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# Inhibition and recovery of cholinesterases in *Odontophrynus americanus* tadpoles exposed to fenitrothion

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***Abstract:*** *We determined the levels of brain acetylcholinesterase (AChE) and tail butyrylcholinesterase (BChE) activities in tadpoles of Odontophrynus americanus exposed to a commercial formulation of fenitrothion. The mean brain AChE activities in the controls tadpoles varied from 6.91 to 6.39 μmol $min^{-1}$ $mg^{-1}$ protein, whereas tail BChE activities ranged among 0.26 to 0.17 μmol $min^{-1}$ $mg^{-1}$ protein; the two sub-lethal concentrations of fenitrothion assayed produced AChE and BChE inhibition ($p < 0.01$). Brain AChE recovered a substantial level of activity with a maximum of 93.2%; after the transference of tadpoles to a free-pesticide solution, whereas tail BChE recovery showed a smaller increase (39%) in the activity at 168 hr after to transference to clear water. According with our results, we suggest that tadpole's tail BChE presents higher sensibility than brain AChE.*

***Key words:*** *Tadpoles, Cholinesterases, Fenitrothion, Recovery*
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## Introduction

In recent years, researches on the effects of agricultural intensification on amphibians have considerably been increased (Relyea *et al*., 2005). This is not surprising, as among amphibian studies, different processes associated to agricultural intensification, ultraviolet radiation and pesticide exposure are regarded as main threats to amphibians and to be partially responsible for global amphibian population declines (Sparling *et al*., 2001; Oromi *et al*., 2008). Particularly, amphibians are likely to be exposed to pesticides during the aquatic development, when it coincides with the timing of pesticide use (Peltzer *et al*., 2008). For example, most anurans species of Argentina breed throughout the spring and summer seasons, and their larval development may extend over long periods overlapping in time with pesticides applications for crop protection (Lajmanovich *et al*., 2004).

Cholinesterases (ChEs) have been the pioneer biomarkers for assessing exposure to organophosphate (OP) and carbamate (CB) pesticides in wildlife (Sanchez-Hernandez, 2001). In vertebrates two types of ChEs were identified based on their distinct substrate specificity and inhibitor sensitivity. The acetylcholinesterase (AChE; EC 3.1.1.7) specifically catalyses the hydrolysis of acetylcholine and is subjected to marked inhibition by its own natural substrate. In contrast, butyrylcholinesterase (BChE; EC 3.1.1.8) is capable of degrading a wider range of choline esters and is not inhibited by its substrate (Quinn, 1987). Caballero de Castro *et al*. (1991) suggested that these enzymes showed, in amphibians tadpoles, different rates of recovery, this fact justify measures of different ChEs (AChE for the brain and BChE for the tail).

Fenitrothion [O, O-Dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate] is an OP insecticide applied to ground or air that remains in use since 1959 to control insects pests on rice, cereals, fruits, vegetables, stored grains, cotton (IPCS, 1992). This pesticide spread the air through volatilization from contaminated surfaces and may drift beyond the intended target area during spraying. It leaches very slowly from most soils, but some runoff can occur (IPCS, 1992). Maximum fenitrothion concentrations observed in lentic waters in New Brunswick (Canada) take place a few hours after spraying averaging 0.2 mg $l^{-1}$ (Fairchild *et al*., 1989) with peak concentrations up to 2.5 mg $l^{-1}$ in the surface layer of small ponds (Ernst *et al*., 1991).

Field studies associated with the aerial application of F did not found acute toxic effects on amphibians (Pearce and Price, 1977). For example, MacAlpine *et al*. (1998) did not record direct mortality effects on mink frog (*Rana septentrionalis*) exposed to aerial applications of fenitrothion (210 g a.i. $ha^{-1}$) in New Brunswick (Canada). The authors pointed out that the low frog numbers recorded in ponds receiving repeated applications of fenitrothion may be consistent with indirect impacts. Both reductions in the available invertebrate food resources and sub-lethal effects leading to increases mortality of tadpoles, were reported in ponds sprayed with fenitrothion (Story and Cox, 2001). On the other hand, Hashimoto and Nishiuchi (1981) demonstrated that lethal fenitrothion concentration ($LC_{50}$) value for anuran tadpoles ranged between 1.2-15 mg $l^{-1}$. Nevertheless, toxicological research emphasizes the lethal effects of short-term exposure of fenitrothion in a few model organisms (mouse, dog cat, Story and Cox, 2001). Moreover, ChE activity depression in plasma of experimental animals (rat and mice) was recorded in red blood cells, brain, and liver tissues (IPCS, 1992).

