Investigation of insecticide-resistance status of *Cydia pomonella* in Chinese populations

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Abstract

The codling moth *Cydia pomonella* (L.) is an economically important fruit pest and it has been directly targeted by insecticides worldwide. Serious resistance to insecticides has been reported in many countries. As one of the most serious invasive pest, the codling moth has populated several areas in China. However, resistance to insecticides has not been reported in China. We investigated the insecticide-resistance status of four field populations from Northwestern China by applying bioassays, enzyme activities, and mutation detections. Diagnostic concentrations of lambda-cyhalothrin, chlorpyrifos-ethyl, carbaryl, and imidacloprid were determined and used in bioassays. Field populations were less susceptible to chlorpyrifos-ethyl and carbaryl than laboratory strain. Insensitive populations displayed an elevated glutathione *S*-transferases (GSTs) activity. Reduced carboxylesterase (CarE) activity was observed in some insecticide insensitive populations and reduced acetylcholinesterase activity was observed only in the Wuw population. The cytochrome P450 polysubstrate monooxygenases activities in four field populations were not found to be different from susceptible strains. Neither the known-resistance mutation F399V in the acetylcholinesterase (AChE) gene, *ace*1, nor mutations in CarE gene *CpCE-1* were found in adult individuals from our field populations. Native-PAGE revealed that various CarE isozymes and AChE insensitivity were occurring among Chinese populations. Our results indicate that codling moth populations from Northwestern China were insensitivity to chlorpyrifos-ethyl and carbaryl. Increased GST activity was responsible for insecticides insensitivity. Decreased CarE activity, as well as the presence of CarE and AChE polymorphisms might also be involved in insecticides insensitivity. New management strategies for managing this pest are discussed.

Keywords: *Cydia pomonella*, insecticides resistance, detoxifying enzymes, management, invasions

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Introduction

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is an economically important pest of pome fruits and walnuts worldwide (Voudouris *et al.*, 2011; Rodríguez *et al.*, 2011*a*, 2011*b*). This invasive species is native to Eurasia. Zhang first reported the presence of *C. pomonella* in Xinjiang Province of China in 1957 (Zhang, 1957). In the following decades, it spread over most cultured regions in Xinjiang. Since this invasive quarantined pest was found in Jiuquan (Gansu Province, China) in 1986, this pest has invaded parts of Gansu, Heilongjiang, and Inner Mongolia Provinces causing

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large losses in pome production in China (Men *et al.*, 2013). This species is difficult to control because the larvae tunnel to the center of the fruit to feed on seeds. In addition, the pest has overlapping generations. The fruit damage by *C. pomonella* includes boring tunnels into the center of fruit to feed on seeds, contaminating fruits with their frass, and causing fruit abscission. According to our observation, in some unmanaged orchards in Wuwei (Gansu Province), the larvae can cause a fruit abscission rate of more than 80%.

To control the codling moth, chemical insecticides including organophosphates (OPs), carbamates (Carbs), synthetic pyrethroids (Pys), neonicotinoids, and insect growth regulators are widely used. However, a major problem associated with chemical control of the codling moth is the development of insecticide-resistance worldwide (Voudouris *et al.*, 2011; Rodríguez *et al.*, 2011a, 2011b). In Southeastern European orchards, this species was first reported to have resistance to chemical insecticides (Waldner, 1993). Failure of deltamethrin use against codling moth was reported in 1995 in France (Bouvier *et al.*, 1995). Azinphos-methyl, the most widely used agent for controlling codling moth (Rodríguez *et al.*, 2010), has been reported now to be ineffective in many countries, including the USA (Varela *et al.*, 1993; Dunley & Welter, 2000), Chile (Reyes *et al.*, 2004; Fuentes-Contreras *et al.*, 2007), South Africa (Giliomee & Riedl, 1998), Australia (Thwaite *et al.*, 1993), France, Spain, and Switzerland (Sauphanor *et al.*, 1998; Reyes *et al.*, 2007).

Insecticide resistance has been related to two major resistance mechanisms in codling moth: increased enzymatic metabolization and the presence of target-site resistance mutations (Reyes *et al.*, 2007, 2011; Voudouris *et al.*, 2011). The metabolization mechanism involves elevated cytochrome P450 monooxygenases (P450s), general esterases (ESTs), and glutathione *S*-transferases (GSTs). In many cases, increased or decreased enzyme activities have been reported in the development of insecticide resistance in the codling moth. Voudouris *et al.*, observed an elevated P450 and GST activities, and a reduced CarE activity in field populations in Greece (Voudouris *et al.*, 2011). In Chile, one field *C. pomonella* population was found to show higher GST activity (Fuentes-Contreras *et al.*, 2007). In the USA, a lower affinity of esterase to non-specific substrates has been implicated in parathion resistance (Bush *et al.*, 1993). The target-site resistance mutations mainly involve mutations in acetylcholinesterase (AChE) and sodium channel genes in the codling moth. A F399V mutation in the AChE protein Cpace1 (Cassanelli *et al.*, 2006) and a knockdown resistance (*kdr*) mutation (Brun-Barale *et al.*, 2005) in the voltage-dependent sodium channel protein have been implicated in resistance to insecticides.

The codling moth invaded China decades ago and intensive control managements such as mating disruption, sex attractant-kill, and the spraying of chemical insecticides and *Cydia pomonella* granulovirus (CpGV) have been used against the codling moth in China. However, effective management of the codling moth has mainly depended on chemical insecticides. Information about the insecticide-resistance status of codling moth in Chinese populations is an important pre-requirement for selection of insecticides and management strategies for managing this pest in China. Bioassays combined with biochemical assays and molecular mutations detection may efficiently detect the incipient resistance.

Unlike several studies of *C. pomonella* in other geographical regions, such as Greece (Voudouris *et al.*, 2011), there have been no studies of insecticide resistance in *C. pomonella* in

China and no analysis is available about its resistance status. The aim of the present study was to investigate the resistance status of *C. pomonella* in Chinese field populations to four insecticides, with different modes of action. Bioassay on fourth-instar larvae was performed by topical application method, the activity of the detoxifying enzymes was measured and the resistance-related mutations in AChE gene, *Cpace*1 (GenBank number: DQ267978.1) and *CarE* gene, and *CpCE-1* (GenBank number: KC832922) were detected.

Materials and methods

Chemicals and insecticides

1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione were obtained from ABCR GmbH & CO. KG (Germany) and Sigma-Aldrich Life Sciences (St Louis, MO), respectively. The α-naphthyl acetate (α-NA), α-naphthol and fast blue B salt were purchased from the National Medicine Group Chemical Reagent Co., Ltd. (Shanghai, China). The *p*-nitroanisole, *p*-nitrophenol, acetylthiocholine iodide (ATChI) and 5,5'-dithiobis-2-nitrobenzoc acid (DTNB) were purchased from Aladdin Reagent (Shanghai, China). Other chemicals and reagents were commercially available. The standard pesticides (>98%) lambda-cyhalothrin, chlorpyrifos-ethyl, carbaryl, and imidacloprid were purchased from Aladdin Reagent (Shanghai, China).

