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ORIGINAL ARTICLE

Effects of chlorpyrifos on enzymatic systems of *Cydia pomonella* (Lepidoptera: Tortricidae) adults

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Abstract The control program of codling moth (*Cydia pomonella* L.) in the Río Negro and Neuquén Valley is intended to neonate larvae. However, adults may be subjected to sublethal pesticide concentrations generating stress which might enhance both mutation rates and activity of the detoxification system. This study assessed the exposure effects of chlorpyrifos on target enzyme and, both detoxifying and antioxidant systems of surviving adults from both a laboratory susceptible strain (LSS) and a field population (FP). The results showed that the FP was as susceptible to chlorpyrifos as the LSS and, both exhibited a similar chlorpyrifos-inhibitory concentration 50 (IC_{50}) of acetylcholinesterase (AChE). The FP displayed higher carboxylesterase (CarE) and 7-ethoxycoumarine O-deethylase (ECOD) activities than LSS. Both LSS and FP showed an increase on CarE activity after the exposure to low-chlorpyrifos concentrations, followed by enzyme inhibition at higher concentrations. There were no significant differences neither in the activities of glutathione *S*-transferases (GST), catalase (CAT) and superoxide dismutase (SOD) nor in the reduced glutathione (GSH) content between LSS and FP. Moreover, these enzymes were unaffected by chlorpyrifos. In conclusion, control adults from the FP exhibited higher CarE and ECOD activities than control adults from the LSS. AChE and CarE activities were the most affected by chlorpyrifos. Control strategies used for *C. pomonella*, such as rotations of insecticides with different modes of action, will probably delay the evolution of insecticide resistance in FPs from the study area.

Key words acetylcholinesterase; antioxidant system; chlorpyrifos; codling moth; detoxification enzymes

Introduction

At the time of pest control, the physiological state of an insect population might be nonuniform and therefore, a concentration that is lethal to one physiological state of the species might be sublethal to another (Gressel, 2011). Among other differences, the detoxification capacity varies with developmental stage (Terriere & Yu, 1974). Moreover, the stress caused by the pesticide might enhance both the mutation rates and the activity of the detoxification system, which might lead to pesticide resistance (Terriere & Yu, 1974; Gressel, 2011). A high insecticide pressure could select for monogenic resistance, while low insecticide pressure could lead to increasingly higher resistance levels as

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a result of many incremental changes (Gardner *et al.*, 1998).

Organophosphates have been one of the most important classes of insecticides used for crop protection (Gupta, 2006). The primary target of organophosphates is the enzyme acetylcholinesterase (AChE). However, these insecticides also have high affinity for other serine hydrolases, such as carboxylesterases (CarE) (Tang *et al.*, 2006). Some organophosphates are bioactivated to oxons by the cytochrome P450 monooxygenases (CYP450) and they are also responsible for pesticide detoxification including organophosphates. The CYP450 enzyme system catalyzes different types of chemical reactions with a substrate specificity that can vary from broad to narrow (Feyereisen, 1999). There is also increasing evidence that organophosphate insecticides generate free radical intermediates leading to the disturbance of the insect homeostasis (Büyükgüzel, 2009). Reactive oxygen species (ROS) could mediate cellular and extracellular injury via the destruction of membranes, lipids, lipoproteins, or alteration of critical enzyme systems, proteins and ion channels. Thus, organophosphates might compromise cellular function amplifying the initial lesion. Antioxidant defenses include enzymes and scavenger molecules such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferases (GST), reduced glutathione (GSH), etc. SOD is the major intracellular antioxidant enzyme in aerobic cells. Both Cu/Zn-SOD in the cytoplasm and Mn-SOD in the mitochondria reduce fast and specifically the superoxide radicals to hydrogen peroxide which is very rapidly converted to water and oxygen by CAT. GST catalyzes the conjugation of GSH to a variety of compounds containing an electrophilic center in all tissues and organisms examined to date. GSH is also capable of participating in nonenzymatic conjugation with some compounds because the thiol group of cysteine is able to donate a reducing equivalent to other unstable molecules, such as ROS (Rice-Evans & Burdon, 1993). Other authors have inferred that the oxidative stress produced by some organophosphates would contribute to the observed neurotoxic effects (Büyükgüzel, 2006, 2009; Wu *et al.*, 2011).

