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Acute toxicity and esterase response to carbaryl exposure in two different populations of amphipods *Hyalella curvispina*

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ABSTRACT

During the last years, a carbaryl insecticide was extensively applied in the valley of Río Negro and Neuquén, North Patagonia Argentina, to manage codling moths (*Cydia pomonella*), the main pest of pear and apple trees. In this study carbaryl susceptibility and B-esterase activity from both insecticide-exposed and non-exposed field populations of amphipods *Hyalella curvispina* were studied. Two subpopulations, one susceptible to carbaryl (LC_{50} = 213 ± 7.5 µg/L carbaryl) and one resistant to it (LC_{50} = 14,663 ± 2379 µg/L carbaryl), were found in the agricultural area selected in this study. Both populations were, in turn, more resistant to carbaryl than the population from a pristine area (LC_{50} = 11.31 ± 2.27 µg/L carbaryl). The *in vivo* 48h-IC_{50} values for cholinesterase (ChE) were close to the corresponding 48h-LC_{50} values as determined for the non-exposed population (IC_{50} = 7.16 ± 0.86 µg/L carbaryl) and for the susceptible subpopulation from the insecticide-exposed site (IC_{50} = 193 ± 99 µg/L carbaryl). Carbaryl exposure of the amphipods from the agricultural area mentioned above produced a significant decrease of carboxylesterase (CabE) activity, at a sublethal concentration (10 µg/L) that was not able to significantly inhibit ChE, thereby showing a protective role of CabE and its usefulness as early biomarker. However, at lethal concentrations the inhibition of ChE activity was higher than that of CabE. On the other hand, CabE of amphipods from the pristine site was less sensitive to carbaryl than ChE, suggesting a different participation of CabE in ChE protection in the susceptible population of *H. curvispina*. Pulse exposure to carbaryl for 2 h caused a significant inhibition of ChE in amphipods from both populations, with a fast recovery as expected for a carbamate insecticide. In conclusion, we proved that amphipods from the said agricultural area have developed resistance to carbaryl and showed the presence of two subpopulations with a different response to the insecticide. Moreover, these results reinforce the use of ChE together with CabE inhibition as indicators of carbamate exposure in *H. curvispina*.

1. Introduction

Carbaryl, a carbamate insecticide, was intensively used during the last years in the valley of Río Negro and Neuquén, North Patagonia Argentina, for pest control of fruit trees. This area produces most of the apples and pears exported by Argentina. As it has a semiarid climate, there is a channel network deriving from the Limay, Neuquén and Negro rivers in order to manage water for agriculture. These channels, as well as rivers and ponds, form a favorable habitat for invertebrate species such as amphipods of the species *Hyalella curvispina* (Amphipoda: Hyalellidae). These amphipods are macrocrustaceans that inhabit different water bodies like lakes, ponds and streams throughout Argentina and South America (Peralta, 2001). *Hyalella* species have benthic habits, being an important link in the aquatic food chain and a food source for fish and various invertebrates; in addition, these amphipods are good indicators of water quality (Tarshis, 2000;

Abbreviations: ChE, cholinesterase; CabE, carboxylesterase; DTNB, 5,5′′-dithio-2-bis-nitrobenzoate; FO, (Fernandez Oro, agricultural site); LB, (Los Barreales, lake pristine site); LC_{50}, lethal concentration fifty; IC_{50}, inhibitory concentration fifty for ChE; IU, international units; OP, organophosphorus insecticide

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Peralta, 2001).

The extensive use of carbaryl in the agricultural area of Río Negro and Neuquén has led to insecticide levels in the aquatic environment that pose a potential risk to organisms (Loewy et al., 2003, 2011). Carbamate compounds are known to inhibit type "B" esterases, which include the cholinesterases (ChE) and the carboxylesterases (CabE), by carbamylation of the active site (Escartin and Porte, 1997; Barata et al., 2004). ChE enzymes are responsible for the removal of the neurotransmitter acetylcholine from the synaptic cleft through hydrolysis (Habig and Di Giulio, 1991). Therefore, ChE inhibition leads to overstimulation of cholinergic receptors and causes hyperactivity, loss of coordination, paralysis and, eventually, death. On the other hand, CabEs are hydrolases that efficiently catalyze the hydrolysis of carboxylic esters to produce the corresponding free acids and alcohols. These enzymes participate in the detoxification of carbamate, organophosphorus (OP) and pyrethroid insecticides (Sogorb and Vilanova, 2002; Jokanovic, 2001). Inhibition of ChE and CabE activity by carbamates and OP has been used as a biomarker of pesticide exposure in many aquatic and terrestrial organisms (Fulton and Key, 2001; Galloway et al., 2002; Wheelock et al., 2005; Gagnaire et al., 2008; Laguerre et al., 2009). However, only a small number of studies have evaluated the effects of carbaryl on these enzymes in aquatic invertebrates; and no studies have been done with this insecticide in amphipods of the genus *Hyalella*. Recently, these macrocrustaceans have been used as biological models in diverse toxicological studies (Lizotte et al., 2012; García et al., 2012; Anguiano et al., 2014; Bartlett et al., 2016; Mugni et al., 2016).