The main objective of present study is evaluate the sub-lethal effects of commercial formulation of fenitrothion in *Odontophrynus americanus* tadpoles (Amphibia: Cycloramphidae), based on observations of relationships between brain AChE and tail BChE and additionally we examine and describe the recovery process.

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## Materials and Methods

**Chemical compound:** Fenitrothion (CAS No. 122-14-5 commercial grade, trade name "Hormigal-L"), was obtained from Agroparque S.R.L., Argentina. The commercial product consisted of 10% w/w F formulated in aqueous solutions.

**Anuran tadpoles:** Tadpoles of *Odontophrynus americanus* were selected as test organisms. This anuran has an extensive Neotropical distribution (IUCN, 2005) and is frequently found in vegetated areas, wetlands, agricultural land, and urban territories. Certainly, this species is easy to handle and acclimatize to laboratory conditions (Cabagna *et al.,* 2006). A total of 108 prometamorphic larvae (stages 26 up 36, Gosner (1960), were collected from a temporary pond in natural parafluvial forests of the Parana river boundary (31° 43´S; 60° 34´W-Argentina). The average total size (snout-tail tip) was 15 ± 0.5 mm (N = 12) and weight was 0.08 ± 0.02 g (N = 12). The tadpoles were acclimatized to a 12:12 hr light: dark cycles in glass tanks (12.5 cm diameter and 13.5 cm high) with dechlorinated tap water of pH 7.4 ± 0.05; conductivity, 175 µmhos $cm^{-1}$; dissolved oxygen concentration, 6.5 ± 1 mg $l^{-1}$; hardness, 50.5 mg $l^{-1}$ of $CO_3Ca$ at 24 ± 2 °C for 7 days.

**Exposure design:** The 24 hr sub-lethal tests were conducted according to USEPA Standard Methods (USEPA, 1989), with 12 replicates per treatment. In the sub-lethal test, the nominal concentrations used were: 0.5 and 1.5 mg $l^{-1}$ of fenitrothion. After 24 hr the tadpoles were transferred to clean water for 3 days. Negative controls with dechlorinated tap water were used. All test solutions were prepared in duplicated immediately before each experiment. Boiled lettuce was added as food source in all treatments and was changed for every 24 hr.

**Enzymatic determinations:** Tadpoles euthanized according with ASIH (2004) criterion. Whole brain and tail were excised under a stereoscope and homogenized in 0.1% octylphenoxypolyethoxy ethanol (triton X-100) in 25 mM tris (hydroxymethyl) aminomethane hydrochloride (pH 8.0) using a polytron over ice. The homogenates were centrifuged at 3500 rpm for 15 min at 4°C. AChE and BChE activities were measured according to method Ellman (1961). The reaction mixture consisted of 0.01 ml of brain and tail homogenate, 2 mM dithio bis 2-nitrobenzoic acid (DTNB), 20 mM acetylthiocholine and butyrylthiocholine iodide (AcSCh and BuSCh, respectively), 25mM Tris-HCl and 1 mM $CaCl_2$ (pH 7.6) (all reagents were from Sigma Aldrich, Germany). Assays were conducted at 20°C. Changes in absorbance at 410 nm were recorded at 10-s intervals for 1 min using a UV-VIS spectrophotometer. All assays were performed at least in duplicate homogenates. Protein concentration in the supernatants was determined according to the Biuret method (Kingbley, 1942). Enzyme activity was expressed as µmol $min^{-1}mg^{-1}$ protein using a molar extinction coefficient of 13.6 x $10^3$ $M^{-1}$ $cm^{-1}$.