The codling moth (*Cydia pomonella* L.), the major pest of apples and pears throughout the world, has developed resistance to organophosphates (Varela *et al.*, 1993; Charmillot *et al.*, 2002; Fuentes-Contreras *et al.*, 2007), pyrethroids, (Varela *et al.*, 1993; Bouvier *et al.*, 1998), insect growth inhibitors (Charmillot *et al.*, 1999, 2002), insect growth regulators (Knight *et al.*, 2001; Charmillot *et al.*, 2002), and biological products (Eberle & Jehle, 2006). Both detoxifying enzymes (Sauphanor *et al.*, 1997; Bouvier *et al.*, 1998, 2002; Fuentes-Contreras *et al.*, 2007; Rodriguez *et al.*, 2011) and target insensitivity (Bouvier *et al.*, 2001; Cassanelli *et al.*, 2006) have been described as resistance mechanisms. Codling moth is also the most important fruit-tree pest in the Río Negro and Neuquén Valley from Northern Patagonia (Argentina). A brief history of pesticide usage against *C. pomonella* in the last 35 years has included an intensive and massive use of pyrethroids for most of a decade until control failures to this pesticide class were reported. Since mid-1990 decade, the recommended and most frequently used pesticide was azinphosmethyl. A regional-scale survey showed that by the beginning of the new century 70% of the applied pesticides corresponded to this organophosphate (Anguiano & Pechen de D'Angelo, 2007). This organophosphate and other conventional pesticides were gradually replaced by the initiation of the National Program of Codling Moth (*C. pomonella*) Control in the Patagonia Region during 2006. This program consists of an integrated pest management (IPM) including, chemical, biological and cultural control (Villarreal *et al.*, 2010). The main choice of chemical sanitation includes some organophosphates (chlorpyrifos, azinphosmethyl, phosmet) neonicotinoids (acetamiprid and thiacloprid), inhibitor of chitin byosintesis (novaluron), chloride channel activators (emamectine benzoate), activator of ryanodine receptors in muscle cells (rynaxypyr), nicotinic acetylcholine receptor allosteric activator (spinetoram) and biopesticides (*Cydia pomonella* granulovirus). Even though large-scale farmers, who are generally fruit exporters, implement a sustainable use of pesticides, small-scale farmers do not always follow this strategy. Field populations (FPs) of codling moth from the area have shown decreased sensitivity to azinphosmethyl, acetamiprid, and thiacloprid (Soleño *et al.*, 2008, 2012; Cichón *et al.*, 2013). Taking into account the current use of chlorpyrifos in the *C. pomonella* control, the first objective of this study was to evaluate whether the decreased sensitivity to azinphosmethyl might be extended to chlorpyrifos in the studied FP. Although IPM is intended to neonate larvae, adults might be exposed to sublethal pesticide concentrations that might enhance the activity of the detoxification system. Therefore, the second objective was to compare the biochemical response of surviving adults exposed to chlorpyrifos from laboratory susceptible strain (LSS) and a FP.

Materials and methods

Chemicals

The organophosphate chlorpyrifos (99.08% pure) was purchased from AccuStandard Inc., New Haven, CT,

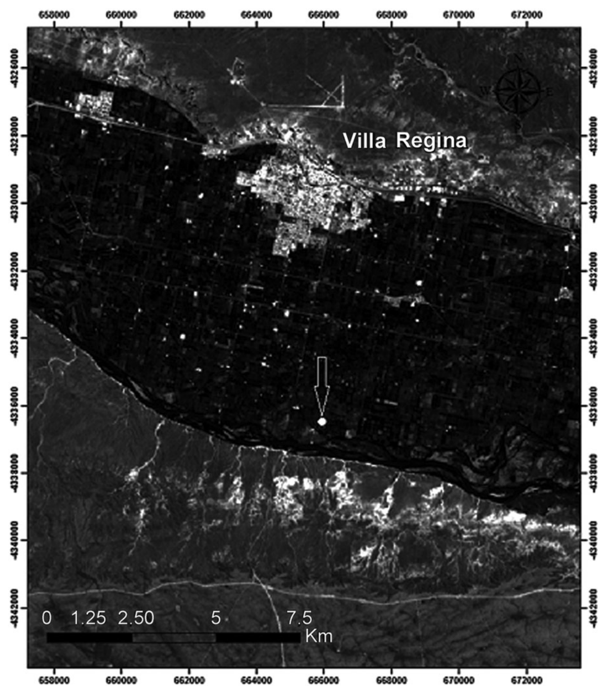

Fig. 1 Regional satellite image of the study area. The arrow points out the collection site of *Cydia pomonella* FP. 80 × 92 mm (300 × 300 DPI)

USA. Reduced glutathione (GSH), epinephrine, 7-ethoxycumarine (7-EC), 7-hidroxycoumarine (7-OHC), 1-chloro-2,4-dinitrobenzene (CDNB), α-naphthyl acetate (α-NA), α-naphthol (α-N), Fast Garnet GBC salt, 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C5), Triton X-100, 5,5'-dithiobis 2 nitrobenzoic acid (DTNB), acetylthiocholine iodide (ATChI) and bovine serum albumin were purchased from Sigma Chemical Co., Saint Louis, MO, USA. All the other reagents used were of analytical grade.

Test species

The FP was collected from a small-scale farmer in the core of the valley at the locality of Villa Regina (Río Negro) (39°09′41.09″S, 67°04′45.84″ W) (Fig. 1). Apple and pear production constitutes one of the most important economic resources of the Río Negro and Neuquén Valley and a variety of pesticides are used to control *C. pomonella* as it was described above. There is no precise information about the phytosanitary history of this population.

Diapausing larvae from FP were collected using corrugated cardboard bands during the summer (from late December 2012 to March 2013). Larvae were transferred to clean corrugated paper and stored in a telgopor box at 4 °C for 3 months and further placed under appropriate conditions for adult emergence (25 °C, 70% RH and a photoperiod of 16 : 8 L : D h). Susceptible adults from LSS were used as reference strain, which was established in June 1991 from 7000 to 8000 diapausing larvae collected from an abandoned apple orchard. The colony has been kept without pesticide pressure under controlled temperature (25 °C), photoperiod (16 : 8 L : D h) and 70% RH.

Bioassays

One milliliter of the insecticide dissolved in acetone was evenly applied on the internal surface of glass cups (212 mL) and their lids and left to dry for 1 h at room temperature (Knight, 2010). Moths of 1−2 d old from both FP and LSS were exposed for 24 h to dry residues of chlorpyrifos under controlled temperature (25 °C) and photoperiod (16 : 8 L : D h). Control groups were placed in bottles coated with 1 mL of acetone. At least 3 replicates of 10 adults each per concentration were used to estimate the lethal concentration fifty (LC_{50}). Mortality was recorded after 24 h of exposure and it was defined as the incapacity to sustain a coordinated flight as individuals from the control group.

