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Resistance in cholinesterase activity after an acute and subchronic exposure to azinphos-methyl in the freshwater gastropod *Biomphalaria straminea*

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ABSTRACT

Organophosphorous and carbamates insecticides are ones of the most popular classes of pesticides used in agriculture. Its success relies on their high acute toxicity and rapid environmental degradation. These insecticides inhibit cholinesterase and cause severe effects on aquatic non-target species, particularly in invertebrates. Since the properties of cholinesterases may differ between species, it is necessary to characterize them before their use as biomarkers. Also organophosphorous and carbamates inhibit carboxylesterases and the use of both enzymes for biomonitoring is suggested. Azinphos-methyl is an organophosphorous insecticide used in several parts of the word. In Argentina, it is the most applied insecticide in fruit production in the north Patagonian region. It was detected with the highest frequency in superficial and groundwater of the region. This work aims to evaluate the sensitivity of *B. straminea* cholinesterases and carboxylesterases to the OP azinphos-methyl including estimations of 48 h NOEC and IC_{50} of the pesticide and subchronic effects at environmentally relevant concentrations. These will allow us to evaluate the possibility of using cholinesterase and carboxylesterase of *B. straminea* as sensitive biomarkers. Previously a partial characterization of these enzymes will be performed. As in most invertebrates, acetylthiocholine was the preferred hydrolyzed substrate of *B. straminea* ChE, followed by propionylthiocholine and being butyrylthiocholine hydrolysis very low. Cholinesterase activity of *B. straminea* was significantly inhibited by the selective cholinesterases inhibitor (eserine) and by the selective inhibitor of mammalian acethylcholinesterase (BW284c51). In contrast, iso-OMPA, a specific inhibitor of butyrylcholinesterase, did not inhibit cholinesterase activity. These results suggest that cholinesterase activity in total soft tissue of *B. straminea* corresponds to acethylcholinesterase. Carboxylesterases activity was one order of magnitude higher than cholinesterase. A greater efficiency (V_{max}/K_m) was obtained using acetylthiocholine and p-nitrophenyl butyrate. Acute exposure to azinphos-methyl did not cause inhibition of cholinesterase activity until 10 mg L^{-1} used. Carboxylesterases towards p-nitrophenyl butyrate was inhibited by azinphos-methyl being the IC_{50} 2.20 ± 0.75 mg L^{-1} of azinphos-methyl. Subchronic exposure to environmental concentrations of azinphos-methyl (0.02 and 0.2 mg L^{-1}) produced a decrease in survival, protein content and carboxylesterases activity despite no inhibition of cholinesterase activity was observed. *B. straminea* cholinesterase is not a sensible biomarker. On the contrary, carboxylesterases activity was inhibited by azinphos-methyl. Carboxylesterases could be protecting cholinesterase activity and therefore, protecting the organism from neurotoxicity. This work confirms the advantages of measuring cholinesterases and carboxylesterases

Abbreviations: AcSCh, acetylthiocholine iodide; BuSCh, butyrylthiocholine iodide; BW284c51, 1,5-bis(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide; CE, carboxylesterase; ChE, cholinesterase; IC_{50}, 50 percent inhibition concentration; DTNB, 5,5′-dithio-2-bis-nitrobenzoate; iso-OMPA, tetraisopropyl pyrophosphoramide; NOEC, the no observed effect concentration; OP, organophosphorous insecticide; p-NPA, p-nitrophenyl acetate; p-NPB, p-nitrophenyl butyrate; PrSCh, propionylthiocholine iodide

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jointly in aquatic biomonitoring of pesticide contamination. This becomes relevant in order to find more sensitive biomarkers and new strategies to protect non-target aquatic organisms from pesticide contamination.

1. Introduction

Organophosphorous (OPs) and carbamates insecticides are ones of the most popular classes of pesticides used in agriculture. Its success relies on their high acute toxicity and rapid environmental degradation (Eto, 1974). They exert acute toxicity by inhibiting acetylcholinesterase (AChE) causing the accumulation of acetylcholine. Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular systems in most species. So, these insecticides cause severe effects on non-target species, particularly in invertebrates (Barata et al., 2004; Fulton and Key, 2001). Therefore, appropriate monitoring strategies are required to protect sensitive non-target invertebrate species against the adverse effect of OPs and carbamates. The term biomarker may be defined as any biochemical, physiological, histological, or behavioral response that is elicited by an organism as a consequence of the exposure to xenobiotics (NRC, 1987). In humans and other vertebrates AChE and butyrilcholinesterase (BChE) are considered useful biomarkers of exposure to OPs and carbamates insecticides (Carlock et al., 1999). In invertebrates, AChE exhibits genetic and molecular polymorphism and their distributions and physiological roles differ amongst species (Forget and Bocquené, 1999; Massoulié et al., 1993). Since the properties of ChEs may differ between species, a characterization of the enzymes can be performed before their use as biomarkers (Kristoff et al., 2006). For this reason, (1) the identification of the preferred hydrolyzed substrate, (2) its optimal concentration to be used in enzyme measurement and (3) its sensitivity to specific inhibitors should be studied. ChEs of aquatic invertebrates generally show a preference for acetylthiocholine (AcSCh) as substrate; however, there are some species that prefer propionylthiocoline (PrSCh) instead of AcSCh (Basack et al., 1998; Hannam et al., 2008; Mora et al., 1999; Talesa et al., 1990; Varó et al., 2002). Generally ChE activity using butyrilthiocholine (BuSCh) as substrate is very low in invertebrates. However, some species show an important hydrolysis of this substrate (Kristoff et al., 2006).

