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# Worldwide variability of insecticide resistance mechanisms in the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae)

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**Abstract**

The activity of detoxifying enzymes (glutathione-S-transferases (GST), mixed-function oxidases (MFO), and esterases (EST)) and the presence of insensitive variants of target proteins (sodium channel and acetylcholinesterase) were examined in individual male and female codling moths. Twenty-nine populations from 11 countries and two laboratory strains were examined. Populations were classified as either unsprayed or sprayed. The ranges of enzyme activities across field populations varied 15-fold, 485-fold and fourfold for GST, MFO and EST, respectively. MFO was the only enzyme whose activity differed in a binomial classification of orchards based on their spray history. Few differences in enzyme activities were found due to sex among populations; and, in these cases, males had higher GST and lower MFO and EST activities than females. Activities of the three enzymatic systems across all populations were positively correlated. Populations from Greece, Argentina and Uruguay had significant percentages of moths with elevated GST and MFO activities. The co-occurrence of moths expressing both elevated MFO and low EST activities was found in conventional orchards from the Czech Republic and France. Chile was the only country where populations from treated orchards did not include a significant proportion of individuals with enhanced enzyme activity. The *kdr* mutation was found at significant levels in ten populations from five countries, including all French and Argentinean populations. The mutation in AChE was only detected in the Spanish population.

**Keywords:** detoxifying enzymes, insecticide resistance mechanisms, apple, pest management

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## Introduction

The codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), is the most serious worldwide insect pest of apple, *Malus domestica* Borkhausen (Rosaceae), and pear, *Pyrus communis* L. (Rosaceae), except in Japan and a few other regions of Asia (Croft, 1982). The control of codling moth is largely achieved with chemical insecticides, such as various neuroactive products (organophosphates, carbamates, synthetic pyrethroids and neonicotinoids) and insect growth regulators (IGR, chitin synthesis inhibitors, juvenile hormone mimics and ecdysone agonists). However, the extensive and continued use of these products has led to the development of resistance by the codling moth to a number of different insecticides in several countries (Thwaite *et al*., 1993; Varela *et al*., 1993; Waldner, 1993; Sauphanor *et al*., 1998; Reuveny & Cohen, 2004; Fuentes-Contreras *et al*., 2007). The resistance is mainly associated to the detoxification systems mixed function oxidases (MFO), glutathione-S-transferases (GST) and esterases (EST) (Bush *et al*., 1993; Sauphanor *et al*., 1997; Soleño *et al*., 2004; Reyes *et al*., 2007). Furthermore, a *kdr* mutation corresponding to a L1014F replacement in the voltage-dependent sodium channel protein is involved in resistance to deltamethrin (Brun-Barale *et al*., 2005), and the F290V replacement in acetylcholinesterase (AChE) is involved in resistance to azinphos-methyl and carbaryl (Cassanelli *et al*., 2006).

The spread of resistance in insect populations depends on multiple factors, including the intensity of insecticide selection pressure, the migration ability of individuals and the fitness costs linked with resistance (May & Dobson, 1986). In *C. pomonella*, resistance to insecticides, based on enhanced GST and MFO activities, are associated with pleiotropic costs with respect to reproduction and development rate (Boivin *et al*., 2001). In addition, a recent microsatellite DNA analysis on samples from different countries suggests that *C. pomonella* populations are structured according to geographical distance at supranational scale and that the intensity of insecticide applications is the main force structuring the populations at the national level (Franck *et al*., 2007). In the same way, Espinoza *et al*. (2007) and Fuentes-Contreras *et al*. (2008) did not detect differentiation by distance between Chilean *C. pomonella* populations, even when they were separated by more than 180 km. In contrast, Timm *et al*. (2006) detected geographical differentiation in South-African populations. The intensity of insecticide applications has an important impact on the level of genetic structure, and insecticide treatments are efficient to reduce population density, even when some cases of insecticide resistance are present (Franck *et al*., 2007). Resistance mechanisms to chemical insecticides were independently analysed in *C. pomonella* populations from several geographical areas (Bush *et al*., 1993; Sauphanor *et al*., 2000; Soleño *et al*., 2004; Fuentes-Contreras *et al*., 2007; Reyes *et al*., 2007). However, it is difficult to assess the geographical variability of the resistance mechanisms due to the absence of similar methodology in these studies. For example, most of the studies have considered only a single mechanism (Bush *et al*., 1993; Soleño *et al*., 2004) and have been limited to populations from a narrow geographical area (one country or one continent). Furthermore, these prior analyses of several mechanisms have been performed in different individuals, which preclude a more careful exploration of the potential linkages among several insecticide resistance mechanisms.

