of the University (CEUA-UEFS protocol no. 02/2012).This study was funded by the Brazilian National Council for Scientific and Technological Development (CNPq), PELD: Chapada Diamantina and the Research Support Foundation of the State of São Paulo (FAPESP): SISBIOTA Project: Tadpoles of anurans from the Atlantic Forest, the Amazon, the Pantanal, Cerrado and Transition Zones: Morphological Characterization, Spatial Distribution and Patterns of Diversity (FAPESP proc. 2010/52321-7 and CNPq proc. 563075/2010-4).

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