Assessment of ChE activity in wildlife population has been proposed as a general method for detecting environmental contamination due to OPs and carbamates, particularly since many of these chemicals have relatively short half lives in the aquatic environment. In the last years, the combined monitoring of ChE and carboxylesterases (CES) activities has been suggested (Kristoff et al., 2012; Vioque-Fernández et al., 2007). According to the classification of esterases by Aldridge (1953), the serine superfamily of esterases that are inhibited by OPs falls into the B-esterase group. This group includes ChE and CES. It has been postulated that CES can protect ChE from OPs-mediated toxicity (Wheelock et al., 2008) by removing a significant amount of insecticide by two main mechanisms: (1) the detoxification by hydrolysis of ester bonds in some of these insecticides and (2) by providing alternative binding sites for OPs (Jokanovic, 2001; Sanchez-Hernandez, 2007). CES comprise a group of isoenzymes with low substrate specificity, so CES activity could be measured with various substrates. For aquatic gastropods, it has been demonstrated that CES from total soft tissue were more sensitive to OPs using p-nitrophenyl acetate (p-NPA) and p-nitrophenyl butyrate (p-NPB) than using 1 and 2 napthyl acetate as substrates (Cacciatore et al., 2013; Kristoff et al., 2012).

In some invertebrates species it has been reported that CES activities were higher or more sensitive than ChE to inhibition by OPs or carbamate acute exposure (Escartín and Porte, 1997; Basack et al., 1998; Barata et al., 2004; Galloway et al., 2002; Vioque-Fernández et al., 2007). However, in others, sensitivity was either similar for ChE and CES (Cacciatore et al., 2013) or ChE resulted more sensitive than CES (Bianco et al., 2013; Kristoff et al., 2006). Only a few freshwater gastropods have been studied in relation to the response of B-esterases (ChEs and CES) to acute exposure to OPs and carbamates (Bianco et al., 2013; Cacciatore et al., 2013; Gagnaire et al., 2008; Kristoff et al., 2006, 2010, 2012). Subchronic or chronic effect on B-esterases was even less studied (Kristoff et al., 2011; Rivadeneira et al., 2013; Tripathi and Singh, 2004).

Azinphos-methyl is an OP insecticide used in several parts of the world. In Argentina, it is the main insecticide applied in the fruit production in the Upper Valley of the Neuquén and Río Negro rivers, in the North Patagonian region. The concentrations found in water of this region were between 0.046 and 22.48 $\mu g\ L^{-1}$ (Loewy et al., 1999, 2011). In Argentina, it is also used in the Mesopotamia Region, fundamentally for the harvest of tobacco (González et al., 2006). In other parts of the world, reported concentrations in surface water range from 0.06 to 420 $\mu g\ L^{-1}$ (Granovsky et al., 1996; Klosterhaus et al., 2003; Schulz, 2004; Wan et al., 1995). *Biomphalaria straminea* is a freshwater gastropod naturally distributed in Argentina (Rumi et al., 2008). Previous studies in our laboratory have characterized the activities of ChE and CES of *Biomphalaria glabrata* and their responses to azinphos-methyl (Kristoff et al., 2006, 2011, 2012). However, *B. glabrata* was not naturally distributed in Argentina. In the Alto Valle region, toxicity studies with local non-target species have been performed mostly in vertebrates (Ferrari et al., 2004a,b, 2007, 2009; Venturino et al., 2001). Amongst not-target invertebrates, these studies are restricted to the amphipod crustacean *Hyalella curvispina* (Anguiano et al., 2008, 2012) and the freshwater gastropod *Chilina gibbosa* (Bianco et al., 2013). *B. straminea* as a sentinel organism has the advantage of being naturally distributed along all the country including Patagonian and Mesopotamian regions of Argentina.

This work aims to evaluate the sensitivity of *B. straminea* ChE and CES to the OP azinphos-methyl including estimations of 48 h NOEC and IC_{50} of the pesticide and subchronic effects at environmentally relevant concentrations. These will allow us to evaluate the possibility of using ChE and CES of *B. straminea* as sensitive biomarkers. Previously a partial characterization of these enzymes will be performed.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (AcSCh), butyrylthiocholine (BuSCh), propionylthiocholine (PrSCh), p-nitrophenyl acetate (p-NPA), p-nitrophenyl butyrate (p-NPB), 5,5′-dithio-2-bis-nitrobenzoate (DTNB), 1,5-bis(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide (BW284c51), azinphos-methyl PESTANAL® (97.2 percent PURE) were purchased from Sigma-Aldrich. All the chemicals used were of analytical reagent grade.

2.2. Organisms

The organisms were provided by Dra. M. Ostrowsky and were then characterized to confirm the species. Then, the snails were raised in our laboratory in aerated glass aquaria (17–20 L), at a temperature of 22 ± 2 °C, and under a 14:10 (L:D) h

photoperiod regime. They were fed with lettuce three times a week (Fried et al., 1992). For all the experiments, similar adult snails (0.079 ± 0.020 g) were used.

2.3. Homogenates

Animals were placed on ice for 6–8 min. The shells were carefully removed and the soft tissue isolated at 0 °C. The body soft tissues were washed in distilled water, placed on filter paper to drain extra fluids, and weighed. Tissues were homogenized mechanically with an electrical homogenizer in 20 mM Tris/HCl buffer, pH 7.5, plus 0.5 mM EDTA (1/10) and homogenates were centrifuged at 11,000 × *g* for 20 min at 4 °C. The resulting supernatants were used for all the determinations.