Codling moth susceptible laboratory strain

The *C. pomonella* diapause larvae were originally collected from abandoned apple orchards with non-managed in Wuwei Botanical Garden (Wuwei City, Gansu Province, China). Before the collection, the orchard was abandoned for more than 10 years. Furthermore, situated in the city center, the orchards are not surrounded by chemically treated orchards. The larvae were reared on an artificial diet (Landolt *et al.*, 2007) in the laboratory in 1.5 ml centrifuge tubes with small holes under standard conditions of $25 \pm 1°C$, $60 \pm 5\%$ relative humidity, and a 16:8 h light: dark photoperiod (Voudouris *et al.*, 2011). Pupae were removed from the diet, sexed, and placed with both sexes together in plastic cases in a 2:1 male/female ratio. Mated female moths laid eggs on filter paper allowing us to obtain the next generation of neonates. Insects reared for eight generations without exposure to any chemicals were used as laboratory strain (Ls) in this study.

Codling moth field populations

The diapause larvae of four field populations – Zha, Wuw, Lan, and Kor – were collected in the August of 2013 at four locations in Northwestern China: Zhangye (100.38°E, 38.85°N), Wuwei (102.61°E, 37.94°N), Lanzhou (103.71°E, 36.03°N), and Korla (86.13°E, 41.60°N) (fig. 1) in apples. These orchards were sprayed chlorpyrifos-ethyl for four times, lambda-cyhalothrin for twice and chlorpyrifos-ethyl for one time, diflubenzuron for one time and deltamethrin for four times, carbaryl for two times, and both chlorpyrifos-ethyl and lambda-cyhalothrin for one time, respectively, to control the codling moth in the last years. The distance between the orchards in Korla and Zhangye is almost 1500 km. The distances among the orchards Zhangye-Wuwei and Wuwei-Lan are 250 and 275 km, respectively. The larvae were placed on the artificial diet under the

Fig. 1. Collection sites of field population of *C pomonella* from Northwestern China. Zha (Zhangye, 100.38°E, 38.85°N), Wuw (Wuwei, 102.61°E, 37.94°N), Lan (Lanzhou, 103.71°E, 36.03°N), and Kor (Korla, 86.13°E, 41.60°N).

conditions described above until they reached the fourth-instar of their next generations.

Bioassays

Dose–response bioassays were performed to determine the LC_{50} and LC_{90} values of the insecticides lambda-cyhalothrin, chlorpyrifos-ethyl, carbaryl, and imidacloprid on fourth-instar larvae from the Ls. Insecticides were dissolved in acetone, and 5–6 different concentrations of each of them were prepared. A 2 µl drop of insecticidal solution was applied on the surface of 0.125 cm^3 artificial diet piece. A minimum of 60 individuals (three replicates of 20 individuals each) per concentration were used. After the treatments, larvae were subsequently placed in the conditions described above. As a control, larvae were applied with acetone using the same method. Mortality was recorded after 36 h. Each larva was considered dead if did not move when stimulated with an ink brush. Fourth-instar larvae from field populations and Ls strain were treated at the diagnostic concentration (LC_{90}) determined in Ls strain in the same way.

Enzyme extract preparation

Three pools of ten larvae of fourth-instar larvae from each population were homogenized on ice in different buffers using a mortar. The crude enzyme for GST, CarE, P450, and AChE activity analysis were homogenized in 2 ml of 50 mM sodium phosphate (pH 7.2), Tris–HCl/CaCl$_2$ (25 mM/1 mM, pH 7.0), 100 mM sodium phosphate (pH 7.8), and 100 mM PBS (containing 0.5% Triton X-100, pH 7.2) buffer, respectively. These homogenates were centrifuged at 14,000 **g** for 30 min at 4°C. Subsequently, supernatants were used as the enzyme sources for the enzymatic activities measured. The protein concentration was determined using bovine serum albumin as a standard according to the Bradford method (Bradford, 1976).

Enzymatic activities

The GST activity was determined as described previously (Rodríguez *et al.*, 2011*b*) using CDNB as substrate in 96-well microplates (Nunc, Roskilde, Denmark) on an Infinite M200 PRO multimode Microplate Reader (Tecan, Männedorf, Switzerland). Each well was supplied with 2 µl of enzymatic extract, 186 µl of sodium phosphate buffer (50 mM, pH 7.2), 2 µl of reduced glutathione (100 mM) and 10 µl of CDNB (40 mM). The absorbances at time zero (t_0) and after 1 min (t_1) at 340 nm at 30°C were recorded, and the absorbance changes ($A_{340}t_1 - A_{340}t_0$) were used to calculate activity using a molar extinction coefficient of 9.6 mM^{-1} cm^{-1}. The enzymatic extract replaced by sodium phosphate buffer was used as the control. Each test was conducted in triplicates.

CarE activity was measured using α-NA as substrate according to our previous study (Yang & Zhang, 2013). Each well consisted of 5 µl of enzymatic extract, 190 µl of Tris–HCl/CaCl$_2$ (25 mM/1 mM, pH 7.0), and the reaction was initiated by supplying 5 µl of α-NA (0.1 mM). After 10 min of incubation, the reaction was terminated by the addition of 25 µl of 0.42% sodium dodecyl sulfate (SDS) and stained by adding 25 µl of 0.2% fast blue B salt at room temperature in the dark for 30 min. Absorbance of the naphthol–fast blue B complex was recorded at 600 nm. A standard curve was conducted with α-naphthol. The heat inactivated enzymatic extract (95°C for 10 min) was used as the control. Each test was conducted in triplicates.

The total P450 activity was determined by measuring *p*-nitroanisole demethylase (PNOD) activity using *p*-nitroanisole as substrate according to a previous study (Yang *et al.*, 2004). Each well contained 10 µl of enzyme and 180 µl of sodium phosphate buffer (100 mM, pH 7.8) containing 2 mM *p*-nitroanisole. The reaction was initiated by adding 10 µl of 9.6 mM of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). After 30 min of reaction, the amount of generated *p*-nitrophenol was measured at 405 nm at 30°C. A standard curve was conducted with vary concentration of *p*-nitrophenol. A negative control reaction was conducted using heat inactivated enzyme (95°C for 10 min) to eliminate any non-enzymatic activity and a no-NADPH-added control was also performed. Each test was conducted in triplicates.

The AChE activity was assessed according to a modified Ellman method (Zhao *et al.*, 2013). The 200 µl reaction mixture consisted of 170 µl of PBS (containing 0.5% Triton X-100, pH 7.2) buffer, 10 µl of enzyme, 10 µl of 10 mM ATChI, and 10 µl of 10 mM DTNB. The heat inactivated enzymatic extract was used as the control. Absorbance was measured after 20 min of incubation at 25°C at 405 nm. The enzyme activity was determined using the extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}. Each test was conducted in triplicates.