Field exposure of aquatic organisms currently occurs in pulses according to pesticide application schedules and also to rainfall events. These pulses may last from minutes to hours depending on the water body and the insecticide (Cold and Forbes, 2004). The impact of such pulses of contaminants over aquatic organisms may be quite different from that of continuous exposure. Moreover, the recovery process after those pulses of exposure varies within species, populations and contaminants. Thus, in order to have a more realistic scenario, it is relevant to evaluate the effect of short pulses of pesticide exposure as well as the potential recovery in the post-exposure period in non-target organism.

One of the objectives of the present study was to evaluate the acute toxicity of carbaryl in *H. curvispina* from both insecticide-exposed and non-exposed areas. Both populations of amphipods have different susceptibility to azinphosmethyl, an anticholinesterasic insecticide, as we observed in a previous study (Anguiano et al., 2008). Another objective was to assess and compare the response to carbaryl of ChE and CabE enzymes from the two different populations of *H. curvispina*, analyzing their potential use as biomarkers. A further aim of the present study was to evaluate the time course of ChE inhibition and recovery after a short pulse of carbaryl exposure in amphipods from both pristine and agricultural areas.

2. Materials and methods

2.1. Test chemicals

The carbamate carbaryl was purchased from Chem Service (99.5% purity, West Chester, PA, USA). Acetylthiocoline iodide, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), Fast Garnet GBC salt (2-methyl-4-([2-methylphenyl]azo)benzenediazonium salt; 4-amino-2′,3-dimethylazobenzene diazotated), 1-naphthyl acetate (α-NA), α-naphthol, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3 one dibromide (BW284C5), Triton X-100 and bovine serum albumin were purchased from Sigma Chemical Company (St. Louis, MO, USA). All chemicals used were of the highest purity grade commercially available.

2.2. Organism collection from agricultural and reference sites

H. curvispina adults potentially exposed were collected from a secondary irrigation channel at Fernández Oro (FO), a town located in an intensive fruit production area in the valley of Río Negro and Neuquén rivers, North Patagonia (agricultural site). Non-insecticide exposed amphipods were collected in Los Barreales lake (LB), North Patagonia Argentina, located 80 km from the nearest fruit production area in the valley, where there is no probability of pesticide exposure and therefore the area was sconsidered as a pristine area (reference site).

2.3. Acute toxicity assays

Differences in response to carbaryl in the two populations studied were evaluated by acute toxicity laboratory tests.

Groups of ten adult amphipods were exposed to a range of carbaryl concentrations in glass bowls containing 1 L of filtered and dechlorinated water with continuous aeration (Anguiano et al., 2014). The tests were carried out according to the guidelines of the U.S. EPA for Gammarid acute toxicity test (USEPA, 1996). A high purity-certified standard of carbaryl (99.5% Chem Service, USA) was dissolved in acetone to prepare a stock standard solution. Test solutions were prepared by diluting the carbaryl stock solution with the appropriate amount of filtered and dechlorinated water in a volumetric flask to obtain the desired final concentrations (Anguiano et al., 2014; Guerreño et al., 2016). The concentrations tested in duplicate were 0.5; 1.0; 2.5; 5; 7.5; 10; 15 and 20 μg/L for amphipods from Los Barreales lake (pristine site) and 10; 65; 160; 300; 400; 1000; 2500; 6000; 9500 and 15,000 μg/L for the amphipods from FO (insecticide-treated site). The acetone vehicle was kept constant at 0.01% in all exposed and control bowls, according to the recommendation of USEPA (1996). The exact concentrations of standard solutions were verified by gas chromatography using an Agilent (Santa Clara, CA, USA) 6980 Series equipment with a HP1 column, coupled to a Nitrogen-Phosphorus detector (Ferrari et al., 2009; Loewy et al., 2011; Anguiano et al., 2014; Guerreño et al., 2016). Assays were carried out during 48 h in static conditions at a constant temperature of 18 ± 1 °C and 12:12 h (L:D) photoperiod. The tests were replicated three times on different days. Survival was monitored daily, and dead animals were recorded and removed at each time. Animals were considered to be dead if no movement of the pleopods under stereoscopic microscope was visible during a 20 s period (Anguiano et al., 2008). Amphipods were not fed during the assays. The average weight of amphipods from LB and FO was 4.2 ± 1.34 mg and 6.4 ± 2.0 mg, respectively.

2.4. Evaluation of time course of ChE recovery after a pulse of carbaryl exposure

For the time course exposure and recovery assays, adults of *H. curvispina* (10 amphipods/L) from FO and LB were exposed during 2 h to 10 μg/L of carbaryl and then transferred to an insecticide-free solution during 48 h to allow recovery. The amphipods were fed with fish food right after they were transferred into a clean medium. ChE enzymatic activity was analyzed at 0, 2 h of exposure and up to 48 h of recovery in a pesticide-free solution.