Herein, a large collaborative project is reported, which aimed to analyze the potential variability of resistance mechanisms among *C. pomonella* populations from different geographical regions. All five known resistance mechanisms (i.e. EST, GST, MFO, *kdr* and modified AChE) were measured on each individual moth. In addition, each metabolic resistance was expressed as activity per milligrams of protein, which avoids the biases of sexual dimorphism in body size. Data are reported for each sex. The association of the recent agricultural practices applied in the orchards surveyed with the resistance mechanisms was also examined.

## Material and methods

### *Insects*

Diapausing larvae of *C. pomonella* were collected in orchards using corrugated cardboard traps during 2005 and 2006 (Audemard, 1992). A total of 958 individuals (a 55 : 45 male : female ratio) were collected from 29 sites in New Zealand (2), South Africa (1), Bulgaria (2), France (4), Greece (4), Spain (1), Czech Republic (3), Chile (5), Argentina (2), Uruguay (2) and United States (3) (table 1). All larvae were shipped via land or air transportation to the INRA laboratory in Avignon, France. Larvae were kept at 2°C and 12 : 12 (light : dark) for three months and then placed under conditions suitable for adult emergence (25°C, 60% RH and 16 : 8 light : dark).

Populations of *C. pomonella* were collected from orchards under a wide range of management practices. Orchards were grouped into two classes (unsprayed and sprayed) based on the number of synthetic insecticides applied in the year that the larvae were collected (table 1). Populations from both sprayed and unsprayed orchards were collected from five countries: Bulgaria, France, Greece, Argentina and Chile. The unsprayed group (< 1 application) included unmanaged orchards, experimental orchards where an unknown proportion of trees were treated with a wide range of insecticides, organic orchards sprayed only with granulosis virus and one orchard treated only with sex pheromone (table 1). Sprayed orchards were treated with 2–7 sprays of synthetic insecticides from among 1 to 4 insecticide classes including organophosphates, carbamates, synthetic pyrethroids, neonicotinyls and insect growth regulators. Organophosphates and carbamates accounted for nearly 78% of all sprays applied in these orchards. Unfortunately, the spray records from four orchards could not be obtained, and these data were not included in the binomial classification.

Individuals from two laboratory strains of *C. pomonella* were included in this study. The *Sv* strain was originally established in 1995 from susceptible individuals identified via bioassays from apple orchards in southern France (Boivin *et al*., 2003). This founding colony has been mass-reared on an artificial diet (Guennelon *et al*., 1981) at INRA Avignon (France) for more than ten years without any exposure to insecticides. The *Raz* strain originated from a pear orchard from the Lerida region in Spain which had been conventionally managed with organophosphate insecticides and control failures had been documented (Sauphanor *et al*., 2000). *Raz* had been mass-reared on artificial diet at the INRA laboratory in Avignon for more

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Table 1. Summary of field populations and laboratory strains of *Cydia pomonella* included in the analyses of enzyme activity and genetic mutations associated with insecticide resistances.