On the other hand, a total of 8 dilutions were arranged starting with the maximum level rate of active ingredient used in field for *C. pomonella* control (about 1.2 kg ai/ha) to a concentration where AChE inhibition was less than 50%. The following concentrations were used: 0.0625, 0.25, 1, 3.9, 15.62, 62.5, 250 and 500 mg/L. All surviving adults (including those with intoxication symptoms) were used for biochemical studies. The number of surviving individuals from each concentration was high enough to obtain at least 4 replicates of 4 individuals each.

Enzyme preparation

Batches of 4 heads each were homogenized in 1200 μL of 0.1 mol/L phosphate buffer (pH 7.5) plus 0.5% Triton X-100 with an electrical homogenizer PRO 200. Homogenates were centrifuged at 5204 × g for 10 min at 4 °C. The supernatants were used as source of AChE activity. Mean AChE activity from each group was obtained from at least 4 independent replicates.

Batches of thoraxes and abdomens from 4 individuals were each homogenized in 1100 μL of 143 mmol/L potassium phosphate buffer (pH 7.5) + 6.3 mmol/L EDTA with an electrical homogenizer PRO 200. The homogenates were centrifuged at 10000 × g for 15 min at 4 °C. From the supernatants, aliquots of 120 and 10 μL were

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separated for GSH content and protein determination, respectively. The remaining volume of each homogenate was centrifuged at 16000 × *g* for 20 min at 4 °C and the resulting supernatants were distributed into aliquots for SOD (300 μL), CAT (100 μL), GST (100 μL), CarE (150 μL) and protein content determination (50 μL). GSH content was immediately determined, and the enzyme source for SOD, CAT, GST, CarE as well as protein content determination was frozen at -80 °C before biochemical assays were performed. Mean enzyme activity from each group was obtained from at least 4 independent replicates. Fresh thoraxes and abdomens of 1−2 d adults were individually used for ECOD determination.

Esterase activity

Acetylcholinesterase activity AChE activity was measured in a final volume of 200 μL, with final concentrations of 1.5 mmol/L ATChI and 0.5 mmol/L of DTNB as described by Wirth and Georghiou (1996) with minor modifications. One hundred microliters of 100 mmol/L sodium phosphate buffer (pH 7.5) plus 0.5% Triton X-l00 were loaded into the first row of the plate. One hundred microliters of each supernatant were loaded by duplicate in the microtiter plate. After that, 100 μL of developing solution (9.4 mL distilled water, 0.2 mL 100 mmol/L ATChI, 0.4 mL 12 mmol/L DTNB) was added to each well and incubated at 30 °C. Absorbance was read in a Microplate Reader at 405 nm at 0, 15, and 30 min after addition of the developing solution. AChE activity was expressed as μmoles/min/mg of protein using a molar extinction coefficient of 13.6×10^3mol/L/cm.

Carboxylesterase activity CarE activity was determined using α-NA as substrate following the technique of Dary *et al.* (1990). Fifty microliters of 100 mmol/L sodium phosphate buffer (pH 6.5) plus 0.5% Triton X-l00 were loaded into the outside wells of the plate. Aliquots of 50 μL of each supernatant were loaded by duplicate in a 96-well microtiter plate held on ice. The reaction was initiated in each well by the addition of 100 μL of the developing solution with final concentrations of 2 mmol/L α-NA and 0.002 mmol/L of BW284C5 (an AChE inhibitor). The microplate was then removed from ice and its content mixed, allowing esterase hydrolysis to continue for 15 min at 25 °C. After that, 100 μL of 2.5 mmol/L Fast Garnet salt was added. Absorbances were recorded 10 min later at 550 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Absorbance values were transformed into μmol of α-N from an α-N standard curve (2−20 nmol) and activity was expressed as μmoles α-N/min/mg of protein.

Ethoxycoumarine O-deethylase activity

Cytochrome P450 monooxygenase (CYP450) activity was assessed by a fluorometric protocol using 7-EC as substrate and expressed as 7-ethoxycoumarine *O*-deethylase activity (ECOD) using black flat bottom 96 multiwell microplate (Bouvier *et al.*, 2002). Thoraxes and abdomens from each adult were cut into 3 fragments to maximize the enzyme source recovery and placed altogether in a well containing 50 μL of 50 mmol/L phosphate buffer (pH 7.2). The reaction was initiated by the addition of 50 μL of developing solution containing 7-EC at a final concentration in the well of 0.2 mmol/L. After 4 h incubation at 30 °C, the reaction was stopped with 100 μL of 1 : 1 glycine (pH 10.4)/ethanol solution with a glycine well concentration of 0.017 mmol/L. Subsequently, plates were centrifuged at 1500 × *g* during 1.5 min to descend the biological tissues. Fluorescence was determined at 380 nm excitation and 460 nm emission in a spectrofluorometer (Wallac 1420 Multilabel, Turku, Finland). A standard curve was measured in every plate with 7-OHC (0.0125−1 nmol) and ECOD activity was expressed as pg of 7-OHC/min/adult.

Antioxidant system

Glutathione *S*-transferase activity GST activity was assayed using CDNB (0.5 mmol/L in acetonitrile) as substrate (Habig *et al.*, 1974). The reaction mixture in a final volume of 1 mL consisted of 0.92 mL of 0.1 mol/L phosphate buffer (pH 6.5), 20 μL of the enzyme source, 10 μL of CDNB (50 mmol/L) and 50 μL of GSH (2.5 mmol/L). Absorbance was recorded continuously at 340 nm for 1 min in a UV/visible spectrophotometer at 25 °C (Shimadzu, Kyoto, Japan). Rate measurements were corrected for the nonenzymatic reaction and transformed into μmol of CDNB conjugated/min/mg of protein using the extinction coefficient 9.6 mmol/L/cm.