2.5. Enzyme preparation

In the acute toxicity experiment, all the amphipods surviving carbaryl exposure were immediately stored frozen until further enzyme analysis, which was performed within 72 h after the exposure was completed. After pulse exposure and recovery, the amphipods were immediately processed and ChE activity was measured. Adult amphipods were individually homogenized in 500 μL ice-cold 100 mM sodium phosphate buffer, pH 6.5 containing 0.5% Triton X-100. Each sample was centrifuged at 10,000 *g* at 4 °C for 10 min and the supernatant was immediately used as enzyme source for ChE and CabE assays.

Total protein content was determined by the method of Lowry et al. (1951). Absorbance values were measured at 750 nm and transformed into protein concentration from a bovine serum albumin standard curve.

2.6. Cholinesterase activity determination

ChE activity from each individual was determined spectrophotometrically following the method of Ellman et al. (1961). Enzymatic reactions were performed at 25 °C in 1 mL of 100 mM phosphate buffer pH 8.0 containing 0.2 mM DTNB, 0.75 mM acetylthiocholine iodide and 50 µL of amphipod 10,000 x *g* supernatant. Activity was continuously recorded at 412 nm using a UV/visible spectrophotometer (Shimadzu, Kyoto, Japan). Each supernatant was assayed in triplicate. Enzyme activity was corrected for spontaneous hydrolysis of the substrate and was expressed as IU mg protein^{-1}.

2.7. Carboxylesterase activity determination

CabE activity from each individual was determined with α-naphtyl acetate as substrate according to the method of Dary et al. (1990). The reaction was initiated by the addition of 2 mM α-NA to the supernatant diluted in the homogenization buffer containing 0.002 mM BW284C5 (an AChE inhibitor). Color development was accomplished after 15 min of enzymatic reaction by the addition of 100 µL of freshly prepared 2.5 mM Fast Garnet GBC solution. The reactions were allowed to stand at room temperature for 10 min, and the absorbance of the α-naphtol-Fast Garnet complex was read at 550 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Each enzyme preparation was assayed by triplicate. Absorbance values were transformed to µmoles of α-naphthol from a α-naphthol standard curve and expressed as IU mg protein^{-1}.

2.7.1. Statistical analysis

All results are expressed as means ± standard error (SE). Lethal concentration for 50% of the animals (LC_{50}) values for both *H curvispina* populations were calculated by non-linear regression fitting (Venturino et al., 1992). One population- and two population-sigmoidal models were fitted to data, as described in Anguiano et al. (2014).

The concentration values that inhibited fifty percent of control ChE enzyme activity (50% inhibitory concentration, IC_{50}) after 48 h of exposure to carbaryl were estimated by non-linear regression (Ferrari et al., 2004a,b). The IC_{50} values obtained for both populations of amphipods were compared by Student's *t*-test, using the standard deviations estimated by the non-linear regression (Anguiano et al., 2014).

ChE and CabE activity data after 48 h of exposure to carbaryl were analyzed by ANOVA. Fisher's Least significant differences (LSD) *post hoc* test was used to assess the statistical differences between control and exposed amphipods.

3. Results

3.1. Acute toxicity of carbaryl

The response to carbaryl of adult *H. curvispina* from both FO and LB sites is presented in Fig. 1A and B. The determined LC_{50} values are presented in Table 1. The LC_{50} value for LB was obtained from one-population model fitting to data (sigmoidal). The non-linear regression of data obtained from acute toxicity tests performed with FO amphipods reveals the coexistence of two subpopulations with different susceptibility to carbaryl, as reported in our previous studies with azinphosmethyl (Anguiano et al., 2008, 2014). Therefore, lethal concentration values for FO were estimated from the two-population model (Fig. 1A, Table 1). The most susceptible subpopulation from the agricultural site FO (18.7% of individuals) was still more resistant to carbaryl (19-fold) than the population from the reference site, LB. The other subpopulation was approximately 68-fold more resistant to this insecticide than the susceptible subpopulation from FO. Furthermore, it was approximately 1200-fold more resistant than the susceptible organisms from LB (Table 1).