Native PAGE and CarE/AChE staining

Non-denaturing polyacrylamide gel electrophoresis (Native PAGE) was performed as described in our previous study (Yang & Zhang, 2013) on an electrophoresis apparatus (Bio-Rad, CA, USA). Twenty microgram of total protein from each sample were loaded on 5% stacking (0.126 M Tris/HCl pH 6.8, 0.1% ammonium persulphate, 30.8% acrylamide, and 2.6% N,N-methylene bisacrylamide) and 12% separating (0.375 M Tris/HCl pH 8.8, 0.1% ammonium persulphate, 30.8% acrylamide, and 2.6% N,N-methylene bisacrylamide) polyacrylamide. The electrophoresis was conducted at 80 V for 30 min and subsequently 140 V until the bromophenol blue reached the bottom of the gel in a cold ice chest. One page for CarE activity staining was incubated in staining solution consisting of 37 µM α-NA and 0.05% fast

Table 1. PCR primers used in this study.

Name	Sequence (5′–3′)	Primer used
*Cpace*1-F399-F	TTTTCTTTGACACGCCCGATGTT	AChE mutation detection
*Cpace*1-F399-R	CGATAATGGGAACGAAAGGAAAT	AChE mutation detection
CpCE-1-A121-F	CAAAGCCACGGACCACATATACAT	CpCE-1 mutation detection
CpCE-1-A121-R	CTACAAGCATAGACAGTGAAGCAGC	CpCE-1 mutation detection
CpCE-1-W233-F	GCTGGCGGCTTTGAGGTGGGTAA	CpCE-1 mutation detection
CpCE-1-W233-R	GGCTCTACGCCTGGAAAAGATTTC	CpCE-1 mutation detection

Table 2. Toxicity of four insecticides on fourth-instar larvae of a *C pomonella* strain (Ss) originally collected from an abandoned apple orchard and reared in laboratory for 8 generations.

Insecticide	n^1	Slope ± SD	χ^2	LC_{50} (mg (a.i.) L^{-1}) 95% confidence intervals	LC_{90} (mg (a.i.) L^{-1}) 95% confidence intervals
Lambda-cyhalothrin	375	2.60 (2.13–3.08)	5.95	0.59 (0.40–0.83)	1.83 (1.209–4.242)
Chlorpyrifos-ethyl	450	2.94 (2.47–3.41)	43.12	445.39 (363.27–543.82)	1217.01 (921.30–1911.52)
Carbaryl	300	2.18 (1.74–2.63)	11.12	730.02 (611.60–870.11)	2819.67 (2140.81–4163.68)
Imidacloprid	300	2.55 (2.06–3.04)	10.94	16.42 (14.05–19.36)	52.28 (40.69–74.13)

[1]Sample size.

blue B salt in Tris-HCl/CaCl$_2$ (25 mM/1 mM, pH 7) buffer. The other page was incubated in 100 mM PBS buffer (pH 7.2) consisting of 0.5 mM ATChI and 0.5 mM DTNB for activity staining. After staining at room temperature in the dark for 60 min, the gels were washed with 30% ethanol and photographed.

Molecular diagnostic assays

The resistance-related mutation F399V (Cassanelli *et al.*, 2006) in codling moth AChE gene, *Cpace*1 (GenBank number: DQ267978.1) and previously reported mutations – A/G137 and W251 ((A121 and W233 in codling moth CarE *CpCE-1* (Yang et al., 2014), GenBank number: KC832922)) – in CarE responsible for resistance in other species (Newcomb *et al.*, 1997), were identified by Polymerase Chain Reaction (PCR). Previous studies for AchE gene cloning (Cassanelli *et al.*, 2006) and microsatellite analysis (Men *et al.*, 2013) in this species used genomic DNA isolated from adult may be the adulthood contains more genomic DNA. Genomic DNA was extracted from whole single individual using DNeasy Tissue Kit (QIAGEN, Hilden, Germany). For each population, ten adults were used. Partial amplification of the DNA region of F399 fragment in the *Cpace*1 gene was performed using primers *Cpace*1-F399-F and *Cpace*1-F399-R (Table 1). For amplification of the DNA regions of A121 and W233 fragments in *CpCE-1* gene, primer pairs *CpCE*-1–A121-F, *CpCE*-1–A121-R and *CpCE*-1–W233-F, *CpCE*-1–W233-R were designed (Table 1). The PCR was carried out in a 25 μl reaction mixture consisting of 3 μl DNA template, 0.4 μM of forward and reverse primers, and 12.5 μl of 2 × *Taq* MasterMix (CWBIO, Beijing, China) on a C1000 PCR Amplifier (Bio-Rad, CA, USA). For AChE fragment amplification, touchdown PCR was conducted under the following cycling program: 1 cycle of 95°C for 3 min, 5 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 40 s, and followed by 30 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 40 s. To amplify the two fragments of *CpCE*-1, PCR was conducted under the following cycling program: 1 cycle of 95°C for 3 min, 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 40 s. The DNA fragments with 417, 416,

and 393 bp long for the F399V in *Cpace*1, A121D, and W233L in *CpCE*-1, respectively, were separated by electrophoresis on 1.0% agarose gel and were visualized by ethidium bromide staining under UV light. The PCR products were sequenced using an ABI3730XL automated DNA Sequencer (Applied Biosystems, CA, USA) by the Shanghai Sunny Biotech Co., Ltd, China.

Data analysis

The results were expressed as the mean of triplicates ±standard deviation (SD). Dose–mortality data were analyzed using the POLO-PC program to calculate the slope and intercept of the dose–response lines, the lethal concentration values (LC_{50} and LC_{90}) and their confidence limits. The significance of enzymatic activities was analyzed using a one way analysis of variance (ANOVA, $P < 0.05$), and the significance of efficacy of insecticides at the diagnostic concentration on fourth-instar larvae was calculated by Abbott's formula (Abbott, 1925) and was compared with its efficacy on Ls strain using χ^2 test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$) using the SPSS statistics software (Version 12, IBM Inc., Chicago, USA). The results were plotted using GraphPad Prism 5 (GraphPad Software, CA, USA).

Results

Determine of the diagnostic concentrations

The probit analyses of the toxicity of four insecticides on fourth-instar larvae are shown in Table 2. The χ^2 values were small, indicating a good fit of the data to the probit model, except for the chlorpyrifos-ethyl ($\chi^2 = 43.12$). The mortality in the controls was lower than 20%. There were large differences in toxicity of the tested insecticides on fourth-instar larvae, with the LC_{50} values ranged from 0.59 to 730.02 mg (a.i.) L^{-1}, and the LC_{90} values ranged from 1.83 to 2819.67 mg (a.i.) L^{-1} (Table 2). The pyrethroid compound, lambda-cyhalothrin was the most effective insecticide, with the LC_{50} and LC_{90} values lower than 0.6 mg (a.i.) L^{-1} and 2 mg (a.i.) L^{-1}, respectively. The

Table 3. Efficacy of insecticides at the diagnostic concentration on fourth-instar larvae of *C. pomonella* from the susceptible strain and field populations.