**Data analysis:** The results were expressed as means ± 1 standard deviation (SD). ANOVA and Dunnet´s post hoc-tests were used to compare ChEs activities from control and exposed animals (Zar, 1999). Moreover, Tukey's post hoc-tests were performed to compare fenitrothion concentrations. Normality and homogeneity of variances were verified using the Kolmogorov-Smirnov test and the Levene test, respectively. The criterion for significance was $p < 0.05$.

## Results and Discussion

The mean values of the brain AChE activities in the control tadpoles varied from 6.91 ± 1.65 µmol $min^{-1}mg^{-1}$ protein at 24 hr of exposition to 6.39 ± 1.60 µmol $min^{-1}$ $mg^{-1}$ of protein at 168 hr (Fig. 1), whereas tail BChE activities ranged among 0.26 ± 0.09 µmol $min^{-1}$ $mg^{-1}$ protein (24 hr) to 0.17 ± 0.07 µmol $min^{-1}$ $mg^{-1}$ of protein (168 hr) (Fig. 2). No tadpole mortality was recorded after 24 hr (exposure) and at the end of recovery times (168 hr). Similarly, we neither observed common visible abnormalities for pesticide expositions (abnormal swimming behavior, altered body shape, edema, paralysis, or abnormal curvature of body axis). Correspondingly, Bain *et al.* (2004) investigating the toxic effects of fenitrothion on the lizard *Pogona vitticeps* concluded that even though plasma BChE activity was inhibited after 24 hr of OP dosing, physiological (body temperature and standard metabolic rate) or behavioural (feeding

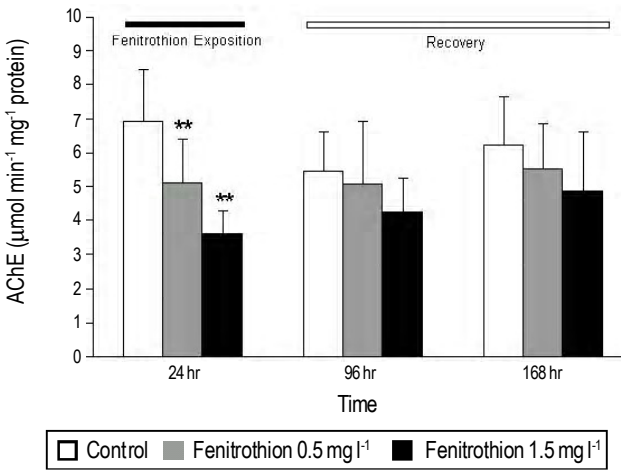


**Fig. 1:** Brain acetylcholinesterase (AChE) activity in *Odontophrynus americanus* tadpoles exposed to sub-lethal concentrations of fenitrothion. The values are mean ± SD (n = 12). (**) indicates significant difference from the respective control ($p < 0.01$)

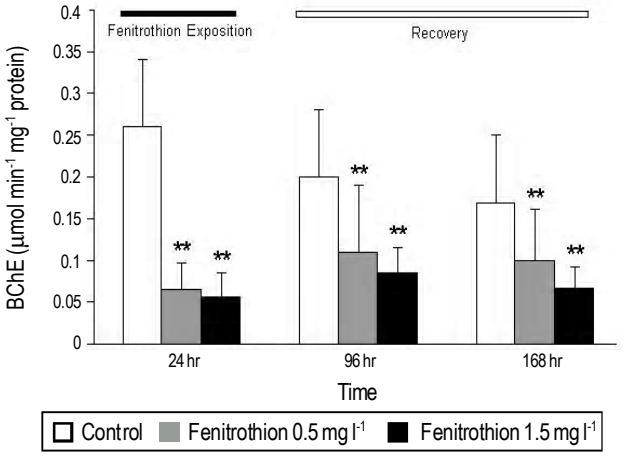


**Fig. 2:** Tail butyrylcholinesterase (BChE) activity in *Odontophrynus americanus* tadpoles exposed to sub-lethal concentrations of fenitrothion. The values are mean ± SD (n = 12). (**) indicate significant difference from the respective control ($p < 0.01$)