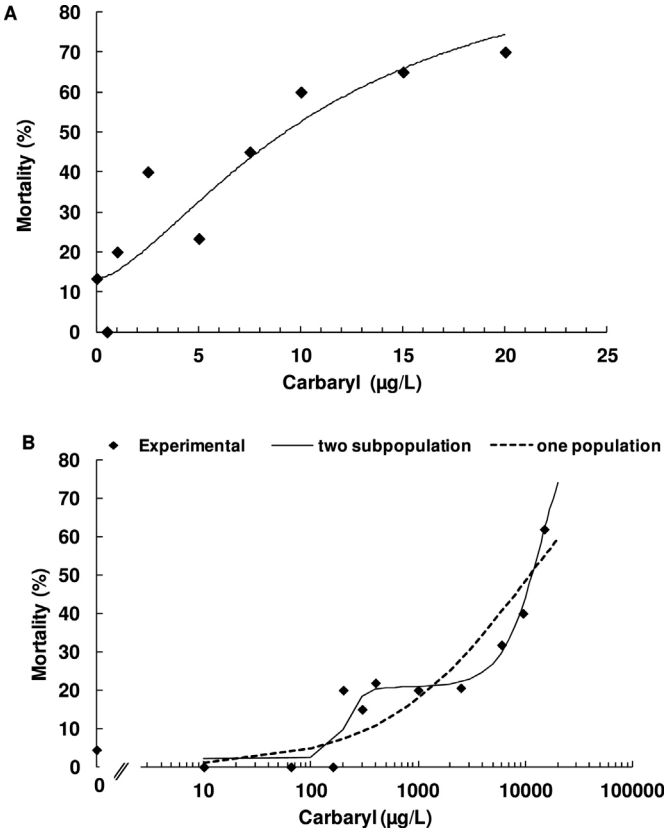

Fig. 1. Response to acute carbaryl exposure in populations of the amphipods *Hyalella curvispina* from A: reference site (Los Barreales lake: LB) and B: agricultural site (Fernandez Oro: FO). In B curves represent the nonlinear fitting of mortality data (sigmoidal) according to one- or two-population models. One-population model AIC (Akaike's coefficient; Akaike, 1974) was 158.5. Two-population model AIC was 156.9.

Table 1
Lethal concentration fifty (LC_{50}) and cholinesterase inhibitory concentration values (IC_{50}) in *Hyalella curvispina* adults from Fernandez Oro and Los Barreales lake acutely exposed to carbaryl.

Parameter	Population		
	FO		LB
	Subpopulation **A**	Subpopulation **B**	
48h-LC_{50} (µg/L)	**213 ± 7.5** P_A: 18.7%	**14,663 ± 2.379** P_B: 79.3%	**11.31 ± 2.27**
48h-IC_{50} (µg/L) for ChE	**193 ± 99**		**7.16 ± 0.86**^{*,a}

P_A: Subpopulation A proportion (%). P_B: Subpopulation B proportion (%) estimated by NRL.
^{*,a} Significantly lesser than FO 48h-IC50 value, $p < 0.005$ (Student's test performed on log transformed data).
FO: Fernández Oro (agricultural site), LB: Los Barreales lake (reference site).

Amphipods of both populations showed symptoms of toxicity due to acute exposure to carbaryl. Amphipods from LB exposed to 5–20 µg/L carbaryl were motionless and with an intermittent movement of pleopods before death occurred. Besides, alterations were observed in the swimming of amphipods exposed to insecticide concentrations between 1 and 2.5 µg/L, with respect to controls. On the other hand, exposure of FO amphipods to 300–400 µg/L carbaryl caused hyper-

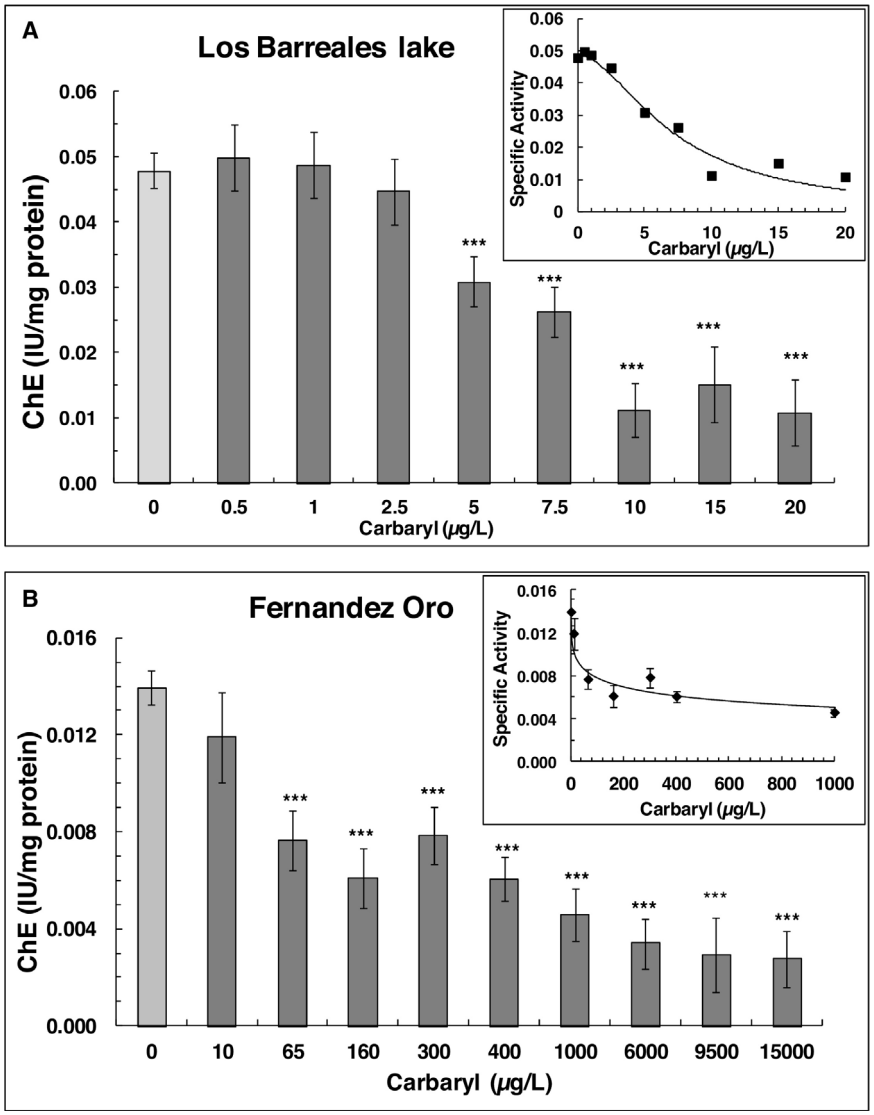

Fig. 2. Cholinesterase activity of *H. curvispina* amphipods exposed 48 h to different concentrations of carbaryl (Cb); A: pristine site (Los Barreales: LB) and B: agricultural sites (Fernandez Oro: FO). Data represent mean ± SE of six replicates. Asterisks indicate significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Inserts correspond to the non-linear fitting of sigmoidal model to data.