Strain	Insecticide efficacy (%)[a]			
	Lambda-cyhalothrin 1.8 mg (a.i.) L^{-1}	Chlorpyrifos-ethyl 1200 mg (a.i.) L^{-1}	Carbaryl 2800 mg (a.i.) L^{-1}	Imidacloprid 52 mg (a.i.) L^{-1}
Ls	98.3	91.7	93.3	88.3
Zha	91.7 (3.789) ns	71.7 (20.632)***	81.7 (5.531)*	85.0 (0.385)ns
Wuw	93.3 (2.909) ns	81.7 (9.557)**	83.3 (4.735)*	86.7 (0.046)ns
Lan	91.7 (3.789) ns	78.3 (13.735)***	90.0 (0.579)ns	81.7 (1.412)ns
Kor	95.0 (1.332) ns	76.7 (14.834)***	90.0 (0.579)ns	88.3 (0.000)ns

[a]Mean of three replicates of 20 larvae per insecticide and per strain.
The mortality of four field population fourth-instar larvae was compared with the mortality measured in the Ls using χ^2 test. The χ^2 value is shown in parentheses (df = 1; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). ns, not significant.

carbaryl was the least effective insecticide, and it exhibited the LC_{50} and LC_{90} values of 730.02 and 2819.67 mg (a.i.) L^{-1}, respectively. The slopes of the dose–response lines of these four insecticides were flat, with the values ranged from 2.184 to 2.936 (Table 2).

Insecticide efficacy on four field populations

The efficacy of four insecticides at their diagnostic concentration (LC_{90}) on fourth-instar larvae of four field populations and Ls are shown in Table 3. The efficacy of four insecticides on Ls at the diagnostic concentration ranged from 88.3% (imidacloprid) to 98.3% (lambda-cyhalothrin). The efficacy of chlorpyrifos-ethyl on four field populations was significantly lower than that on Ls (Table 3). Two populations from Zhangye and Wuw apple orchards (Zha and Wuw) were resistant to carbaryl, but no difference of efficacy was observed on Lan and Kor populations from Lanzhou and Xinjiang, respectively. The efficacy of lambda-cyhalothrin and imidacloprid was not different between four field populations and Ls at the diagnostic concentrations, suggesting both lambda-cyhalothrin and imidacloprid are effective control options currently (Table 3).

Enzymatic activities

The GST, CarE, P450, and AChE activities from four field populations and one susceptible strain were determined (fig. 1). All four field populations showed a GST activity significantly higher ($F = 88.892$, df = 4–10, $P < 0.001$) than that of the Ls (fig. 2a). The GST mean activities measured were 540.2, 505.0, 762.1, 518.0, and 452.7 nmol glutathione-conjugated mg protein^{-1} min^{-1} for Zha, Wuw, Lan, Kor, and Ls, respectively. The highest GST activity was observed in the Lan population, with a value of 762.1 nmol glutathione-conjugated mg protein^{-1} min^{-1}. These four field populations had GST activity ratios higher than the Ls and the range among populations was 1.1–1.7.

For CarE activities, the values detected in fourth-instar larvae from Zha, Wuw, and Lan populations were 216.6, 209.3, and 218.9 nmol mg protein^{-1} min^{-1}; these three populations showed significantly lower activity levels than Kor ($F = 60.896$, df = 3–8, $P < 0.001$) and Ls ($F = 70.350$, df = 3–8, $P < 0.001$) (fig. 2b). Furthermore, the activity ratio was significantly lower than the Ls, with a value of about 0.5 for all three populations compared with the Ls. It is worth pointing out that the Kor and Ls strains exhibited similar activity, with

activity values of 406.4 and 454.1 nmol mg protein^{-1} min^{-1}, respectively.

Concerning P450 activity, there was no significant difference between four field populations and the Ls strain (fig. 2c). The activity values calculated were 10.7, 10.5, 9.6, 10.8, and 9.9 pmol *p*-nitrophenol mg protein^{-1} min^{-1} for Zha, Wuw, Lan, Kor, and Ls, respectively.

For AChE activity, the Wuw population with a value of 1089.3 µmol mg protein^{-1} min^{-1} and activity ratio value of 0.52 was a significantly lower activity than for Ls ($F = 26.780$, df = 1–4, $P = 0.007$) (fig. 2d). Furthermore, the field populations Zha, Lan, Kor, and the Ls exhibited AChE activity values of 1772.4, 2065.4, 1823.2, and 2094.0 µmol mg protein^{-1} min^{-1}, respectively. The Zha, Lan, and Kor populations had AChE activity ratios lower than the Ls, with the activity ratio range between 0.85 and 0.99.

CarE polymorphisms

To determine the presence and the numbers of CarE isozymes in different populations, native PAGE was carried out (fig. 3a). A total of eight bands (A–H) were clearly detected in the native gel. Bands B, C, E, and H were present in all populations, while band D was only visualized in the Kor population. The band G was not observed in Kor and Ls, but these two populations were detected to have band F, which was also observed in Wuw but absent in the Zha and Lan populations. Among these bands, E was stronger than the other bands.

AChE polymorphisms

We determined the molecular forms of AChE in various populations; only one AChE band was detected in the native gel stained with ATChI (fig. 3b). The band was weaker in the Wuw population than in the Ls, but significantly stronger in the Zha, Lan, and Kor populations than in the Ls.