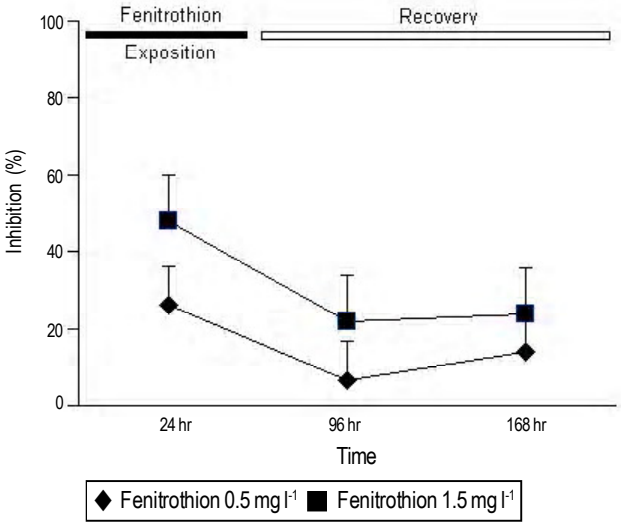


**Fig. 3:** Inhibition percentages mean (± SD), in relation to controls, of brain acetylcholinesterase (AChE) activity in *Odontophrynus americanus* tadpoles exposed to sub-lethal concentrations of fenitrothion

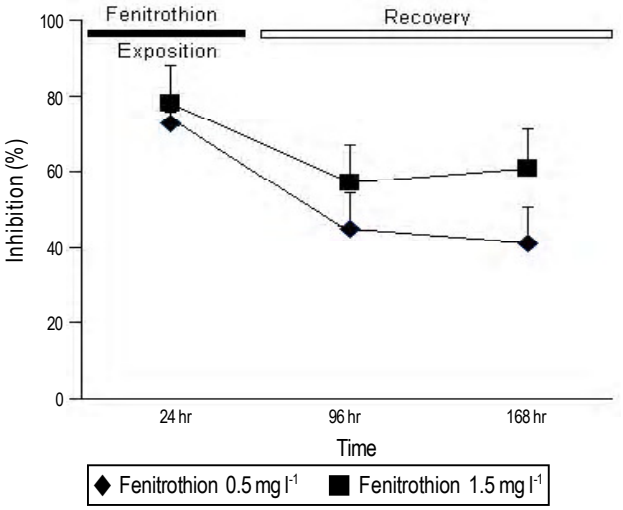


**Fig. 4:** Inhibition percentages mean (± SD), in relation to controls, of tail butyrylcholinesterase (BChE) activity in *Odontophrynus americanus* tadpoles exposed to sub-lethal concentrations of fenitrothion

rate) endpoints were not affected by fenitrothion exposure. Moreover, the authors suggested that the ability of plasma BChE to sequester circulating OP could explain the absence of toxic effects.

On the basis of these results we could conclude that AChE activity in ectotherms was relatively insensitive to inhibition by OP compounds compared to that found in endotherms (Lotti and Johnson, 1978), especially brain AChE in amphibian species appeared especially insensitive (Fulton and Chambers, 1985). However, present results, suggest that in our assays the two sub-lethal concentrations of F produced AChE and BChE inhibition ($p < 0.01$; fenitrothion = 16.92 and 48.48, respectively), whereas the higher concentration (1.5 mg $l^{-1}$ fenitrothion) produced more than 50% of their inhibition and differed significantly to the control (Fig. 1, 2). The brain AChE presented a maximum inhibition percentage of 47.9% at 1.5 mg $l^{-1}$ fenitrothion