activity and a greater mobility compared to controls. However, the exposure to higher concentrations of carbaryl, between 9000 and 15,000 μg/L, caused general immobility of amphipods together with scarce pleopod movement. Moreover, the exposure to 1000 μg/L or higher concentrations of carbaryl caused the separation of couples during the first 24 h, while control couples were not affected.

3.2. Effect of acute carbaryl exposure on H. curvispina esterase activities

The remnant ChE activity was determined in amphipods after 48 h of exposure to different carbaryl concentrations (Fig. 2). In both populations, carbaryl concentration-dependent ChE inhibition was observed. ChE activity in amphipods from the reference area decreased with increasing concentrations of carbaryl and reached a 77% of inhibition ($p < 0.001$) with 20 μg/L insecticide (Fig. 2A). The 48h-IC_{50} value determined by non-linear regression was 7.16 ± 0.86 μg/L of carbaryl; this value was 0.6× the 48h-LC_{50} value determined for this population. Likewise, *H. curvispina* from the agricultural area (FO) showed a significant inhibition of the enzyme activity in organisms exposed from 65 μg carbaryl/L to 15,000 μg carbaryl/L compared to controls (Fig. 2B). The highest inhibition achieved, when compared to the control, was 76% ($p < 0.001$) at 15,000 μg carbaryl/L. The 48h-IC_{50} calculated was 193 ± 99 μg/L, a value 0.9× the 48h-LC_{50} estimated for the most sensitive subpopulation of FO and 75 times less than the LC_{50} value for the most tolerant subpopulation of FO. There was a significant difference between ChE-IC_{50} values for carbaryl between both LB and FO populations of *H. curvispina*, ($p < 0.05$, determined by Student's *t*-test; Table 1).

The response of CabE activity was investigated after 48 h of exposure to carbaryl. Exposure of the amphipods from LB (pristine site) to different concentrations of carbaryl did not show a significant reduction of CabE activity compared to control activity, except for the concentrations of 5 and 10 μg/L (Fig. 3A). The percentages of inhibition observed in amphipods exposed to those concentrations were 24% and 27% with respect to control value. On the other hand, exposure of amphipods from FO to 10–6000 μg/L of carbaryl led to significant inhibition of CabE activity. However, the inhibition was not concentration-dependent (Fig. 3B). The sublethal concentration of 10 μg/L carbaryl, the lowest concentration assayed, produced a 48.5% significant inhibition of CabE activity compared to the control activity ($p < 0.001$) (Fig. 3B). Unexpectedly, increasing concentrations of the insecticide did not lead to a subsequent increase of the inhibition percentage of enzyme activity. Moreover, at the two highest concentrations assayed (9500 and 15,000 μg/L carbaryl) no inhibition of CabE

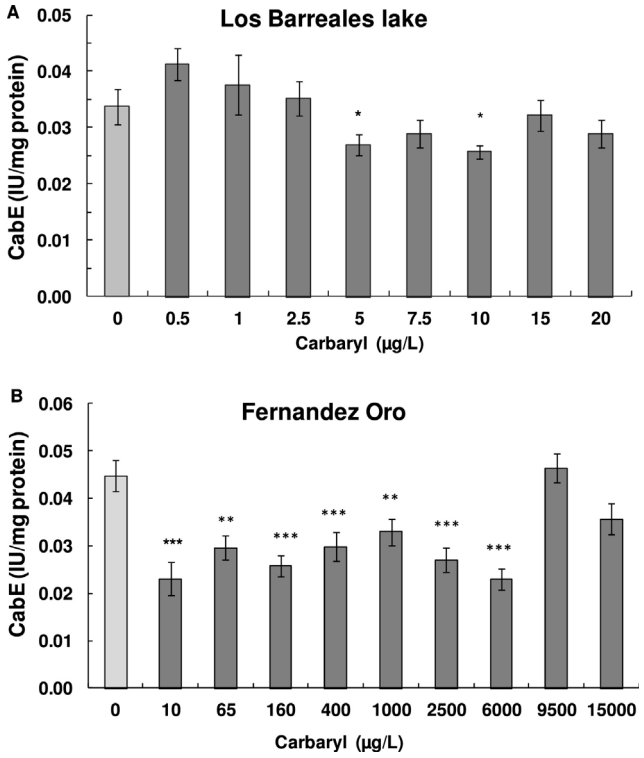

Fig. 3. Carboxylesterase activity of *H. curvispina* exposed to carbaryl for 48 h; A: pristine (Los Barreales: LB) and B: agricultural (Fernandez Oro: FO) sites. Data represent mean ± SE of six replicates. Asterisks indicate significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

activity compared with control values was observed (Fig. 3B).