Frequency of known CarE and AChE mutations

Molecular diagnostic assays were performed for analyzing the presence and frequency of the insecticide-resistant mutation F399V in the AChE gene *Cpace1* and the A121D and W233L in the CarE gene *CpCE-1* in codling moth. DNA sequencing showed that none of the adult samples from our four field populations and the susceptible strain had the F399V mutation in the *Cpace1* gene (fig. 4), and A121D and W233L in *CpCE-1* gene (fig. 5).
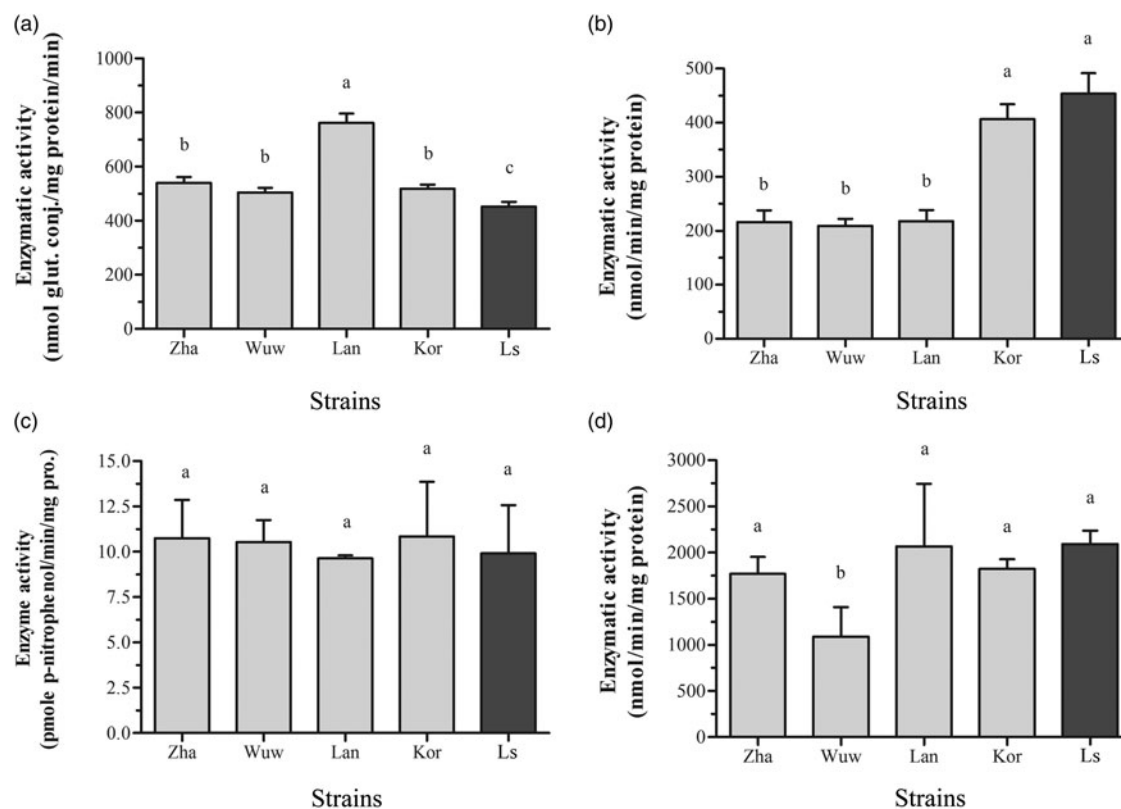
Fig. 2. Enzymatic activities in one susceptible and four field (Zha, Wuw, Lan, and Kor) populations of *C. pomonella*. (A) GTS activities (B) CarE activities (C) P450 activities (D) AChE activities measured in fourth-instar larvae ($n = 10$). The error bars represent the standard error across three replicates. Letters on the error bars indicate significant differences between field populations and Ls by ANOVA analysis ($P < 0.05$).

Discussion

Our present study is the first investigation of insecticide resistance status of *C. pomonella* from the main infected areas and pome growing regions of China. To date, *C. pomonella* has been reported in Gansu and Heilongjiang Provinces, and Xinjiang and Inner Mongolia Autonomous Regions. This invasive species was first reported in Heilongjiang and Inner Mongolia in 2006 (Men *et al.*, 2013). We suspect that the insecticide resistance is not highly developed in these two areas. Although this pest was reported in Xinjiang for more than 50 years, most areas in Xinjiang are deserts and unsuitable for its colonization. However, *C. pomonella* populations have been established for decades in Xinjiang and Gansu. In these two Provinces, the Korla, Wuwei, Zhangye, and Lanzhou cities are pome cultivating regions. Spraying of chemical insecticides has been the primary management practice against *C. pomonella* in these regions as it has proven to be an effective approach for pest control. So, investigation of insecticide resistance of *C. pomonella* populations in these four regions provides guidelines for management of this quarantine pest in China. However, no information is available about its pesticide-resistance in China, although widespread use of insecticides for *C. pomonella* control over the last few decades has led to serious insecticide resistance in many countries (Rodríguez *et al.*, 2010).

In previous resistance studies, eggs (Rodríguez *et al.*, 2011*b*), newly hatched larvae (Rodríguez *et al.*, 2011*b*; Reyes

et al., 2011), non-diapausing larvae, dapausing larvae (Reyes *et al.*, 2007), and adults (Dunley and Welter 2000; Reuveny and Cohen 2004; Reyes and Sauphanor 2008) were used in bioassay and enzymatic assays. It is best to use neonate larvae for investigating resistance because they are the main target of insecticides (Voudouris *et al.*, 2011), and after that, the larvae tunnel to the center of the fruit to feed on seeds. However, collection of accurate stage of codling moth (eggs, neonate larvae, diaspausing or non-diapausing larvae, and adults) in the field is unfeasible. Furthermore, in China, this pest occurs 2–3 generations per year, and it has overlapping generations, thus it is difficult to obtain same generation of non-diapausing larvae in the field. In addition, the vitality of first-instar larvae is weak and will perish very easily under standard conditions. Thus the diapause larvae were collected in the field and reared on artificial diet in the laboratory, and the fourth-instar larvae of their next generation were used in bioassays and enzymatic assays in this study. Therefore, there is no comparability between the susceptibility of our Ls with the susceptibility of other Ls, as well as the activities of detoxifying enzymes.

Bioassay was performed on a Ls, and the LC$_{50}$ and LC$_{90}$ of tested insecticides were obtained. The LC$_{50}$ and LC$_{90}$ values of lambda-cyhalothrin were 0.59 and 1.83 mg(a.i) L^{-1}, and were 445.39 and 1217.01 mg(a.i) L^{-1} for chlorpyrifos-ethyl, 730.02 and 2819.67 mg(a.i) L^{-1} for carbaryl, and 16.42 and 52.28 mg (a.i) L^{-1} for imidacloprid (Table 2). Using the LC$_{90}$ as a diagnostic concentration, insecticide efficacy on fourth-instar
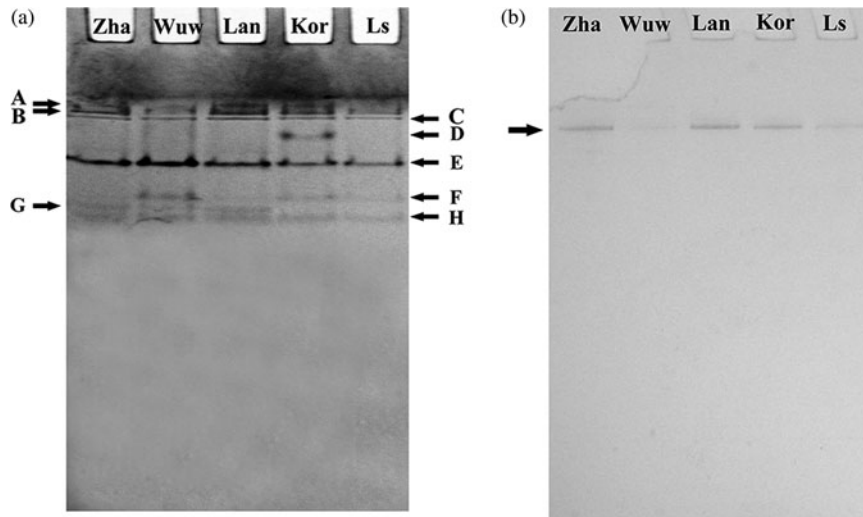
Fig. 3. Polymorphisms of CarE and AChE in larvae of four field populations (Zha, Wuw, Lan, and Kor) and Ls of *C. pomonella*. 20 μg total protein samples were loaded on a non-denaturing polyacrylamide gel and the CarE activity (a) and AChE (b) was stained using α-NA and ATChI as substrates, respectively. A total of 8 bands were observed (numbered from A to H according to its mobility) for CarE (a) and only one band was observed for AChE (b).