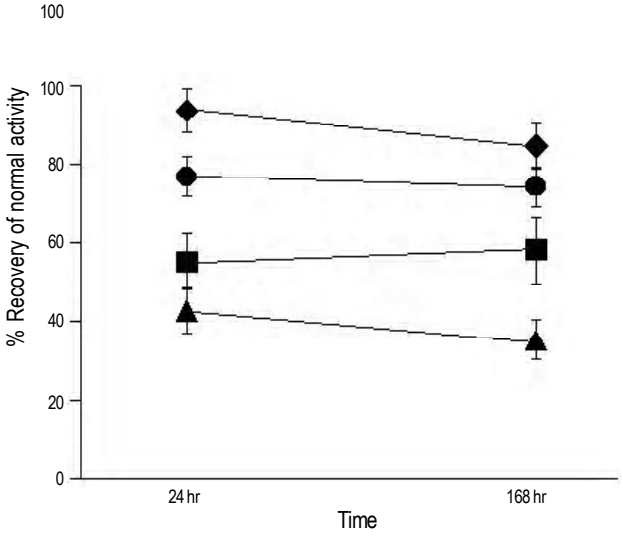


**Fig. 5:** Recovery percentages mean (± SD), in relation to controls, of brain acetylcholinesterase (AChE) and tail butyrylcholinesterase (BChE) activities in *Odontophrynus americanus* tadpoles exposed to sub-lethal concentrations of fenitrothion

concentration at 24 hr although this value decreased to 23.7% at 168 hr in relation to controls groups (Fig. 3). In contrast, tail BChE activity presents the higher inhibition percentage at 24 hr (78%) decreasing only until 61% at 168 hr (Fig. 4). Thus, for this enzyme the inhibition was kept significant throughout all treatments (Fig. 2).

In general, inhibition of ChEs depends on OP toxicity, with BChE being typically more sensitive as a biomarker. However, AChE is considered more relevant to toxicity and often differing in the structure-activity relationships (Wilson, 2001). Nevertheless, BChE is considerably more sensitive to cholinesterase-inhibiting pesticides than AChE in most vertebrates, fish (Sturm *et al*., 1999), birds (Thompson *et al*., 1991), mammals (Ecobichon and Comeau, 1973), and reptiles (Sanchez-Hernandez *et al*., 1997). Likewise, amphibian plasma BChE appears more sensitive to pesticide exposure than brain AChE (Sparling *et al*., 1997). Thus, the greater depression in the activity of BChE observed in *O. americanus* compared to AChE agree with former suggestions.

Many toxicological studies have focused on measuring ChE activity in tadpoles exposed to anti-ChEs pesticides (Widder and Bidwell, 2008; Ezemonye and Ilechie, 2007; Sparling and Fellers, 2007), however few researches measures at the same time AChE and BChE activities to provide a powerful approach for rapid detection of exposure (Sparling *et al*., 1997). In *O. americanus* tadpoles exposed to fenitrothion, brain AChE recovered a substantial level with a maximum of 93.2% of its activity after the transference to a free-pesticide solution, whereas tail BChE recovery showed a smaller increase in its activity (39% at 1.5 mg $l^{-1}$ fenitrothion ) at 168 hr, after to clear water (Fig. 5). According with our result AChE and BChE responded differently to increased levels of fenitrothion exposure. Brain AChE was not modified substantially showing smaller inhibition ($F = 1.97$; $p > 0.05$; *post-hoc*

Tukey comparison of 0.5 vs. 1.5 mg $l^{-1}$ fenitrothion; $p > 0.05$). In contrast, the activity rate for BChE declined considerably at both concentration of fenitrothion assayed ($F = 5.35$; $p<0.01$; *post-hoc* Tukey comparison of 0.5 vs. 1.5 mg $l^{-1}$ fenitrothion; $p<0.05$). We concluded that tail BChE inhibition is a sensitive indicator of OP insecticide exposure for this anuran species and that significant inhibition can be detected at sub-lethal concentrations.

AChE activity was recovered more than 90% at 96 hr for both F concentrations. Conversely, the BChE activity did not recover the normal level when tadpoles were transferred to free fenitrothion water. In this sense, we suggest a higher sensitivity of tail BChE than brain AChE.

Johal *et al*. (2007) suggested that low dose of organophosphate affecting the water environments. Considering that, our results, and the massive use of insecticides for control insect pest of crops in Argentina near to waterbodies (Jergentz *et al*., 2005), more researches are urgently needed to be conducted, both under laboratory conditions and agricultural systems with other larval anuran native species. These studies are important to determine the use of both ChEs' enzymes as reliable bioindicators to monitor OP organophosphate contamination.

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