Catalase activity CAT activity was determined by recording the decomposition of H_2O_2 at 240 nm (Beers & Sizer, 1952). The reaction was performed in 3 mL of 50 mmol/L sodium phosphate buffer (pH 7.0) containing 25 mmol/L H_2O_2 with initial absorbance of 1.0. Ten microliters of supernatant were added to initiate the catalyzed reaction. Absorbances were recorded at 240 nm for 1 min in an UV/visible spectrophotometer at 25 °C. Specific activity was expressed as mmoles/min/mg of protein of protein using a molar extinction coefficient of 40 mmol/L/cm.

Superoxide dismutase activity SOD activity was determined by its ability to inhibit the auto-oxidation of

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epinephrine (Misra & Fridovich, 1972). The reaction volume was 1 mL containing 50 mmol/L buffer glycine (pH 10.2), 60 mmol/L epinephrine (pH 2.0), and different volumes of supernatant (10, 20, 40, and 60 μL). Absorbances were recorded at 480 nm for 1 min in an UV/visible spectrophotometer at 30 °C. One unit of SOD activity was defined as the amount of enzyme (mg of protein) necessary to decrease by 50% the rate of epinephrine auto-oxidation.

GSH content GSH content was measured by the method of Ferrari *et al.* (2009) (modified from Ellman, 1959). Fresh 120 μL of supernatant were mixed with 120 μL of 10% trichloroacetic acid and centrifuged at 10000 $\times$ g for 10 min at 4 °C. Then, a mixture of 1 mL of the Ellman reagent (1.5 mmol/L 5, 5'-dithiobis [2 nitrobenzoic acid] in 0.25 mol/L sodium phosphate buffer, pH 8) plus 100 μL of supernatant were left to settle for 5 min at room temperature and absorbances were recorded at 412 nm. Reduced GSH was quantified using a standard curve of GSH (1−8 nmol) and expressed as nmoles/mg of protein.

Protein determination

Protein concentration was determined using bovine serum albumin as the standard curve (5−40 μg) (Lowry *et al.*, 1951).

Statistical analysis

Dose-response bioassay data from the LSS and the FP were subjected to PROBIT analysis and the regression lines were compared by Likelihood Ratio Chi-square Test using Dr. Sakuma's PriProbit NM software (http://www.ars.usda.gov/Services/docs.htm?docid=11281). Values of LC_{50} and LC_{95} between LSS and FP were significantly different if their 95% confidence limits (95% CL) did not overlap. Both AChE and CarE IC_{50} values and their Standard Error (SE) were calculated using the GraphPad 5.0 software (Graphpad Software, San Diego, CA, USA). The data were tested for normality and homogeneity of variance using Kolmogorov–Smirnov test and the Levene median test; respectively. Comparisons within each population that satisfied the above 2 assumptions, were analyzed by one-way ANOVA with Bonferroni's multiple comparison test. When data failed normality or homogeneity of variance tests, they were transformed in order to stabilize variability. When common transformations were unsuccessful, data were analyzed by the nonparametric Kruskal–Wallis test followed by the Dunn's multiple comparison test. At last, differences between FP and LSS control groups were analyzed by unpaired Student's *t*-test or unpaired *t*-test with Welch's correction. Both comparisons within each population and between LSS and FP control groups were analyzed with STATISTICA version 7 (StatSoft Inc. 2004).

Results

Bioassays

Chlorpyrifos concentration-mortality data from both LSS and FP fitted the PROBIT model as indicated by the goodness-of-fit test ($P > 0.05$). Both LC_{50} and LC_{95} obtained from the PROBIT analysis are presented in Table 1. Based on overlapping of their 95% CL, neither the LC_{50} nor the LC_{95} from LSS (0.65 and 3.85 mg/L) and FP (0.36 and 4.80 mg/L) were significantly different. Moreover, the regression lines were parallel (Likelihood Ratio Chi-square Test: $\chi^2 = 3.45$; df = 1; $P = 0.063$). No adult mortality was observed in glass cups coated with acetone only.

Esterase activity

Acetylcholinesterase activity The response of AChE activities to several concentrations of chlorpyrifos on adults of *C. pomonella* from both LSS and FP is shown in Fig. 2A. The AChE activities (mean ± SE) from control groups LSS (0.036 ± 0.0031 μmoles/min/mg of protein) and FP (0.041 ± 0.0027 μmoles/min/mg of protein) were not statistically significant (Student's *t*-test: $t_8 = 1.13$; $P = 0.29$). A concentration-dependent inhibition of AChE by chlorpyrifos was observed in the LSS with an IC_{50} (± SE) of 0.39 ± 0.10 mg/L. Even though AChE inhibition was more than 95% from 3.9 mg/L, the enzyme activity became statistically significant at 15.62 mg/L (Kruskal–Wallis: $H = 36.78$; df = 8; $P = 0.00$). The IC_{50} (± SE) value for chlorpyrifos in the FP was 0.35 ± 0.094 mg/L and, AChE activity was significantly inhibited from 15.62 mg/L (Kruskal–Wallis: $H = 39.88$; df = 8; $P = 0.00$). Furthermore, at the chlorpyrifos concentrations of 250 and 500 mg/L, the enzyme was completely inhibited.

Carboxylesterase activity Mean (± SE) CarE activity from FP (0.070 ± 0.0068 μmoles/min/mg of protein) was significantly higher (Student's *t*-test with Welch's correction: $t_4 = 3.62$; $P = 0.022$) than the one from LSS (0.031 ± 0.0034 μmoles/min/mg of protein) (Fig. 2B). Both LSS and FP showed a similar profile of CarE activity after the treatment with increasing concentrations of chlorpyrifos. Adults from LSS treated with 0.0625 and 0.25 mg/L showed a significant increase in the CarE activity (one-way ANOVA: $F_{8,30} = 130.60$;

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Table 1 Toxicity of chlorpyrifos in adults of *Cydia pomonella* from LSS and FP.