| Origin | Population | Year | Number of moths tested (male/female) | Insecticide history[a] | | |
|---|---|---|---|---|---|---|
| | | | | Management practices | Number of chemical sprays | Orchard class |
| Laboratory[b] | *Sv* | – | 17/17 | | | |
| | *Raz* | – | 19/8 | – | – | – |
| South Africa | SA1 | 2005 | 7/6 | Experimental | N.A. | – |
| New Zealand | NZ1 | 2005 | 12/10 | Organic | 0 | Unsprayed |
| | NZ2 | 2005 | 20/20 | Experimental | N.A. | – |
| Bulgaria | Bu1 | 2005 | 29/12 | Pheromone | 0 | Unsprayed |
| | Bu2 | 2005 | 8/4 | Conventional | 4 | Sprayed |
| Czech Republic | CR1 | 2005 | 9/9 | Conventional | N.A. | Sprayed |
| | CR2 | 2006 | 33/20 | Conventional | 7 | Sprayed |
| | CR3 | 2006 | 31/16 | Conventional | 7 | Sprayed |
| France | Fr1 | 2005 | 15/14 | Conventional | 7 | Sprayed |
| | Fr2 | 2005 | 15/15 | Organic | 0 | Unsprayed |
| | Fr3 | 2005 | 17/16 | Conventional | 6 | Sprayed |
| | Fr4 | 2005 | 15/15 | Organic | 0 | Unsprayed |
| Greece | Gr1 | 2005 | 34/22 | Conventional | 5 | Sprayed |
| | Gr2 | 2005 | 7/9 | Unmanaged | 0 | Unsprayed |
| | Gr3 | 2005 | 35/29 | Conventional | 7 | Sprayed |
| | Gr4 | 2005 | 6/7 | Organic | 0 | Unsprayed |
| Spain | Sp1 | 2005 | 12/17 | Conventional | 2 | Sprayed |
| United States | US1 | 2006 | 15/19 | Pheromone | <1 | Unsprayed |
| | US2 | 2006 | 16/17 | Unmanaged | 0 | Unsprayed |
| | US3 | 2006 | 8/9 | Pheromone | <1 | Unsprayed |
| Argentina | Ar1 | 2005 | 22/14 | Unmanaged | 0 | Unsprayed |
| | Ar2 | 2005 | 16/7 | Conventional | 7 | Sprayed |
| Chile | Ch1 | 2005 | 20/20 | Experimental | N.A. | – |
| | Ch2 | 2005 | 26/20 | Conventional | 6 | Sprayed |
| | Ch3 | 2005 | 20/20 | Unmanaged | 0 | Unsprayed |
| | Ch4 | 2005 | 35/20 | Conventional | 4 | Sprayed |
| | Ch5 | 2005 | 20/20 | Unmanaged | 0 | Unsprayed |
| Uruguay | Ur1 | 2005 | 8/10 | Pheromone | 3 | Sprayed |
| | Ur2 | 2005 | 9/10 | Conventional | 7 | Sprayed |

[a] Conventional, conventional insecticide programs; pheromone, sex pheromone-based programs with and without the addition of insecticides; organic, organic with or without the supplemental use of granulosis virus; unsprayed, no insecticides used; experimental, research sites where various plots were treated with an array of products.
[b] *Sv*, susceptible lab strain; *Raz*, azinphos-methyl resistant laboratory strain.

than ten years and has regularly been submitted to selection pressure by exposing the larvae to diet surface-treated with 375 mg $l^{-1}$ azinphos-methyl. While *Sv* strain is susceptible to insecticides, *Raz* is resistant to most of the insecticides currently used against codling moth. Both strains are clearly differentiable by their GST and MFO activities (Reyes *et al*., 2007).

### *Analytical procedures*

Adult moths were sexed before analysis. All five resistance mechanisms were analyzed in each individual moth. GST, MFO and EST activities were evaluated using adult abdomens which were dissected and divided longitudinally into two equal parts in NaCl (6 g $l^{-1}$). Activities are expressed per mg protein. Target site modifications were investigated using DNA extracts from a hind leg. Fluorescence and absorbance were measured using a microplate reader (HTS 7000, Perkin Elmer, Massachusetts, USA).

### *Biochemical assays*

GST and EST activities were determined using the same extract. One half of each adult abdomen was individually homogenized on ice in 75 µl of Hepes buffer (50 mM, pH 7.0) and centrifuged at 15,000 *g* for 15 minutes at 4°C. The supernatants were used as enzyme source. Protein content of each sample was measured in 10 µl of extract diluted ten times, according to Bradford (1976) using bovine serum albumin to build the standard curve.