2.4. Protein content

Protein content was determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

2.5. Cholinesterase activity (ChE)

ChE activity was measured in the supernatant fraction (100 μl), in 100 mM phosphate buffer, pH 8, 0.2 mM DTNB and 0.75 mM AcSCh, PrSCh or BuSCh as a substrate according to the method of Ellman et al. (1961). Optimal substrate concentration (0.75 mM) was obtained after estimation of kinetic parameters. Absorbance was continuously recorded at 412 nm. Rates were corrected for spontaneous hydrolysis of the substrate and non-specific reduction of the chromogen by tissue extracts. ChE activity was calculated using the molar extinction coefficient of the 2-nitro-5-thiobenzoate anion, formed from the reaction (13.6 mM^{-1} cm^{-1}). Absolute activity was expressed as μmoles of 2-nitro-5-thiobenzoate anion produced per min per ml of supernatant. The specific activity was expressed as μmol of the 2-nitro-5-thiobenzoate anion produced per min per mg of protein.

2.6. Carboxylesterases activity (CES)

Hydrolysis of p-NPA and p-NPB by CES was measured, according to Kristoff et al. (2010). Reactions were performed in 100 mM phosphate buffer pH 8.0 containing 5 percent acetone (2.5 ml) and 1 mM p-NPA or p-NPB (100 μl). Optimal substrate concentration was obtained after estimation of kinetic parameters (1 mM). 150 μl of the supernatant fraction were used for the determination. Absorbance was continuously recorded at 400 nm. CES activity was calculated using the molar extinction coefficient for p-nitrophenol (18.6 mM^{-1} cm^{-1}). Absolute activity was expressed a μmoles of p-nitrophenol produced per min per ml of supernatant. The specific activity was expressed as μmol of p-nitrophenol produced per min per mg protein.

2.7. Kinetic constants of ChE and CES

Rates of hydrolysis of 8 AcSCh, PrSCh, BuSCh, p-NPA or p-NPB concentrations (0.03–1.5 mM), were used to calculate K_m and V_{max}. The values of K_m and V_{max} were estimated from hyperbolic regression analysis using OriginPro 7.5 (OriginLab, Northampton, MA). Six snails per homogenate and four homogenates for the determinations were used ($N=4$).

2.8. In vitro inhibition of ChE with eserine, BW284c51 and iso-OMPA

To assay the *in vitro* effects of eserine (a selective ChE inhibitor), BW284c51 (a selective inhibitor of mammalian AChE activity) and iso-OMPA (a specific inhibitor of mammalian BuChE activity) on enzyme activities, the compounds were preincubated with the supernatant fraction for 15 min at 25 °C before substrate addition. Eserine and iso-OMPA were dissolved in ethanol while BW284c51 was prepared in distilled water. Preincubation concentrations of the inhibitors were 0.001; 0.01 and 1 mM for eserine, 0.01 and 1 mM for BW284c51 and 1 mM for iso-OMPA. Four snails for each homogenate were required and eight homogenates were used for the assays ($N=8$). Controls with ethanol and distilled water were also included. After the preincubations, ChE activity using AcSCh, PrSCh and BcSCh as substrates was measured.

2.9. General conditions of bioassays

All the bioassays were performed at 22 ± 2 °C under a photoperiod of 10:14 (L:D) h. The following physico-chemical parameters were recorded: total hardness = 67 ± 3 mg $CaCO_3$ L^{-1}; alkalinity = 29 ± 2 mg $CaCO_3$ L^{-1}; pH 7.0 ± 0.2 and conductivity = 250 ± 17 μS cm^{-1}. The stock solution of the insecticide (4000 mg L^{-1}) was prepared in acetone. Azinphos-methyl concentration was tested by HPLC with UV detector (detection wavelength: 230 nm). The concentration values measured were always within the range 95–102 percent of the nominal values. Each concentration of azinphos-methyl was obtained by diluting the stock solution with dechlorinated tap water. To avoid insecticide degradation, test media was renewed every 24 h (Cacciatore et al., 2013). The constancy of the azinphos-methyl concentration in the aqueous solution has been previously demonstrated in stability studies conducted in our laboratory with several concentrations of the insecticide. The 24 h average measured insecticide concentration remained in all cases within the range 82–98 percent of the initial concentration (Cacciatore et al., 2013).

2.10. Acute bioassays

To perform the bioassays, 1 L glass vessels were used, containing 800 ml for each solution (dechlorinated tap water, 0.05 percent of acetone in dechlorinated tap water as the solvent control and eight concentrations of azinphos-methyl in dechlorinated tap water). Pesticide concentrations used were 0.05; 0.1; 0.25; 0.5; 2.5; 5; 6.5 and 10 mg L^{-1}. For each control and for each concentration of the pesticide six glass vessels containing two snails were used. During the bioassay animals were not fed. After 48 h, two snails were homogenized together and six homogenates for each group were used for ChE, CES and protein determinations.

2.11. Subchronic bioassays

To perform the bioassays, 1 L glass vessels were used containing 800 ml for each solution (dechlorinated tap water, 0.05 percent of acetone in dechlorinated tap water as the solvent control and two concentrations of azinphos-methyl in dechlorinated tap water). The concentrations of azinphos-methyl used were (1) an environmental concentration found in Argentina (0.02 mg L^{-1}) and (2) a higher concentration found in other parts of the world (0.2 mg L^{-1}). For each control and for each concentration of the insecticide, nine glass vessels containing three snails were used. During the bioassay snails were fed with lettuce once a week. After 21 days, two snails were homogenized together and at least six homogenates for each group were used for ChE, CES and protein determinations.