3.3. Time course of ChE recovery after a pulse exposure of carbaryl

Enzyme activity of ChE of the amphipods from both sites showed a significant inhibition after 2 h of exposure to 10 µg/L carbaryl. The percentages of inhibition achieved, with respect to the corresponding control values, were 38% and 55% for LB and FO, respectively (Fig. 4A and B).

ChE activity of the amphipods from LB showed no recovery after 2 h in a pesticide-free solution but a significant recovery after 5 h in clean water, as the value obtained was significantly different from the one achieved after 2 h of exposure. However, at this point the activity was still significantly different from the control one (Fig. 4A). After 24 and 48 h of recovery, the ChE activity reached control values.

On the other hand, after 2 and 5 h in a pesticide-free solution the enzyme activity of *H. curvispina* from FO showed no statistically significant differences to the control values; although the mean values were 32% and 22% below control ones, indicating a faster recovery of ChE activity. ChE activity of FO amphipods recovered completely after 48 h in clear water.

4. Discussion

The present study demonstrates high differences in the acute toxicity of carbaryl for *H. curvispina* from two different areas, pristine and agricultural, confirmed by the highly significant differences in the corresponding LC_{50} values. In addition, this study demonstrates the presence of two subpopulations of amphipods with different susceptibility to carbaryl coexisting in the agricultural site. Amphipods *H. curvispina* from the pristine area were 20 and 1200 times more sensitive to carbaryl than subpopulations A (susceptible) and B (tolerant) from the agricultural area, indicating that these organisms were considerably more resistant than those from the pristine region. Similar results were

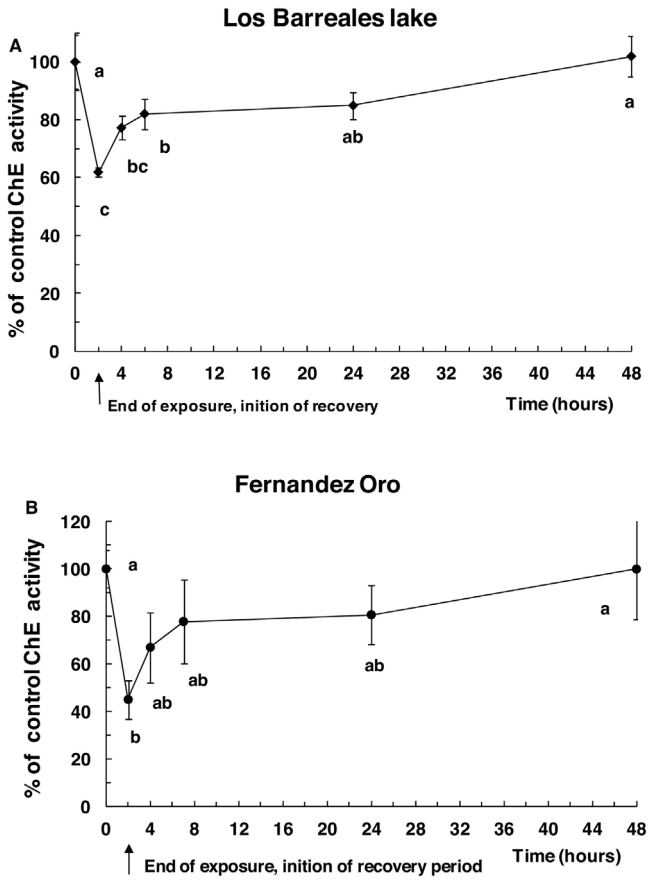

Fig. 4. Time course of cholinesterase inhibition and recovery following a pulse of 2 h of exposure to 10 µg/L carbaryl. Each point represents the mean ± S.E.M. of quadruplicate analysis. Amphipods were exposed for 2 h and then transferred to clean medium for 48 h. The arrow indicates the time at which the organisms were transferred. Different uppercase letters indicate significant differences at $p < 0.05$. A: pristine site (Los Barreales lake: LB) and B: agricultural site (Fernandez Oro: FO).

reported in our previous works (Anguiano et al., 2008, 2014) with *H. curvispina* exposed to the anticholinesterasic insecticide azinphos-methyl. As discussed in our previous studies, amphipods inhabiting irrigation channels within the agricultural area of Río Negro valley are abundant during spring and summer but diminish during winter when the irrigation is cut off. The organisms that are able to survive pesticide exposure may find refuge in the persistent ponds during autumn and winter. Once water flow returns into channels, new individuals coming from unexposed areas are introduced in these channels, representing the most susceptible subpopulation present in the irrigation channel of FO. Besides, *H. curvispina* from both pristine and agricultural sites were more tolerant to carbaryl than to the OP insecticide azinphosmethyl as revealed by the LC_{50} values calculated in a previous study (Anguiano et al., 2008) and in the present work.

In particular, amphipods from LB were 23.3-fold more tolerant to carbaryl than to azinphosmethyl, whereas subpopulations A and B from FO were 83.9- and 38.1-fold more tolerant to the carbamate insecticide than to azinphosmethyl, respectively.