#### *Glutathione-S-transferase (GST)*

GST activities were determined using monochlorobimane (MCB) as substrate (Nauen & Stumpf, 2002). Each well was supplied with 30 µl of enzyme extract, 168 µl of 100 mM reduced glutathione (GSH) in Hepes buffer (50 mM, pH 7.0) and 2 µl of 30 mM MCB (Reyes *et al*., 2007). Twelve wells in each 96-well plate loaded with Hepes buffer instead of protein extract were used as controls. Fluorescence was

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measured after 20 min of incubation at 22°C with 380 nm excitation and 450 nm emission filters. Enzyme activity was expressed as fluorescence units per mg of total protein.

*Esterases*

Total esterase activities were measured using β-naphthyl acetate as substrate (Sauphanor *et al*., 1997). Each well was supplied with 0.1 mM substrate in 50 mM of phosphate buffer (pH 6.5), 0.5 µl of enzyme extract and 89.5 µl of Hepes buffer (50 mM, pH 7.0). After 15 min of incubation at 30°C, 20 µl of a staining reagent containing 3 g $l^{-1}$ Fast Garnet and 35 g $l^{-1}$ sodium dodecyl sulfate were added to the solution, and the absorbance was measured at 492 nm after 15 min at room temperature. Twelve wells without enzyme were included as controls. EST activities are expressed as nm of β-napthol/mg protein/min.

*Mixed function oxidases (MFO)*

MFO activities were determined using 7-ethoxycoumarin O-deethylation (ECOD) (Ulrich & Weber, 1972) adapted for *in vivo* analysis with a microplate (De Sousa *et al*., 1995). Each half of the adult abdomens was individually introduced into a well containing 100 µl of phosphate buffer (50 mM, pH 7.2) and ethoxycoumarin (0.4 mM). After four hours of incubation at 30°C, the reaction was stopped by adding 100 µl of 0.1 mM glycine buffer (pH 10.4)/ethanol (v/v). The 7-hydroxycoumarin fluorescence was quantified with 380 nm excitation and 450 nm emission filters. Twelve wells filled with only glycine buffer prior to incubation were used for controls on each plate. MFO activities are reported as pg of 7OH formed/mg protein/min.

### *DNA diagnostics*

Target mutations in sodium channel and AChE proteins were detected by PCR-RFLP (Reyes *et al*., 2007). Total DNA was extracted from an adult leg with 200 µl of 10% Chelex 100 (Biorad, California, USA) solution (Walsh *et al*., 1991) and 3 µl of 10 mg $ml^{-1}$ of proteinase K. After a four times dilution, extracts were used as DNA templates for partial PCR amplifications of the sodium channel and *ace1* genes. PCR amplifications were carried out separately for each gene in a 25 µl reaction volume containing 1× reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of Taq DNA polymerase and 2 µl of DNA template. Restrictions were performed by pooling the PCR products from both genes (5 µl each) with 0.2 units of Tsp509I (NEB) in 20 µl reaction volume for 16 h incubation at 65°C. DNA fragments were separated by electrophoresis on 6% polyacrylamide gel and visualized after silver staining. The *kdr* and sensitive alleles for the sodium channel were identified by DNA fragments of 77 bp and 112 bp, respectively. The resistant V290 and sensitive F290 alleles for *ace1* were identified by DNA fragments of 190 bp and 152 bp, respectively (Reyes *et al*., 2007).