Fig. 4. Sequences obtained from direct sequencing of partial amplification of the DNA region of F399V in the *Cpace*1 gene from four field populations (Zha, Wuw, Lan, and Kor) and Ls. The boxed region highlights the first nucleotide of the codons encodes the Phe399. Asterisks (*) indicate identical residues among sequences.

larvae of four field populations and Ls was performed (Table 3). The result shows that the efficacy of chlorpyrifos-ethyl on four field populations (71.7–81.7%) are significantly smaller than those of Ls (91.7%), and the efficacy of carbaryl on Zha and Wuw populations (81.7 and 83.3%) are significantly smaller than its efficacy on the Ls (93.3%). Although the magnitude of the differences is rather small, the results suggest that a decrease in chlorpyrifos-ethyl and carbaryl efficacy to the field populations, indicating that low-level insecticide resistance has developed in the tested *C. pomonella* field populations. Fortunately, both lambda-cyhalothrin and imidacloprid are effective control options currently.

Insecticide resistance has been related to two major resistance mechanisms in codling moth, including increased enzymatic metabolization and target site insensitivity (Reyes et al., 2007, 2011; Voudouris et al., 2011). For the metabolic mechanism, the activities of detoxifying enzymes in fourth instar larvae of the codling moth from four field populations were determined. We also detected the presence or absence of the F399V mutation in *Cpace*1 gene in adults from field populations. These results suggested that low-level insecticide resistance has developed in the tested *C. pomonella* field populations, and an increase or decrease of detoxifying enzyme activity is the main reason for development of insecticide resistance. However, to answer which mechanism confers resistance to these insecticides are difficult. The GST seems to be the main mechanism because the enzymatic activities in all the field populations were significantly higher than the
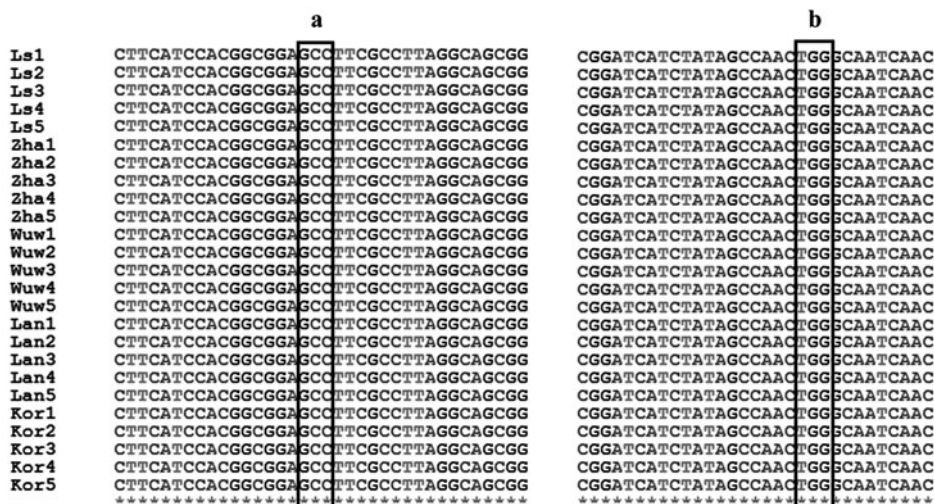
Fig. 5. Sequences obtained from direct sequencing of partial amplification of the DNA regions including the A121D (a) and W233L (b) in the *CpCE-1* gene from four field populations (Zha, Wuw, Lan, and Kor) and Ls. The boxed regions highlight the codons of A121 (a) and W233 (b), respectively. Asterisks (*) indicate identical residues among sequences.

Ls. The CarE and AChE are somewhat associated with developing resistance as reduced activities were detected in some populations compared with the Ls. For instance, populations resistance to carbaryl (Zha and Wuw) showed reduced CarE activity, but a non-carbaryl-resistance strain, Lan also showed a lower CarE activity. It is strange that there was no difference of P450 activity observed across all of the field populations. The correlation between GST activity and insecticide resistance has been reported in many previous works. Voudouris *et al.* demonstrated that elevated GST activity was related to resistance for several insecticides (Voudouris *et al.*, 2011). In an azinphos-methyl-resistant population in Chile, the GST activity was increased compared with the susceptible strain (Fuentes-Contreras *et al.*, 2007). Similar results reported that increased GST activity was associated with insecticide resistance in European populations (Reyes *et al.*, 2007; Rodríguez *et al.*, 2010).

Many reports suggest that reduced CarE activity is involved in insecticide resistance. In Spanish field resistant populations, the CarE activity from adults was significantly lower than in the susceptible strain (Rodríguez *et al.*, 2010). Another resistant adult population from Europe also exhibited reduced CarE activity (Reyes *et al.*, 2007). In insecticide-resistant population larvae, a similar result was also reported associating reduced CarE activity with resistance (Reyes *et al.*, 2011). In the present study, the insensitivity to chlorpyrifos-ethyl was observed in Zha, Wuw, Lan, and Kor populations and the insensitivity to carbaryl was only detected in Zha and Wuw populations. The activities of populations resistance to chlorpyrifos-ethyl were significant lower than the Ls, suggesting that CarE was responsible for the observed chlorpyrifos-ethyl insensitivity in these field populations. Significant lower CarE activities were also observed in populations resistance to carbaryl (Zha and Wuw). However, it is unable to conclude that reduced CarE activity was responsible for carbaryl insensitivity because lower CarE activity was also detected in a non-carbaryl-resistant strain, Lan. The reduced CarE activity may result from a mutation in a CarE (Bush *et al.*, 1993). Previously reported mutations (Newcomb *et al.*, 1997) in CarE from other species, such as A/G137 and W251 (A121 and

W233 in *CpCE-1* gene from codling moth), have been tested in all the populations, however, neither A121 nor W233 mutations were observed in the tested codling moth populations. It has been indicated that the presence and the number of isozymes are involved in resistance (Wheelock *et al.*, 2008). Thus native PAGE was carried out to investigate the differences of isozymes from various populations. Our results suggests that different populations appear to have different numbers of isozymes, demonstrating that *C. pomonella* in the detected populations have developed different levels of resistance. Our results suggest the presence of multiple CarE isozymes among resistant populations, and this finding is consistent with a previous report (Reyes *et al.*, 2011). The classical mutant aliesterase hypothesis indicated that reduced CarE activity may be the result of a mutation in a CarE (Newcomb *et al.*, 1997), and these mutations could result in altering CarE activities of model substrates and improving the ability to hydrolyze OP insecticides (Reyes *et al.*, 2011; Bush *et al.*, 1993). In this study, no mutations were detected on the *CpCE-1* gene, suggesting that the mutations may occur on other CarE genes.