Comparison of our results with 48h-LC_{50} values for carbaryl reported in the literature for some aquatic invertebrate species are indicated in Table 2. *H. curvispina* from the pristine area presented a similar susceptibility to carbaryl compared to the amphipod *Gammarus pseudolimnaeus* (Bluzat and Seuge, 1979), but was 1.8 and 2.4 times more sensitive than *Gammarus lacustris* (Sanders, 1969) and *Gammarus pulex* (Mayer and Ellersieck, 1986). The 48h-LC_{50} values of carbaryl reported for several Daphnid species were between 6.4 and 17 µg/L carbaryl (Schäfer, 2004), showing a susceptibility to the insecticide

Table 2
LC_{50} values of carbaryl in some aquatic invertebrates.

Species	48 h-LC_{50}	References
Macrocrustaceans (amphipods)		
Gammarus lacustris	22 µg/L	Sanders (1969)
Gammarus pseudolimnaeus	13 µg/L	Mayer and Ellersieck (1986)
Gammarus pulex	29 µg/L	Bluzat and Seuge (1979)
H. curvispina (L. Barreales)	11.31 µg/L	Present study
H. curvispina (FO)	A: 240 µg/L	Present study
	B: 14,633 µg/L	Present study
Microcrustaceans (cladocerans)		
Daphnia magna	17.0 µg/L	Sch & fer (2004)
Chydorus sphaericus	12.4 µg/L	Sch & fer (2004)
Daphnia obtusa	11.5 µg/L	Sch & fer (2004)
Daphnia longispina	7.8 µg/L	Sch & fer (2004)

similar to *H. curvispina* from the pristine site. On the contrary, the reported LC_{50} values of carbaryl for the gammarid species in Table 2 were 28 and 1600 times lower than the values estimated for subpopulations A and B from FO, indicating that *H curvispina* from this agricultural site had developed considerable resistance to this carbamate. Taking into account our previous results for the OP azinphosmethyl, a cross-resistance to both families of pesticides may be possible. Tolerance of non-target aquatic organisms to pesticides has been previously reported. Brausch and Smith (2009a,b) determined that the shrimp *Thamnocephalus platyurus,* living in agricultural regions, has also developed some degree of resistance to a variety of agrochemicals, including anticholinesterasic insecticides, in response to historical usage. Likewise, Clark et al. (2015) reported that amphipods *Hyalella azteca* collected from six different sites influenced by agricultural runoff were two orders of magnitude more tolerant to pyrethroid insecticides than amphipods reared in laboratory. Similarly, the microcrustacean *Daphnia magna* collected from rice fields with massive application of the OP fenitrothion developed resistance to this insecticide (Damásio et al., 2007). In addition, the macrocrustaceans *Carcinus maenas* from a moderately contaminated estuary were less sensitive to the same OP insecticide than those from the low impacted site (Rodrigues et al., 2013). The authors also reported a significant difference in the sensitivity of ChE enzyme after *in vivo* insecticide exposure. In the present work, populations of *H. curvispina* from both sites also presented a different sensitivity of ChE to carbaryl exposure as the 48h-IC_{50} value obtained for amphipods from LB was 25-fold lower than the corresponding value calculated for the amphipods from the FO agricultural site (Table 1). It should be considered that differences in ChE response after *in vivo* exposure may be caused by several factors including differences in the toxicokinetics and/or the toxicodynamics of the insecticide (Ramírez Mora et al., 2000; Rodrigues et al., 2013).

On the other hand, the relationship between the inhibition percentage of ChE and mortality is variable among different invertebrate species (Fulton and Key, 2001; Dominguez et al., 2010). In the present study, the IC_{50} value obtained for the pristine population of *H. curvispina* was slightly smaller than the corresponding LC_{50} and high inhibitory percentages of ChE could only be observed with lethal concentrations of carbaryl. In turn, the IC_{50} value obtained for FO amphipods was slightly smaller than the LC_{50} of the susceptible subpopulation but much less (76 fold) than the LC_{50} value of the tolerant subpopulation. As discussed in Anguiano et al. (2014) control ChE activity in amphipods from the agricultural site did not reflect a pattern which suggests the presence of different subpopulations in terms of enzymatic activity. In fact, control ChE activity in these individuals was lower than control ChE in the amphipods collected in the pristine site (3 times lower approximately), suggesting that there might be a baseline difference driven by pesticide (OP, carbamate) pressure. The results obtained in the present study indicate that 50% of mortality in the most tolerant subpopulation of *H. curvispina* from the agricultural site occurs with an 80% of ChE inhibition. Therefore, some individuals from the most tolerant subpopulation were able to survive high levels of ChE inhibition. ChE has been used as biomarker of OP and carbamate exposure in some aquatic invertebrates (Vioque-Fernández et al., 2007). For *H. curvispina,* the usefulness of ChE activity as an early biomarker of carbaryl exposure is stronger for the tolerant subpopulation of FO because inhibition is significant before death occurs. This was also previously observed in the azinphosmethyl tolerant population of *H. curvispina* (Anguiano et al., 2014).