2.12. Data analysis

Results were expressed as mean ± S.D. Data were analyzed by one-way ANOVA. When overall F statistic was significant, pair wise comparisons among treatment groups were performed by Tukey HSD post-test by using VassarStats (http://vassarstats.net). The level of significance used was 0.05.

48 h NOEC was experimentally estimated as the maximum concentration causing no significant effect on ChE or CES tested by ANOVA ($p>0.05$) (Kristoff et al., 2006). 48 h IC_{50} values were calculated using the 4-parameter logistic model using OriginPro 7.5 (OriginLab, Northampton, MA) as it was described by Cacciatore et al. (2013) and Laguerre et al. (2009).

3. Results

3.1. Characterization of B-esterases

Table 1 shows the results of the estimations of apparent K_m and V_{max} of ChE activity towards AcSCh and PrSCh and CEs activity towards p-NPA and p-NPB. In the case of BuSCh, K_m could not be determined since ChE activity measured with this substrate was very low. Regarding ChE, the highest activity was obtained with AcSCh as substrate, followed by PrSCh (31 percent respect to AcSCh) and BuSCh (16 percent respect to AcSCh). K_m value was

Table 1
Kinetic parameters of different cholinesterases and carboxylesterases substrates.

Enzyme	Substrate	V_{max} (μmol min^{-1} ml^{-1})	V_{max} (μmol min^{-1} mg $protein^{-1}$)	K_m (mM)	V_{max}/K_m
ChE	AcSCh	0.158 ± 0.055	0.051 ± 0.018	0.17 ± 0.07	0.3×10^{-3}
ChE	PrSCh	0.047 ± 0.003	0.016 ± 0.001	0.08 ± 0.04	0.2×10^{-3}
ChE	BuSCh	0.025 ± 0.003	0.008 ± 0.001	–	–
CE	p-NPA	1.925 ± 0.415	0.621 ± 0.134	0.91 ± 0.31	0.7×10^{-3}
CE	p-NPB	1.221 ± 0.418	0.394 ± 0.135	0.20 ± 0.09	0.2×10^{-2}

V_{max}/K_m: efficiency; K_m is expressed as mM.