### *Statistical analysis*

Nonparametric statistics were used to compare classes of orchards, origin, moth sex and *C. pomonella* field populations and laboratory strains because several standard transformations could not consistently normalize the variances (Shapiro-Wilkes test). A Wilcoxon rank sum test was used to compare the enzyme activities among the susceptible and resistant laboratory strains (Analytical Software, 2003). Pearson correlations were used to evaluate the relationships of mean enzyme activity levels across all field-collected individuals. A Wilcoxon rank sum test was also used to compare enzyme activities due to moth sex and to compare the various enzyme activities in each field population versus the *Sv* strain. The same statistical test was used to evaluate mean enzyme activities in a binomial classification of orchards based on their spray history and their geographic origin (European vs. Non European orchards). A one-tailed Fisher exact test was used to compare the frequency of resistant individuals in each population with the *Sv* strain. All these statistical tests were run with Statistix 8 (Analytical Software, 2003). Moths were classified as resistant if their enzyme activities exceeded the upper 95% confidence limit for GST activity (42,589.2 fluorescence units/mg protein) and MFO activity (95.6 pg of 7OH formed/mg protein/min) of the *Sv* strain. With EST, individuals were classified as resistant if either their enzyme activity was greater than (EST >) the upper (552.78 nm of β napthol/mg protein/min) or less than (EST <) the lower (183.1 nm of β napthol/mg protein/min) 95% confidence limit for the *Sv* strain. Finally, Fisher's exact tests were performed using the GENEPOP software (Rousset, 2008) to test departure to the Hardy-Weinberg equilibrium in each population for the sodium channel and AChE loci.

## Results

Significant differences in the mean activity levels of each of the three enzymes were found among the two laboratory strains of *C. pomonella* (table 2). As expected, the insecticide-resistant *Raz* strain had significantly higher MFO and GST activities than the susceptible strain. *Sv* male moths had significantly higher GST activity than *Sv* females.

When the whole sample was analyzed, MFO and EST activities were higher in females, while GST activity was higher in males. For each 29 samples, activities of at least one enzymatic system significantly differed with those reported in the susceptible strain *Sv*. The mean GST activity values among field populations ranged 17.6- and 14.6-fold for female and male moths, respectively (table 3). However, a clear assessment of each population's resistance ratio (R/S) was complicated by the significant differences in GST activity between sexes within *Sv*. For example, 21 versus six field populations had GST levels significantly higher than *Sv* for the female versus male moths (table 3). The mean GST activity was higher in male than in female moths in three populations (Gr3, Ar1 and Ur2). Both sexes in six field populations (SA, Gr4, Ar1, Ar2 and Ur1) had significantly higher GST activity than *Sv*.

The mean MFO activity among field populations varied 147-fold for females and >10,000-fold for male moths (table 4). The mean MFO activities were significantly higher than in the susceptible strain for eight samples (Ur1, Ar2, Sp1, Gr3, Fr1, Fr3, CR3 and Bu1) out of the 29 orchards analyzed with resistant ratios up to 25 in both female and male sub-samples (table 4). No significant differences in MFO activities between sexes were observed in these eight samples. All of them, except Bu1, were from orchards sprayed with chemical insecticides (table 1).

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Table 2. Mean enzyme activities expressed in two laboratory strains of *Cydia pomonella*.

| Strain[b] | Sex | *n* | Mean (SE) enzyme activity[a] | | |
|---|---|---|---|---|---|
| | | | GST | MFO | EST |
| *Sv* | F | 17 | 9380 (2202)b | 26 (8)a | 368 (23)a |
| | M | 17 | 21,863 (3637)a | 37 (26)a | 374 (35)a |
| | F+M | 34 | 15,622 (2359)B | 32 (14)B | 371 (20)A |
| *Raz* | F | 8 | 35,707 (6300)a | 1502 (421)a | 324 (19)a |
| | M | 19 | 58,165 (6914)a | 2129 (548)a | 417 (41)a |
| | F+M | 27 | 51,510 (5522)A | 1943 (404)A | 390 (30)A |

Column means for each strain (both sexes) followed by a different uppercase letter and means for each sex within each strain followed by a different lowercase letter, were significantly different; $P < 0.05$, Wilcoxon rank sum test.
[a] The activities of glutathione S-transferase (GST), mixed-function oxidases (MFO) and esterases (EST) are reported as fluorescence units/mg protein, pg of 7OH formed/mg protein/min, and nm of β napthol/mg protein/min, respectively.
[b] The *Sv* strain is susceptible to insecticides, while the *Raz* strain has been selected with azinphos-methyl for nearly ten years and is resistant to most of the insecticides currently used against codling moth.