Population	*n*	LC_{50} (CL) (mg/L)	LC_{95} (CL) (mg/L)	Slope	χ^2	df
LSS	217	0.65 (0.35–1.00)	3.85 (1.97–31.49)	2.12	11.07	5
FP	321	0.36 (0.24–0.48)	4.80 (3.10–9.42)	1.42	5.74	5

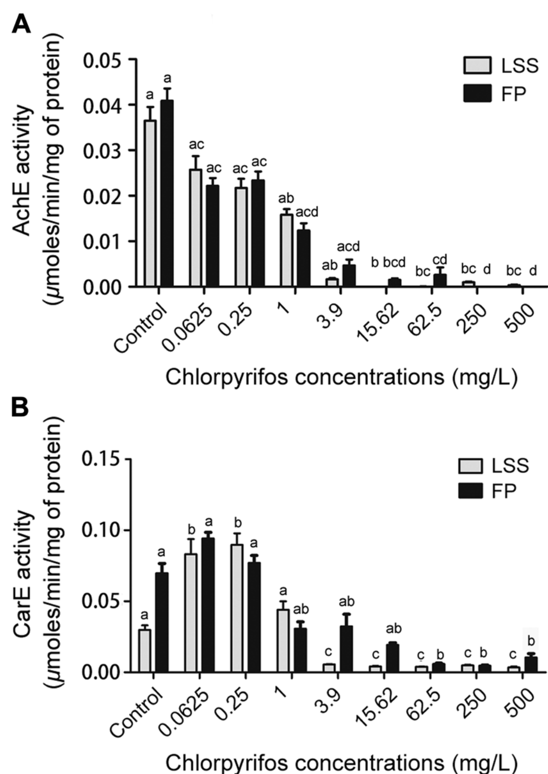

Fig. 2 Enzymatic activity of (A) AChE and (B) CarE of adults of *Cydia pomonella* from both LSS and FP exposed to chlorpyrifos. Each column and bar represents the mean and SE of at least 4 independent replicates. AChE activities from both LSS and FP were analyzed by the nonparametric Kruskal–Wallis test followed by the Dunn's multiple comparison test. Log-transformed data of CarE activities from LSS were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. CarE activities from FP were analyzed by the nonparametric Kruskal–Wallis test followed by the Dunn's multiple comparison test. Within each population, columns labeled with a different letter are significantly different ($P < 0.05$).

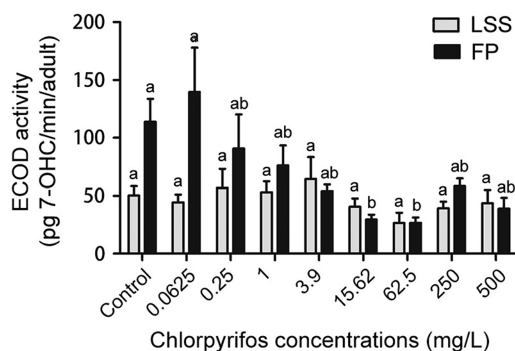

Fig. 3 Effect of chlorpyrifos on ECOD activities of adults of *Cydia pomonella* from both LSS (n = 99) and FP (n = 201). Each column and bar represents the mean CYP450 activities and SE. CYP450 activities from LSS were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. CYP450 activities from FP were analyzed by the nonparametric Kruskal–Wallis test followed by the Dunn's multiple comparison test. Within each population, columns labeled with a different letter are significantly different ($P < 0.05$).

P = 0.00). The CarE activities were 127.44% and 156.16% higher than the control group at chlorpyrifos concentrations of 0.0625 and 0.25 mg/L, respectively. Subsequently, adults from this strain treated with 1 mg/L showed no significant differences in mean CarE activity with respect to the control group. From 3.9 mg/L chlorpyrifos, the enzyme activity was drastically and significantly inhibited compared to the control group (more than 82% inhibition). The IC_{50} (± SE) value for chlorpyrifos in LSS was 2.21 ± 1.24 mg/L. Although the differences were no significant, adults from FP treated with 0.0625 and 0.25 mg/L showed an enhancement of the CarE activity of 35.62% and 10.37%, respectively. Groups of treated adults with chlorpyrifos concentrations from 62.5 mg/L showed significant CarE inhibition (Kruskal–Wallis: H = 36.07; df = 8; P = 0.00). The IC_{50} (± SE) value for chlorpyrifos in FP was 4.33 ± 2.6 mg/L.

Ethoxycoumarine O-deethylase activity

Mean (± SE) ECOD activities from LSS (50.26 ± 8.03 pg 7-OHC/min/adult) and FP (113.90 ± 19.80 pg 7-OHC/min/adult) were significantly different (Student's t-test with Welch's correction: t_{33} = 2.97; P = 0.0054) (Fig. 3). The ECOD activities in LSS between control and treated groups were no statistically significant (one-way ANOVA: $F_{8,90}$ = 0.73; P = 0.66). On the other hand,

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ECOD activities from FP treated groups showed a different profile compared to LSS. There was a trend towards a decrease in mean ECOD activity as chlorpyrifos concentrations increased. Moreover, the differences between treated adults at 15.62 and 62.5 mg/L (Kruskal–Wallis: $H = 30.58$; df = 8; $P = 0.00$) were statistically significant from the ones in the control group. Almost 60% of FP exhibited individual ECOD activities above the mean upper 95% CL (23.72 pg 7-OHC/min/adult) of LSS.