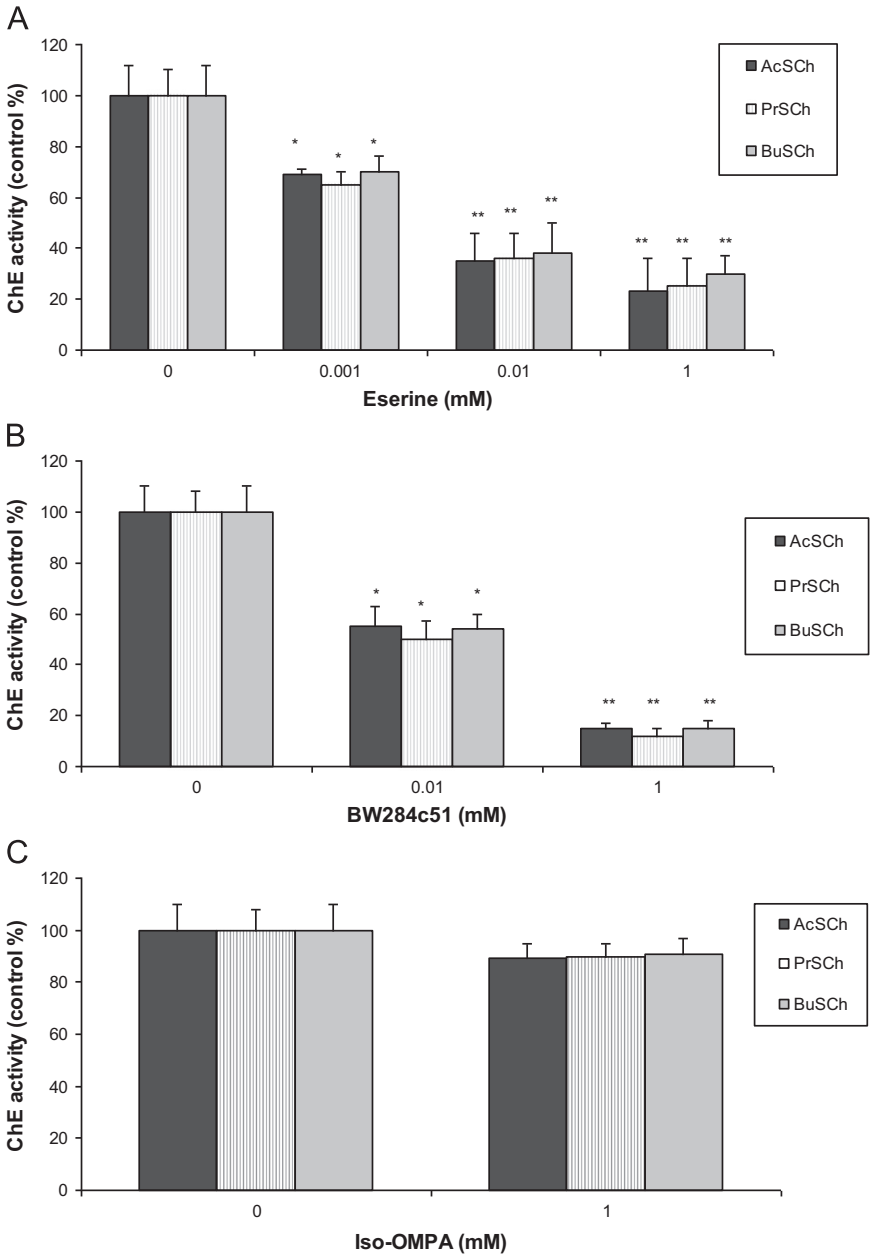

Fig. 1. ChE inhibition by (A) eserine, (B) BW284c51 and (c) iso-OMPA in adult *Biomphalaria straminea*. The activity was measured with AcSCh, PrSCh and BuSCh in the supernatant fraction after the preincubation with the drugs for 15 min. Activities were expressed as percent of the control. Each bar represents the mean ± S.D. of eight replicates. The asterisk indicates significant differences from controls ($p < 0.05$) and two asterisks indicate significant differences from controls and from the other concentrations ($p < 0.05$).

lower for PrSCh than to AcSCh but the efficiency (V_{max}/K_m) was higher for AcSCh. V_{max} was expressed as absolute activity (μmoles of product produced per min per ml of supernatant) and specific activity (μmoles of product produced per min per mg of protein).

Effects of eserine, BW284c51 and iso-OMPA on ChE activity were shown in Fig. 1. Eserine induced inhibition of ChE measuring with AcSCh, PrSCh and BuSCh. Eserine 1 mM inhibited approximately 80 percent of ChE activity. Regardless the used substrate, similar results were obtained with BW284c51 (1 mM), showing approximately 90 percent of inhibition of ChE. On the contrary, with iso-OMPA, no inhibition of ChE was observed.

Considering CES activity, V_{max} was higher with p-NPA than with p-NPB as substrate while the apparent K_m was lower with p-NPB. Basal CES activity was one order of magnitude higher than basal ChE activity (Table 1).

3.2. Acute exposure

ChE (Fig. 2) and CES activities (Fig. 3) were measured in the supernatants of the whole organism soft tissue of snails exposed 48 h to different concentration of azinphos-methyl. AcSCh was chosen for ChE studies because it has been suggested that the most hydrolyzed substrate should be used in its measurements (Cacciatore et al., 2013; Kristoff et al., 2012). Regarding CES, since both activity and sensitivity to OPs have been reported as dependant on the substrate (Kristoff et al., 2012), the studies were carried out with both substrates: p-NPA and p-NPB. No statistically significant differences were observed between the control with water and the control with 0.05 percent acetone ($p > 0.05$). ChE and CES towards p-NPA were not inhibited by the insecticide, not even at the highest used concentration. CES activity using p-NPB as substrate was inhibited from 0.5 mg L^{-1}. In this case, NOEC

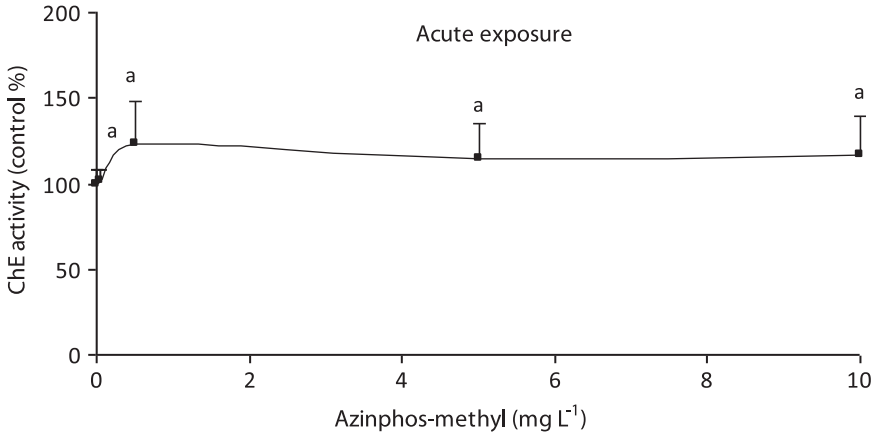

Fig. 2. Cholinesterase (ChE) activity using AcSCh as substrate in adult *Biomphalaria straminea* exposed 48 h to different concentrations of azinphos-methyl. Absolute activities were expressed as percent of the control. Data represent the mean ± S.D. of six homogenates.