Besides GST and CarE, our results show that AChE is also involved in resistance. This is evident in the Wuw population where reduced AChE activity is detected. However, reduced AChE activity may not be the main mechanism in the insecticides insensitive populations. Previous study suggested that reduced AChE activity is due to a replacement of phenylalanine by valine at position 399 of the *Cpace1* gene (Cassanelli *et al.*, 2006). However, detection of the presence of F399V mutation in field adults from four populations indicates that there are no mutations at or around the F399 region. The codling moth is reported to contain two *ace* genes, *ace1*, and *ace2* (Cassanelli *et al.*, 2006), but their specific function remains unclear. In other insects, mutations in the *ace1* gene are related to resistance to OP and Carb insecticides (Baek *et al.*, 2005; Alon *et al.*, 2008; Kim & Lee, 2013). No mutations correlate with resistance in the *ace2* gene. Furthermore, only one band observed in the AChE activity staining gel, suggesting that only one molecular form of the AChE protein is present in all populations, suggesting that AChE1 is the major catalytic enzyme. The weaker band in the Wuw population observed

in native-PAGE was consistent with the activity assay, suggesting a reduced AChE activity occurs in this population. Similar results (Kim & Lee, 2013) were found in the Lepidoptera species *Papilio bianor*, *Plutella xylostella*, *Polygonia c-album*, and *Vanessa cardui*. This suggests that *Cpace*2 plays some unknown function and should be further studied.

Many reports show that elevated P450s are involved in insecticide resistance. Increased P450 activity is correlated with resistance to azinphos-methyl in the Rdfb population from Greece (Reyes *et al.*, 2011). Research by Reyes *et al.* suggests that increased P450 activity is involved in resistance to many insecticides including azinphos-methyl, diflubenzuron, spinosad, tebufenozide, and thiacloprid (Reyes *et al.*, 2007). In our study, no difference in P450 activity was observed by detecting PNOD activity, while Reyes *et al.*, determined P450 activity by measuring 7-eth-oxycoumarin O-deethylation activity (Reyes *et al.*, 2007, 2011). Therefore, more substrates should be investigated. Our previous study reported that chlorpyrifos-ethyl and lambda-cyhalothrin induced the mRNA levels of *CYP9A61*, indicating that *CYP9A61* has a potential role in detoxification of insecticides and plant toxins (Yang *et al.*, 2013). However, P450 is a superfamily of enzymes which are under polygenic control. Induced expression of one gene may not directly prove its role in insecticide resistance. Toxicological bioassay and synergy tests should be performed to confirm the role of P450 in development of insecticide-resistance in Chinese *C. pomonella* populations.

Apart from what is already mentioned above, the knock-down resistance (*kdr*) mutation also participates in the development of insecticide resistance in several populations of codling moth from France, Italy, Switzerland, and Armenia (Reyes *et al.*, 2007). In Greece, no *kdr* mutation was detected in resistant populations (Voudouris *et al.*, 2011). The presence or absence of the *kdr* mutation in China's *C. pomonella* populations should be investigated in future studies.

To prevent this invasive species from invading into Shaanxi, a main apple production area in China, the government has designated several regions for elimination of this pest centralized in Gansu Province, especially those adjacent to Shaanxi Province. Focusing on the apple and pear growing regions in Zhangye and Wuwei, various management practices have been implemented to control the codling moth. Although some methods such as control by spraying CpGV and the attract-and-kill strategy are also used against the codling moth, effective management of the codling moth continues to mainly depend on chemical insecticides. There are a broad range of effective insecticides for the control of codling moth, unfortunately, as a consequence of the misuse or overuse these insecticides, serious resistance to these insecticides have developed in this species. Our data demonstrate that codling moth populations from Northwestern China have developed a low-level of resistance to several groups of insecticides due to overuse and misuse of chemical pesticides over the long term. The insensitivity to chlorpyrifos-ethyl was predominantly correlated with increased GST activity. The insensitivity to carbaryl was not obviously associated with any enzyme activity measured, CarE and AChE polymorphisms or mutation evaluated. Thus resistance management is also an urgent issue in China. New types of insecticides with novel modes of action need to be developed (Hollomon, 2012); however, this process is becoming ever harder and more costly. Hence, it is vital that integrated pest management strategies are implemented to manage the developed insecticide resistance or to prevent or delay the incidence of resistance.

Among the insecticides investigated, the imidacloprid has a poor contact activity in fruits following foliar sprays, and it reaches too low concentration in reproductive organs after irrigation or drenching treatments in apple trees. Other insecticides from this chemical group, such as acetamiprid or thiacloprid should have been used instead (Brunner *et al.*, 2005). By alternations of insecticides from different modes of action, and other management strategies, such as mating disruption, sex attractant-kill and spraying CpGV should be advocated and the use of chemical insecticides should be appropriately supervised and restrained by government.

References

Abbott, W.S. (1925) A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology* **18**, 265–267.

Alon, M., Alon, F., Nauen, R. & Morin, S. (2008) Organophosphates resistance in the B-biotype of *Bemisia tabaci* (Hemiptera: Aleyrodidae) is associated with a point mutation in an ace1-type acetylcholinesterase and overexpression of carboxylesterase. *Insect Biochemistry and Molecular Biology* **38**, 940–949.

Baek, J.H., Kim, J.I., Lee, D.W., Chung, B.K., Miyata, T. & Lee, S.H. (2005) Identification and characterization of ace1-type acetylcholine sterase likely associated with organophosphate resistance in *Plutella xylostella*. *Pesticide Biochemistry and Physiology* **81**, 164–175.

Bouvier, J.C., Brosse, V. & Sauphanor, B. (1995) La résistance du carpocapse. *L' Arboriculture Fruitière* **479**, 21–23.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Brun-Barale, A., Bouvie, J.C., Pauron, D., Bergé, J.B. & Sauphanor, B. (2005) Involvement of a sodium channel mutation in pyrethroid resistance in *Cydia pomonella* L. and development of a diagnostic test. *Pest Management Science* **61**, 549–554.

Brunner, J.F., Beers, E.H., Dunley, J.E., Doerr, M. & Granger, K. (2005) Role of neonicotinyl insecticides in Washington apple integrated pest management. Part I. Control of lepidopteran pests. *Journal of Insect Science* **5**, 14–0.

Bush, M.R., Abdel-All, Y.A.I. & Rock, G.C. (1993) Parathion resistance and esterase activity in codling moth (Lepidoptera: Tortricidae) from North Carolina. *Journal of Economic Entomology* **86**, 660–666.

Cassanelli, S., Reyes, M., Rault, M., Manicardi, G.C. & Sauphanor, B. (2006) Acetylcholinesterase mutation in an insecticide-resistant population of the codling moth *Cydia pomonella* (L.). *Insect Biochemistry and Molecular Biology* **36**, 642–653.

Dunley, J.E. & Welter, S.C. (2000) Correlated insecticide cross-resistance in azinphosmethyl resistant codling moth

(Lepidoptera: Tortricidae). *Journal of Economic Entomology* **93**, 955–962.