Antioxidant system

Glutathione *S*-transferase activity Mean GST activities from control and chlorpyrifos-treated adults of *C. pomonella* from both LSS and FP is presented in Fig. 4A. Mean activities of GST from LSS (0.086 ± 0.013 μmol of CDNB conjugates/min/mg of protein) and FP (0.065 ± 0.0066 μmol of CDNB conjugates/min/mg of protein) were not significantly different (Student's *t*-test: $t_7 = 1.31$; $P = 0.22$). There was about 50% GST increased activity in LSS treated with 0.25; 250 and 500 mg/L chlorpyrifos. Despite the variability, none of the concentrations of chlorpyrifos evaluated on the LSS (one-way ANOVA: $F_{8,33} = 1.87$; $P = 0.098$) and FP (one-way ANOVA: $F_{8,34} = 3.48$; $P = 0.049$) elicited any significant change of GST activities compared to controls.

Catalase activity No significant differences were found on mean (± SE) CAT activities between LSS (0.14 ± 0.028 mmoles/min/mg of protein) and FP (0.11 ± 0.0075 mmoles/min/mg protein) (Student's *t*-test with Welch's correction: $t_4 = 1.09$; $P = 0.33$) (Fig. 4B). Moreover, CAT activities from adults of both LSS (one-way ANOVA: $F_{8,32} = 0.94$; $P = 0.49$) and FP (one-way ANOVA: $F_{8,34} = 2.97$; $P = 0.012$) were not significantly different from the control.

Superoxide dismutase activity The effect of several concentrations of chlorpyrifos on SOD activities in adults of *C. pomonella* from both LSS and FP is shown in Fig. 4C. Mean activities (± SE) of SOD from LSS (8.20 ± 0.74 U/mg of protein) and FP (7.20 ± 0.68 U/mg of protein) were not significantly different (Student's *t*-test: $t_6 = 0.95$; $P = 0.37$). Neither LSS (one-way ANOVA: $F_{8,29} = 2.91$; $P = 0.016$) nor the FP (one-way ANOVA: $F_{8,31} = 1.34$; $P = 0.26$) showed statistically significant effects of chlorpyrifos treatments on SOD activities compared to control group.

GSH content Mean content (± SE) of GSH from LSS (14.45 ± 2.30 nmoles/mg protein) and FP (16.47 ± 1.15 nmoles/mg of protein) were not significantly different (Student's *t*-test: $t_8 = 0.78$; $P = 0.45$) (Fig. 4D). A significant increase of GSH content was observed on LSS treated with chlorpyrifos at the concentrations of 0.25, 250, and 500 mg/L compared to the control mean (one-way ANOVA: $F_{8,32} = 9.24$; $P = 0.00$). Conversely, nearly all chlorpyrifos treatments on FP significantly decreased mean GSH compared to control group (one-way ANOVA: $F_{8,35} = 26.56$; $P = 0.00$).

Discussion

The results from the current study showed that the FP was as susceptible to chlorpyrifos as the LSS. Previous research on neonates from this FP showed significant lower mortality to azinphosmethyl, acetamiprid and thiacloprid compared to the LSS. At the diagnostic concentrations (LC_{99}) of azinphosmethyl (2 mg/L), acetamiprid (0.7 mg/L) and thiacloprid (1 mg/L), the percentage of FP mortalities was 16.9, 51.1, and 14.8; respectively (Cichón *et al.*, 2013). Decreased toxicity to azinphosmethyl was also found in diapausing larvae of *C. pomonella* across the Río Negro and Neuquén Valley compared to the LSS (Soleño *et al.*, 2008, 2012). Although life-stage variation in susceptibility to insecticides has been described in *C. pomonella* (Bouvier *et al.*, 2002), resistance to insecticides is expressed in both adults and neonates (Varela *et al.*, 1993) as well as in diapausing larvae (Bouvier *et al.*, 1998). For example, *C. pomonella* from a pear orchard showed 4.6 and 6.2 fold resistance to azinphosmethyl at the LC_{50} level in larvae and adult bioassay, respectively (Varela *et al.*, 1993). Cross-resistance between azinphosmethyl and other organophosphates such as phosalone (Reyes *et al.*, 2007), chlorpyrifos-ethyl (Reyes *et al.*, 2015), and phosmet (Mota-Sanchez *et al.*, 2008) has been reported in codling moth populations. Other authors have found negatively correlated cross-resistance for both chlorpyrifos and methyl parathion when the azinphosmethyl-resistant colonies were compared to the susceptible strain.

Results from this study showed no significant differences in mean AChE activity between LSS and FP control and, both LSS and FP exhibited similar IC_{50} values of chlorpyrifos. Up to day, codling moth resistance to organophosphates by AChE insensitivity has only been described in a laboratory-selected strain (Cassanelli *et al.*, 2006).

In this study, we found significantly higher mean CarE activity in the FP compared to the LSS (see control groups from Fig. 2B). Previous studies on several diapausing larvae populations at the Río Negro and Neuquén Valley have also shown significantly higher CarE activity than the LSS (Soleño *et al.*, 2008, 2012). However, it is worth noting that the increase percentage of mean CarE