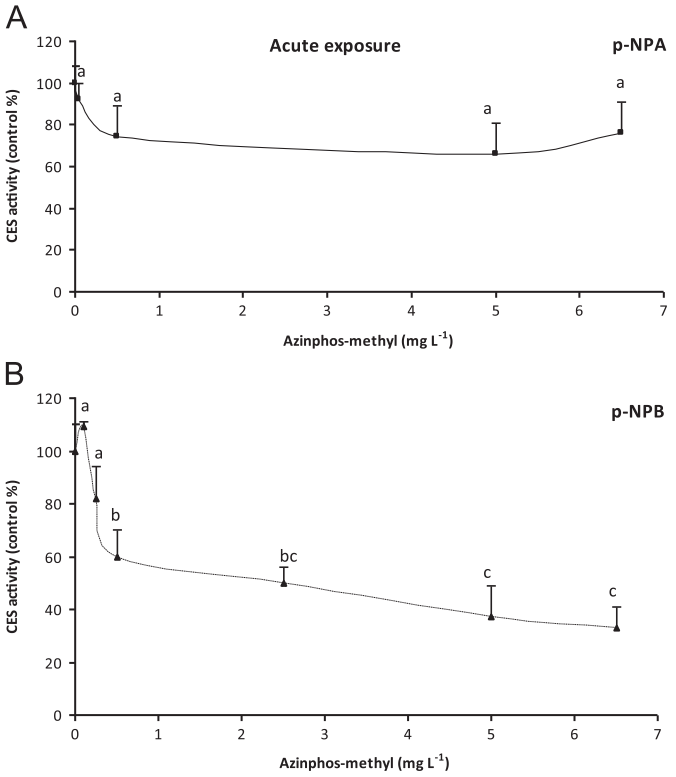

Fig. 3. Carboxylesterases (CES) activity using p-NPA (A) and p-NPB (B) as substrates in adult *Biomphalaria straminea* exposed 48 h to different concentrations of azinphos-methyl. Absolute activities were expressed as percent of the control. Data represent the mean ± S.D. of six homogenates. Data with different letters is statistically different ($p < 0.05$).

corresponded to 0.25 mg L^{-1} and IC_{50} to 2.20 ± 0.75 mg L^{-1} of azinphos-methyl. In controls and treated snails similar protein content was obtained ($p > 0.05$). Also, no mortality or visible signs of neurotoxicity were observed.

3.3. Subchronic exposure

To study the effect on B-esterases after a 21 day exposure, environmental concentrations of azinphos-methyl were used. No mortality was observed in control snails, but treated groups had a low survival. Percentage of survival corresponds to 84 percent (0.02 mg L^{-1}) and to 73 percent (0.2 mg L^{-1}) in exposed snails. Also,

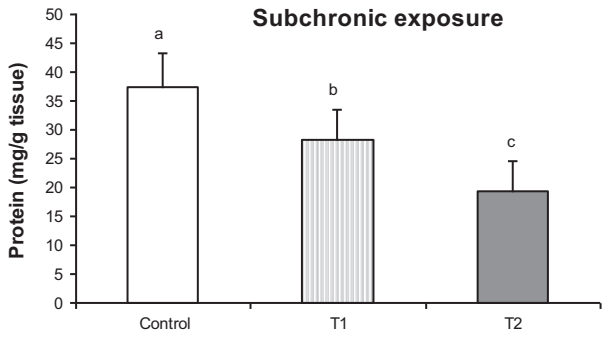

Fig. 4. Protein content (mg per g of tissue) in control group of *Biomphalaria straminea* and groups exposed 21 days to 0.02 (T1) and 0.2 mg L^{-1} (T2) of azinphos-methyl. Data represent the mean ± S.D. of at least six homogenates. The data with different letters is statistically different ($p < 0.05$).

the subchronic exposure produced a decrease of the protein content. Snails exposed to 0.02 mg L^{-1} and 0.2 mg L^{-1} showed a decrease of 25 percent and 49 percent respect to the control group (Fig. 4).

Since subchronic exposure to azinphos-methyl produced a decrease in protein content, data (Fig. 5) was expressed as percent of absolute control activity (μmoles of product produced per min per ml of supernatant). In regards to ChE activity, no statistically significant differences were observed between the control and exposed groups ($p > 0.05$). CES activity with p-NPB as substrates was strongly inhibited by the subchronic exposure to 0.2 mg L^{-1} of the insecticide (approximately 90 percent of inhibition). Using p-NPA as substrate, inhibition of approximately 50 percent was obtained with 0.02 and 0.2 mg L^{-1} in the absolute activity (p<0.05) (Fig. 5).

4. Discussion

In this work, a partial characterization of ChE and CES of the whole organism soft tissue of *B. straminea* was first performed. Then, the effects on ChE and CES by the acute and subchronic exposure to azinphos-methyl were investigated.

Most of the works that measure ChE activity in invertebrate species express the activity normalized by the protein content (Anguiano et al., 2012; Bianco et al., 2013; Bonacci et al., 2009; Cacciatore et al., 2013; Collange et al., 2010; Gagnaire et al., 2008; González Vejares et al., 2010; Kristoff et al., 2006; Rivadeneira et al., 2013; Xuereb et al., 2007). The normalization using protein content is the only way to become independent of the prepared