On the other hand, CabE enzymes present in FO amphipods (agricultural site) were sensitive to carbaryl exposure. The sublethal concentration of 10 µg/L carbaryl caused a significant inhibition of CabE without altering ChE activity. This demonstrates a higher sensitivity of this enzyme at sublethal concentrations and a great potential for sequestering available carbaryl insecticide and hence protecting ChE from being inhibited as observed in the previous study with *H. curvispina* and azinphosmethyl (Anguiano et al., 2014) and reported by other authors for some anticholinesterasic insecticides (Barata et al., 2004; Vioque-Fernández et al., 2009; Ferrari et al., 2011). On the other hand, the lack of significant inhibition observed at the higher concentrations assayed (9500 and 15,000 µg/L carbaryl) could be related to the induction of some isoenzymes that are refractory to the insecticide or to the augmentation of total CabE activity as reported in other studies (Perez-Mendoza et al., 2000; Gong et al., 2016). It is relevant to note that in the present study, as in others (Galloway et al., 2002; Ferrari et al., 2011; Anguiano et al., 2014), a proportion of CabE activity was not inhibited by pesticide exposure regardless of the concentration used. For FO population, the highest inhibition of CabE reached was about 50%; therefore at lethal concentrations of carbaryl ChE was inhibited more than CabE.

Alternatively, CabE of amphipods from LB showed less sensitivity to carbaryl compared to ChE. Moreover, only two lethal concentrations were able to inhibit CabE. Thus CabE of the susceptible population of *H. curvispina* from the pristine site shows a lower potential to protect ChE towards this carbamate. A minor response after carbaryl exposure of CabE activity compared to ChE was also reported in the aquatic annelid *Lumbriucus variegatus* (Kristoff et al., 2010). In addition, other authors reported a similar inhibition of both B-esterases in invertebrates exposed to carbamates (Barata et al., 2004; Malagnoux et al., 2014), and also a lack of CabE inhibition (Laguerre et al., 2009). Considering the role of CabE activity in *H. curvispina* as biomarkers of carbaryl exposure, it would be more useful for FO amphipods than LB ones. In that sense, some studies performed with invertebrates proposed CabE as good indicators of anticholinesterasic exposure (Malagnoux et al., 2014; Ochoa et al., 2013; Vioque-Fernández et al., 2007).

Pulse exposure simulates the entry of the insecticide into the water bodies after application in the field. The impact of such pulses of contaminants on aquatic organisms may be quite different from a continuous exposure. In the present work, a significant inhibition of ChE activity was observed in both populations of amphipods from FO and LB exposed to 10 µg/L carbaryl for two hours. This was expected for amphipods from the pristine site but not for those from FO as the 48h-IC_{50} values are significantly different. The results point out differences in ChE response to 2 h or 48 h of exposure, and reinforce the idea that impact of pesticides pulses could be in occasions more harmful for aquatic organisms than expected, mainly under field conditions where ChE activity is relevant for animal behaviour. It is important to note that the concentration of carbaryl causing significant effect on ChE activity after a pulse exposure was 4.57 times less than the highest concentration reported by Loewy et al. (2011) in superficial and shallow groundwater within the agricultural area. This reveals a real risk to both populations of amphipods. On the other hand, recovery of ChE activity was relatively fast for both populations. Nevertheless, ChE of FO amphipods seemed to recover slightly faster to control values. This may be associated to the presence of a range of individuals with different tolerance, which may be the cause of higher dispersion

values that were observed in the exposed and recovered amphipods (compare Fig. 4A and B). ChE recovery after carbamate exposure is often faster than that after OP exposure as reported in many other species (Barata et al., 2004; Ferrari et al., 2004a,b; Kallander et al., 1997; Moulton et al., 1996) and is frequently associated to reactivation of the enzyme (Domingues et al., 2010). Besides, it depends on the pesticide concentration, duration of exposure and the percentage of esterase inhibition achieved. Similar to our results, in many invertebrates exposed to carbaryl the ChE activity recovered after a short period in clean water (Kallander et al., 1997; Barata et al., 2004; Kumar et al., 2010; Kristoff et al., 2010). However, Ramírez Mora et al. (2000) observed a lack of ChE recovery in snails when transferred to clean water although the elimination of the insecticide was rapid. Also, Kristoff et al. (2010) observed a delay in ChE recovery after carbaryl exposure in the gasteropod that was more tolerant to the insecticide.

5. Conclusions

In conclusion, the present work presents for the first time acute effects of carbaryl exposure in the amphipod *H. curvispina* and demonstrates the existence of a high difference in susceptibility to this insecticide between two populations, from pesticide exposed and non-exposed areas. Besides, this study confirms the coexistence of two subpopulations of *H. curvispina* with different susceptibilities to carbaryl in the irrigation channels of the agricultural area of Rio Negro valley, as was previously reported for the OP azinphosmethyl. This reveals an impact of pesticide use in aquatic environments within this region and highlights the need to assess adverse effects in different populations.

In addition, the results obtained after both continuous and pulse exposure to carbaryl, indicate the usefulness of using both esterases of *H. curvispina* as biomarkers of carbamate exposure/effect.

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