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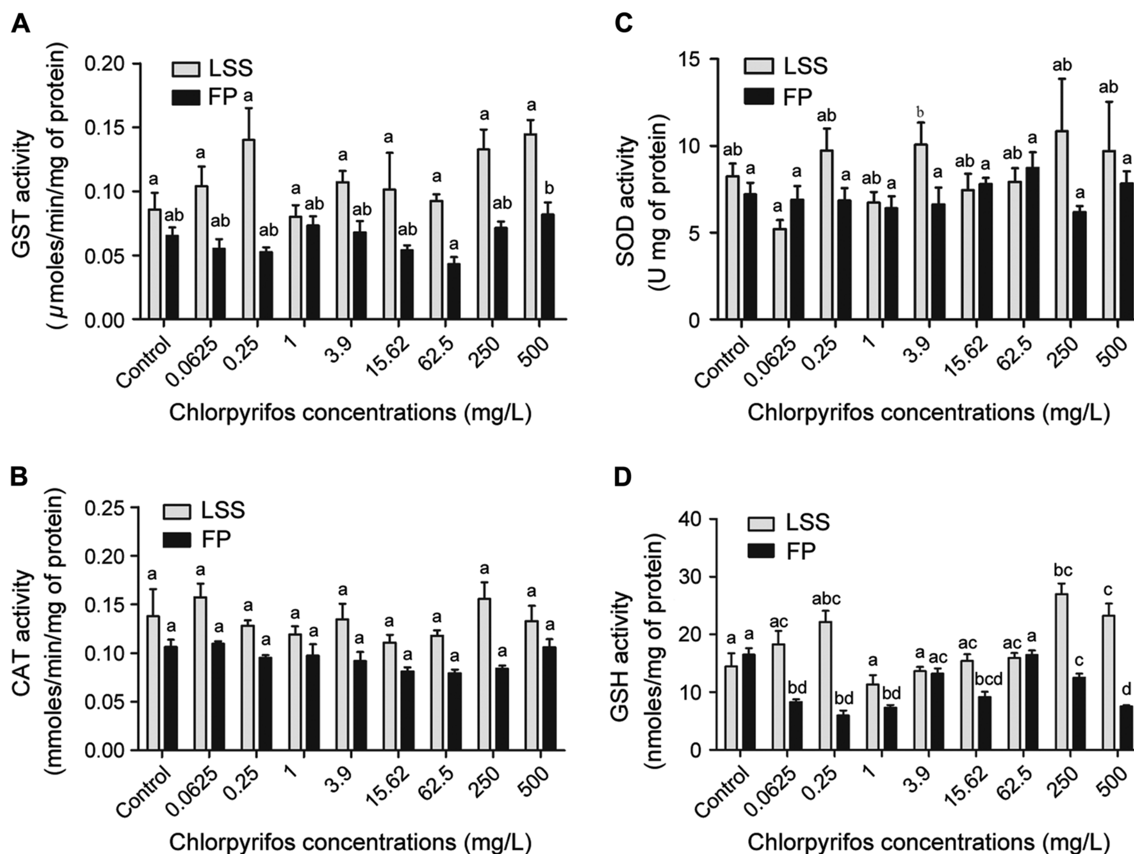

Fig. 4 Effects of chlorpyrifos concentrations on the antioxidant system of adults of *Cydia pomonella*. (A) GST, (B) CAT, (C) SOD, and (D) GSH content. Each column and bar represents the mean and SE of at least 4 independent replicates. Enzymes activities and GSH content were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. Within each population, columns labeled with a different letter are significantly different ($P < 0.05$).

activities at the 2 lower chlorpyrifos concentrations was much higher in LSS than in FP. According to Plapp and Wang (1983), higher amounts of detoxifying enzymes are often found in resistant insects and they are frequently inducible. The induction of these enzymes in resistant insects is often to a lesser degree, at least on a basis percentage, than in susceptible insects. The enzymes CarE "protect" the AChE by acting as an alternative phosphorylation site for organophosphates (Jokanović, 2001). Considering the CarE protector role, the differential enzyme induction might explain the comparable chlorpyrifos toxicity to both LSS and FP. As it was observed in Fig. 2B, the mean CarE activities at the bioassay concentration's range (0.0625−3 mg/L chlorpyrifos) were quite similar between LSS and FP. Resistance to organophosphates was associated with both increased and decreased esterase activity in other FPs of *C. pomonella* (Reyes *et al.*, 2007; Rodríguez *et al.*, 2011). On the other hand, FPs of *C. pomonella* from Chile that were resistant to chlorpyrifos showed a significant negative correlation between chlorpyrifos mortality and esterase activity (Reyes *et al.*, 2015).

In this study, adults from FP exhibited a significantly higher mean ECOD activity than the one determined from LSS (see control groups from Fig. 3). Previous results on neonates from this FP showed increased mean ECOD activity, although not statistically significant (Cichón *et al.*, 2013). However, both FPs of diapausing larvae and neonates from the region have shown more than 4 fold higher activity than the one from LSS. Resistance to organophosphates in FPs of *C. pomonella* was also associated with increased activity of ECOD (Reyes *et al.*, 2011; Rodríguez *et al.*, 2011). The CYP450 enzymes are found in almost all insect tissues and they are involved in the metabolism of both endogenous and exogenous compounds. CYP450 genes are under complex regulation, with induction playing a central role in the adaptation to plant chemicals; and regulatory mutations playing a

central role in insecticide resistance. The chlorpyrifos concentrations evaluated in this study produced no significant effects on ECOD activity from LSS. However, in adults from FP there was a trend for ECOD activities to decrease as chlorpyrifos concentrations increased with significant differences between control group and treated adults at 15.62 and 62.5 mg/L. It has been observed that besides metabolic activation of the organophosphate parathion by CYP450, this insecticide also induces a suicidal inhibition. It has been proposed that the reactive sulfur formed during activation of parathion to paraoxon inhibits the enzyme that participates in its formation by its reaction with SH groups of cysteine placed in an apoprotein part of the enzyme (De Matteis, 1974). Despite the differences found in ECOD activities of control adults and ECOD response to chlorpyrifos treatment between LSS and FP, it seems that these enzymes did not play an apparent role in the FP susceptibility to chlorpyrifos.