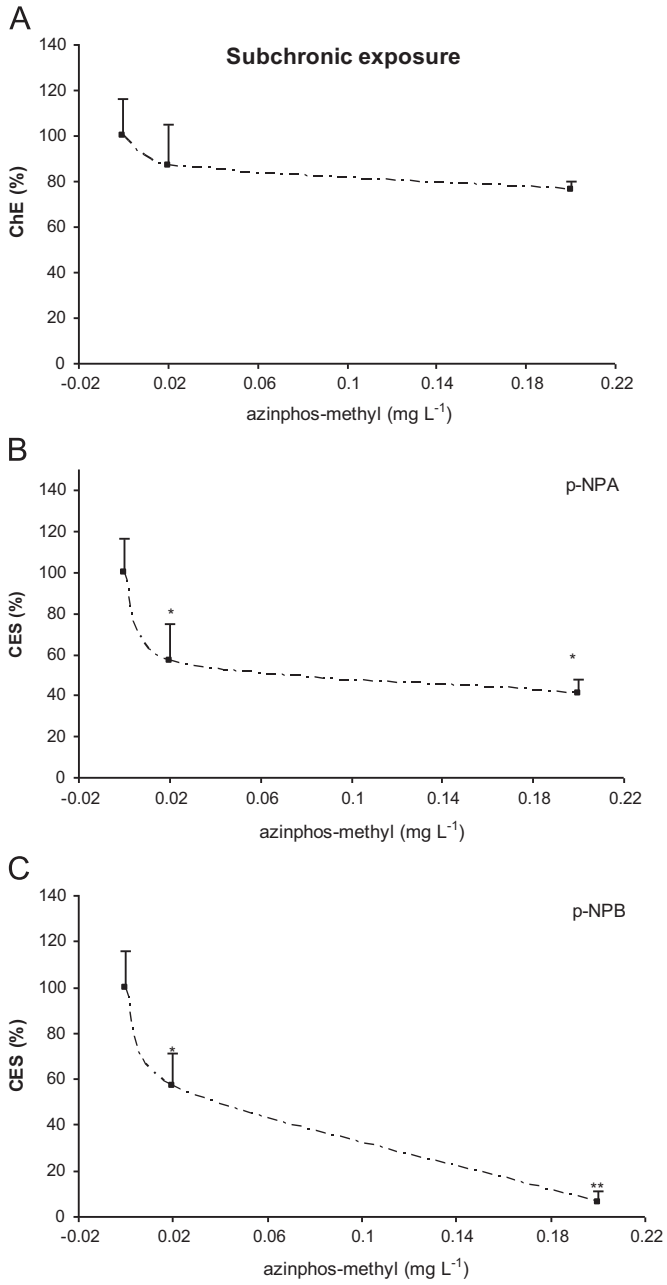

Fig. 5. Absolute ChE using AcSCh as substrate and CES using p-NPA and p-NPB as substrates in adult *Biomphalaria straminea* exposed 21 days to 0.02 and 0.2 mg $L^{-1}$of azinphos-methyl. Activities were expressed as percent of the respective control. Data represent the mean ± S.D. of at least six homogenates. The asterisk indicates significant differences from controls ($p < 0.05$) and two asterisks indicate significant differences from controls and from the other concentrations ($p < 0.05$).

homogenate. It is also the only way to know if the activity of the studied enzyme is the one that increases or decreases and not the total protein content. Generally, the specific activity of an enzyme (enzyme activity/total protein content) is used for the comparison between species, tissues, ages and sexes (Bonacci et al., 2004; Brown et al., 2004; Ferrari et al., 2004b, 2007; Kristoff et al., 2006, 2012; Di Salvatore et al., 2013; Laguerre et al.,2009). Xuereb et al. (2009) have discussed the normalization by protein content, referring to the advantages and disadvantages of its use. The authors have recommended the expression without normalization. However, they also state that if the normalization by protein content is not performed, AChE depends directly on the sample weight and the technical process used to prepare the homogenate (Xuereb et al., 2009). In the use of B-esterase activities as biomarkers, comparing control and exposed organisms, the normalization by proteins is correct if the protein content is equal in control and exposed organism. In the present work, basal B-esterase activities were presented in both forms allowing the comparison with other invertebrates. However, since organisms exposed 21 days to the pesticide showed a decrease in protein content, both acute and subchronic results were presented as percent of absolute activity respect to control.

As in most invertebrates, AcSCh was the preferred hydrolyzed substrate of *B. straminea* ChE, followed by PrSCh and being BuSCh hydrolysis very low. In other studied gastropods: *B. glabrata*, *C. gibbosa*, *P. antipodarum*, *P. corneus* and *V. piscinalis*, the percent of activity using PrSCh was greater than in *B. straminea*. ChE activity using BuSCh was very low in studied gastropods with the exception of *P. corneus* in which ChE activity was similar using BuSCh and PrSCh (Bianco et al., 2013; Cacciatore et al., 2012; Gagnaire et al., 2008; Kristoff et al., 2012, this paper).

ChE activity of *B. straminea* was significantly inhibited by the selective ChE inhibitor (eserine) and by the selective inhibitor of mammalian AChE (BW284c51). In contrast, iso-OMPA, a specific inhibitor of BChE did not inhibit ChE activity. These results suggest that ChE activity in total soft tissue of *B. straminea* corresponds to AChE. Preincubation with 1 mM of eserine inhibited 80 percent of ChE activity suggesting that approximately twenty percent of the activity corresponds to non-specific esterases. Similar results were obtained in *B. glabrata* (Kristoff et al., 2012).

Basal *B. straminea* CES was one order of magnitude higher than ChE activity. Barata et al. (2004) have also described a greater basal activity of CES in regards to ChE in the crustacean *Daphnia magna*. In *B. straminea*, V_{max} and K_m were higher for p-NPA than for p-NPB as substrate. However, efficiency (V_{max}/K_m) was greater for p-NPB in accordance with the obtained results for the gastopods *C. gibbosa*, *B. glabrata* and *P. corneus* (Bianco et al., 2013; Cacciatore et al., 2012; Kristoff et al., 2010).