Fuentes-Contreras, E., Reyes, M., Barros, W. & Sauphanor, B. (2007) Evaluation of azinphos-methyl resistance and activity of detoxifying enzymes in codling moth (Lepidoptera: Tortricidae) from central Chile. *Journal of Economic Entomology* **100**, 551–556.

Giliomee, J.H. & Riedl, H. (1998) A century of codling moth control in South Africa. I. Historical perspective. *Journal of the Southern African Society for Horticultural Sciences (South Africa)* **8**, 27–31.

Hollomon, D. (2012) Do we have the tools to manage resistance in the future? *Pest Management Science* **68**, 149–154.

Kim, Y.H. & Lee, S.H. (2013) Which acetylcholinesterase functions as the main catalytic enzyme in the Class Insecta? *Insect Biochemistry and Molecular Biology* **43**, 47–53.

Landolt, P.J., Suckling, D.M. & Judd, G.J.R. (2007) Positive interaction of a feeding attractant and a host kairomone for trapping the codling moth, *Cydia pomonella* (L.). *Journal of Chemical Ecology* **33**, 2236–2244.

Men, Q.L., Chen, M.H., Zhang, Y.L. & Feng, J.N. (2013) Genetic structure and diversity of a newly invasive species, the codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) in China. *Biological Invasions* **15**, 447–458.

Newcomb, R.D., Campbell, P.M., Ollis, D.L., Cheah, E., Russell, R.J. & Oakeshott, J.G. (1997) A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proceedings of the National Academy of Science of the United States of America* **94**, 7464–7468.

Reuveny, H. & Cohen, E. (2004) Resistance of the codling moth *Cydia pomonella* (L.) (Lep., Tortricidae) to pesticides in Israel. *Journal of Economic Entomology* **128**, 645–651.

Reyes, M. & Sauphanor, B. (2008) Resistance monitoring in codling moth: a need for standardization. *Pest Management Science* **64**, 945–953.

Reyes, M., Bouvier, J.C., Bouvin, T., Fuentes-Contreras, E. & Sauphanor, B. (2004) Susceptibilidad a insecticidas y actividad enzimática de *Cydia pomonella* L. (Lepidop-tera: Tortricidae) provenientes de tres huertos de manzano de la región del Maule. *Chile Agric Técn (Chile)* **64**, 229–237.

Reyes, M., Frank, P., Charmillot, P.J., Ioriatti, C., Olivares, J., Pasqualini, E. & Sauphanor, B. (2007) Diversity of insecticide resistance mechanisms and spectrum in European populations of codling moth, *Cydia pomonella*. *Pest Management Science* **63**, 890–902.

Reyes, M., Collange, B., Rault, M., Casanelli, S. & Sauphanor, B. (2011) Combined detoxification mechanisms and target mutation fail to confer a high level of resistance to organophosphates in *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). *Pesticide Biochemistry and Physiology* **99**, 25–32.

Rodríguez, M.A., Bosch, D. & Sauphanor, B. (2010) Avila J, Susceptibility to organophosphate insecticides and activity of detoxifying enzymes in Spanish populations of *Cydia pomonella* (Lepidoptera: Tortricidae). *Journal of Economic Entomology* **103**, 482–491.

Rodríguez, M.A., Bosch, D. & Avilla, J. (2011a) Resistance of Spanish codling moth (*Cydia pomonella*) populations to

insecticides and activity of detoxifying enzymatic systems. *Entomologia Experimentalis et Applicata* **138**, 184–192.

Rodríguez, M.A., Marques, T., Bosch, D. & Avilla, J. (2011b) Assessment of insecticide resistance in eggs and neonate larvae of *Cydia pomonella* (Lepidoptera: Tortricidae). *Pesticide Biochemistry and Physiology* **100**, 151–159.

Sauphanor, B., Bouvier, J. & Brosse, V. (1998) Spectrum of insecticide resistance in *Cydia pomonella* (Lepidoptera: Tortricidae) in Southeastern Franc. *Journal of Economic Entomology* **91**, 1225–1231.

Thwaite, W.G., Williams, D.G. & Hately, A.M. (1993) Extent and significance of azinphos-methyl resistance in codling moth in Australia. pp. 166–168 in Corey, S., Dall, D. & Milne, W. (*Eds*) *Pest Control and Sustainable Agriculture*. Melbourne, Australia, Commonwealth Scientific and Industrial Research Organization.

Varela, L.G., Welter, S.C., Jones, V.P., Brunner, J.F. & Riedl, H. (1993) Monitoring and characterization of insecticide resistance in codling moth (Lepidoptera, Tortricidae) in four western states. *Journal of Economic Entomology* **86**, 1–10.

Voudouris, C.C., Sauphanor, B., Frank, P., Reyes, M., Mamuris, Z., Tsitsipis, J.A., Vontas, J. & Margaritopoulos, J.T. (2011) Insecticide resistance status of the codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) from Greece. *Pesticide Biochemistry and Physiology* **100**, 229–238.

Waldner, W. (1993) Rückblick und Vorschau auf die Bekämpfung des Apfelwicklers. *Obstbau-Weinbau* **12**, 355–357.

Wheelock, C.E., Phillips, B.M., Anderson, B.S., Miller, J.L. & Hammock, B.D. (2008) Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). pp. 117–178 in Whitacre, D.M. (ed.) *Reviews of Environmental Contamination and Toxicology*, New York, Springer.

Yang, X.Q. & Zhang, Y.L. (2013) Effect of temperature and sorbitol in improving the solubility of carboxylesterases protein CpCE-1 from *Cydia pomonella* and biochemical characterization. *Applied Microbiology and Biotechnology* **97**, 10423–10433.

Yang, X.Q., Li, X.C. & Zhang, Y.L. (2013) Molecular cloning and expression of *CYP9A61*: a chlorpyrifos-ethyl and lambda-cyhalothrin-inducible cytochrome P450 cDNA from *Cydia pomonella*. *International Journal of Molecular Science* **14**, 24211–24229.

Yang, X.Q., Liu, J.Y., Li, X.C., Chen, M.H. & Zhang, Y.L. (2014) Key amino acid associated with acephate detoxification by Cydia pomonella carboxylesterase based on molecular dynamics with alanine scanning and site-directed mutagenesis. *Journal of Chemical Information and Modeling* **54**, 1350–1370.

Yang, Y., Wu, Y., Chen, S., Devine, G.J., Denholm, I., Jewess, P. & Moores, G.D. (2004) The involvement of microsomal oxidases in pyrethroid resistance in *Helicoverpa armigera* from Asia. *Insect Biochemistry and Molecular Biology* **34**, 763–773.

Zhang, X.Z. (1957) Taxonomic notes on the codling moth, *Carpocapsa pomonella* L. in Sinkiang. *Acta Entomologica Sinica* **7**, 467–472.

Zhao, P., Wang, Y. & Jiang, H. (2013) Biochemical properties, expression profiles, and tissue localization of orthologous acetylcholinesterase-2 in the mosquito, *Anopheles gambiae*. *Insect Biochemistry and Molecular Biology* **43**, 260–271.