No significant differences were found between mean GST activities from both LSS and FP. Previous results on diapausing larvae of *C. pomonella* from the Río Negro and Neuquén Valley showed a poor association between GST activity and azinphosmethyl mortality (Soleño *et al.*, 2008). On the other hand, increased activity of GST was correlated with organophosphate resistance in other FPs of *C. pomonella* (Rodríguez *et al.*, 2011). In the present study, neither the LSS nor the FP was affected by any of the chlorpyrifos concentrations despite the observed variability of GST activities. Likewise, exposure of *Oxya chinensis* (Thunberg) for 24 h to chlorpyrifos concentrations between 0.080 and 0.28 μg/L had not influenced GST activities (Wu *et al.*, 2011). GST enzymes are members of a multifunctional superfamily that play a dual role in both detoxification and protection against oxidative damage caused by ROS (Yan *et al.*, 2013). Treatments of chlorpyrifos in *Locusta migratoria* (L.) caused a significant decrease of GST activities toward 1,2-dichloro-4-nitrobenzene (DCNB) and *p*-nitro-benzyl chloride (pNBC) in a dose-dependent manner. Conversely, GST activity toward CDNB was not significantly affected by chlorpyrifos in this species (Qin *et al.*, 2014). These authors also found that chlorpyrifos increased mRNA and protein expression of some GST classes, whereas mRNA and protein expression of other GST decreased. The authors conclude that the insect differentially regulate the expression of enzymes involved in the detoxification of insecticides at the expense of others.

Our results did not show significant differences in the activities of CAT and SOD from both LSS and FP. Additionally, none of both enzymes were significantly affected by chlorpyrifos concentrations. Changes in the antioxidant enzymes observed in insects after acute (Akbar *et al.*, 2012) and long-term exposure (Adamski *et al.*, 2003; Büyükgüzel, 2006; Wu *et al.*, 2011) contribute to neurotoxic and physiological effects of some pesticides. Long-term exposure of *Spodoptera exigua* (Hübner) to LC_{10} and LC_{30} of the organophosphate fenitrothion revealed an association between SOD activity and survival. When SOD activity was low, survival was low; when SOD activity was high, survival was also high (Adamski *et al.*, 2003). The authors concluded that the toxicity of the pesticide may be enhanced by the unbalance between free radicals and antioxidants. It has also been suggested that the pyrethroid tolerance in *Anopheles arabiensis* (Patton) would be associated to an increased gene expression of SOD (Muller *et al.*, 2008). In another study, *O. chinensis* treated with different concentrations of the organophosphates malathion and chlorpyrifos exhibited changes in both SOD and CAT activities. However, these changes were not dose-correlated (Wu *et al.*, 2011). SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, while CAT converts hydrogen peroxide into water alleviating the toxic effects of ROS (Rice-Evans & Burdon, 1993). Many insects also possess general peroxidases (POD) that convert hydrogen peroxide into water using many substrates such as ascorbate or glutathione. Peroxidase activity of nonselenium GST in insects is able to replace the lipid hydroperoxide reduction activity of selenium-enzyme glutathione peroxidase (GPOX) which is absent in these organisms (Felton & Summers, 1995). These authors also proposed that enzymatic recycling of ascorbate replaces GPOX for removal of hydrogen peroxide in insects. The ascorbate-recycling enzymes include POD, dehydroascorbic acid reductase and ascorbate free radical reductase. It was also proposed that these enzymes comprise an important antioxidant system that scavenge many other oxidizing agents such as lipid hydroperoxides, singlet oxygen, hydroxyl radical, quinones, phenolics, etc. (Felton, 1995).

The content of GSH between LSS and FP were also not significantly different. However, substantial differences were found between the LSS and FP when they were treated with different concentrations of chlorpyrifos. A comparable pattern between GSH content and GST was found in adults from the LSS treated with chlorpyrifos. Higher GST activity and GSH content were determined on groups treated with 0.25, 250, and 500 mg/L chlorpyrifos. These results might indicate that GSH is regenerated from the oxidized form (GSSG) by glutathione reductase (GR). In contrast, mean GSH content in the FP was significantly decreased nearly at all chlorpyrifos treatments. GSH depletion is indicative that it has been used either as direct ROS scavenger or as cosubstrate of the

GSH-dependent enzymes as well as a poor recycling of GSSG by GR.

In conclusion, both AChE and CarE activities were the most affected by chlorpyrifos. The antioxidant system of adult *C. pomonella* was mostly unaffected by the concentrations studied. Further research is required to evaluate whether other enzymes or antioxidant molecules are affected by chlorpyrifos exposure in *C. pomonella*. Despite the selection of *C. pomonella* individuals with high CarE and ECOD activities due to the long history of insecticide pressure in the area, this FP was as susceptible to chlorpyrifos as the LSS. These two mechanisms, previously associated with decreased azinphosmethyl sensitivity, do not confer cross-resistance to chlorpyrifos. The present resistance management strategies include rotation of insecticides with different modes of action. This might delay the evolution of insecticide resistance in FPs from the study area. There are currently about seven pesticide applications during the production season. Avoiding treatment of successive pest generations with compounds from the same mode of action group is fundamental. An example of pesticide rotation strategy to keep the products efficacy might include an organophosphate (Group 1B), a neonicotinoid (Group 4A), an insect growth regulator such as novaluron (Group 15) or methoxyfenozide (Group 18), spinetoram (Group 5), chlorantraniliprole (Group 28), emamectin benzoate (Group 6), and a biopesticide. Chemical applications should be supplemented with proper sprayer calibration and cultural control. At last, small-scale farmers should also follow the regional IPM to reduce the selection pressure of conventional pesticides.

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