In invertebrate species, differences in ChEs sensitivity to anticholinesterase insecticides were observed. The exposure of *B. straminea* to azinphos-methyl for 48 h did not cause inhibition of ChE until 10 mg L^{-1} used. Others authors have also reported the presence of ChEs relatively insensitive to OPs in some aquatic invertebrates. Gagnaire et al. (2008) have reported that the OP chlorpyrifos did not have any effect over ChE activity of *Valvata piscinalis* while ChE of *Potamopyrgus antipodarum* was inhibited by this insecticide. Mora et al. (1999) have shown that ChEs of *Mytilus galloprovincialis* and *Mytilus edulis* were poorly inhibited by OPs. Regarding azinphos-methyl, Kristoff et al. (2006) have shown in *B. glabrata* a low sensitivity of ChE; however, in contrast to *B. straminea*, inhibition appeared from 0.5 mg L^{-1} of azinphos-methyl. In other invertebrates exposed to azinphos-methyl for 48 h, strong inhibition of ChE was observed. For example, in the oligochaete *L. variegatus* and the gastropod *C. gibbosa*, IC_{50} of 6 μg L^{-1} and 0.02 μg L^{-1} were obtained (Bianco et al., 2013; Kristoff et al., 2006). *In vitro* sensitivity to eserine of ChEs may be related to *in vivo* inhibition after the exposure to an anticholinesterase insecticide. More sensitive organisms may be related to greater *in vitro* eserine inhibition of ChE. In *C. gibbosa* and in *L. variegatus* an inhibition of 90 percent with 1 μM of eserine was obtained coinciding with a NOEC on ChE of 0.001 and 1 μg L^{-1} of azinphos-methyl respectively (Bianco et al., 2013; Kristoff et al., 2006). In contrast, *B. straminea* ChE was less sensitive to eserine than *C. gibbosa* ChE and *L. variegatus* ChE and also less sensitive to inhibition by azinphos-methyl *in vivo*.

Regarding CES, these enzymes were more sensitive than ChE in *B. straminea* as in most of the studied invertebrates (Anguiano et al., 2012; Basack et al., 1998; Barata et al., 2004; Cacciatore et al., 2013;

Escartín and Porte, 1997; Kristoff et al., 2012). Bianco et al. (2013) described that those species in which CES are more sensitive than ChE to azinphos-methyl, show no signs of neurotoxicity after exposure to the OP. In contrast, *C. gibbosa* and *L.variegatus* showed CES less sensitive than ChE and the appearance of signs of neurotoxicity (Bianco et al., 2013; Kristoff et al., 2006). Several authors proposed that CES would be protecting the organisms from neurotoxic effects by competing with ChE for the binding to the OP (Bianco et al., 2013; Sanchez-Hernandez, 2007).

Concering subchronic exposures, low concentrations of azinphos-methyl caused a decrease in survival and in protein content in *B. straminea*. It has been reported that OPs can produce reactive oxygen species (ROS), effects on antioxidant defenses and oxidative stress damage in aquatic organisms (Bianco et al., 2013; Ferrari et al., 2007; Kristoff et al., 2008; Öruc et al., 2004; Peña-Llopis et al., 2002). ROS may react with critical cellular macromolecules, leading to oxidation of proteins, enzyme inactivation, lipid peroxidation, DNA damage and, ultimately, cell death (Winston and Di Giulio, 1991). Also, Peña-Llopis et al. (2002, 2003) have described that the impairment of glutathione redox status in organisms exposed to OPs was associated with a decrease in survival. Therefore, future works become necessary to elucidate if ROS contributes to the decrease in the amount of proteins and in the lethality of *B. straminea* exposed to azinphos-methyl for several days.

Furthermore, despite being the target of OPs, ChE activity was not inhibited after a subchronic exposure to low concentrations of azinphos-methyl. On the other hand, CES with p-NPB as substrate was strongly inhibited with 0.2 mg L^{-1} (90 percent). In concordance with these results, Rivadeneira et al. (2013) described in *P. corneus* exposed 14 days to 0.4 μg L^{-1} of chlorpyrifos a decrease in CES measured with p-NPB but no inhibition of ChE was reported.

In *B. straminea*, CES with p-NPB as substrate were the most sensitive biomarker for acute and subchronic exposure. Both basal CES activity and sensitivity to OPs might vary with the substrate used, probably as a consequence of differences in CE isoenzymes (González Vejares et al., 2010; Kristoff et al., 2012; Laguerre et al., 2009; Sanchez-Hernandez and Wheelock, 2009).

Besides giving protection on neurotoxicity, CES may be involved in physiological processes such as reproduction. Mikhailov et al. (1997) found in a mollusk a CE whose expression increased in the reproductive stage. Korochkin (1980) and Kubli (1992) have reported the presence of a CE in the seminal fluid of *Drosophila* involved in sperm protection and activation. González Vejares et al. (2010) also suggested that the inhibition of CE in the seminal vesicles of *Lumbricus terrestris* should be investigated in relation to its potential role in spermatozoid differentiation and maturation. Other authors (Yang et al., 1995; Mikhailov and Torrado, 1999) have proposed that CES activity in the male reproduction system may be involved in tissue protection against OP toxicity. Future works in *B. straminea* should be performed in order to investigate the possible correlation between CES inhibition and toxic effects on reproduction.

Within the invertebrates and even within gastropods, there are a wide variety of responses to OPs regarding ChEs, CES and neurotoxicity. Even within the same family, some differences can be seen in basal ChE, CES and its sensitivity to OPs existing sensitive and resistant species.

5. Conclusions

ChE of *B. straminea* was not a sensible biomarker; its activity was not inhibited by the OP even at the highest exposure concentration. On the contrary, CES activity was inhibited by azinphos-methyl. CES could be protecting ChE and therefore, protecting the organism from neurotoxicity. Despite no inhibition of ChE was observed, subchronic exposure to local environmental concentrations of azinphos-methyl produced a decrease in CES activity, protein content and survival of *B. straminea*.

This work confirms the advantages of measuring ChE and CES jointly in aquatic biomonitoring of pesticide contamination. It also shows the importance of evaluating several toxic effects in acute and subchronic exposed organisms. This becomes relevant in order to find more sensitive biomarkers and new strategies to protect non-target aquatic organisms from pesticide contamination.

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