Table 3. Mean activities of glutathione S-transferase activities (fluorescence units/mg protein) and their resistance ratios (R/S) compared with the insecticide-susceptible laboratory strain (S*v*) for 29 field populations of male and female *Cydia pomonella*.

| Population | Orchard class | Male | | Female | |
|---|---|---|---|---|---|
| | | Mean (SE) activity | R/S[a] | Mean (SE) activity | R/S[a] |
| SAI | – | 80,728 (53,687) | 8.6** | 63,129 (15,032) | 2.9* |
| NZ1 | Unsprayed | 23,221 (3090) | 2.5** | 25,900 (4285) | 1.2 |
| NZ2 | – | 19,006 (2331) | 2.0** | 17,221 (1796) | 0.8 |
| Bu1 | Unsprayed | 20,863 (3312) | 2.2** | 18,682 (3380) | 0.9 |
| Bu2 | Sprayed | 9127 (5984) | 1.0 | 11,720 (3998) | 0.5 |
| CR1 | Sprayed | 23,053 (3406) | 2.5** | 21,304 (4501) | 1.0 |
| CR2 | Sprayed | 21,416 (2731) | 2.3** | 18,843 (1593) | 0.9 |
| CR3 | Sprayed | 31,022 (4307) | 3.3*** | 31,212 (3020) | 1.4 |
| Fr1 | Sprayed | 17,488 (3033) | 1.9 | 36,630 (12,775) | 1.7 |
| Fr2 | Unsprayed | 13,720 (2548) | 1.5 | 15,191 (4719) | 0.7 |
| Fr3 | Sprayed | 17,465 (3259) | 1.9* | 21,956 (4632) | 1.0 |
| Fr4 | Unsprayed | 4576 (1647) | 0.5 | 5004 (2118) | 0.2*** |
| Gr1 | Sprayed | 12,606 (2788) | 1.3 | 10,099 (1818) | 0.5** |
| Gr2 | Unsprayed | 13,144 (1366) | 1.4 | 17,118 (4450) | 0.8 |
| Gr3 | Sprayed | 13,766 (2561)b | 1.5 | 29,199 (3843)a | 1.3 |
| Gr4 | Unsprayed | 23,076 (7267) | 2.5* | 43,622 (3841) | 2.0** |
| Sp1 | Sprayed | 13,946 (3465) | 1.5 | 7653 (2180) | 0.4* |
| US1 | Unsprayed | 29,089 (3183) | 3.1*** | 21,579 (1912) | 1.0 |
| US2 | Unsprayed | 34,228 (3083) | 3.6*** | 32,660 (3655) | 1.5* |
| US3 | Unsprayed | 21,601 (3062) | 2.3** | 25,884 (3327) | 1.2 |
| Ar1 | Unsprayed | 25,651 (2365)b | 2.7*** | 41,938 (2677)a | 1.9*** |
| Ar2 | Sprayed | 45,228 (3764) | 4.8*** | 73,319 (9191) | 3.4*** |
| Ch1 | – | 25,846 (2841) | 2.8*** | 29,725 (1527) | 1.4 |
| Ch2 | Sprayed | 30,049 (5291) | 3.2*** | 30,974 (3379) | 1.4 |
| Ch3 | Unsprayed | 24,096 (2611) | 2.6*** | 28,673 (2679) | 1.3 |
| Ch4 | Sprayed | 20,972 (1941) | 2.2*** | 23,645 (1810) | 1.1 |
| Ch5 | Unsprayed | 17,089 (1963) | 1.8** | 20,577 (1804) | 0.9 |
| Ur1 | Sprayed | 46,706 (6042) | 5.0*** | 54,662 (13,062) | 2.5* |
| Ur2 | Sprayed | 20,702 (2326)b | 2.2** | 29,248 (3269)a | 1.3 |

Differences between field and laboratory populations were tested with the nonparametric, Wilcoxon rank sum test: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Row means followed by different letters were significantly different, $P < 0.05$, Wilcoxon rank sum test.
[a] R/S ratio equals the mean enzyme activity of the field population versus the laboratory, insecticide-susceptible strain (*Sv*).

The mean EST activity among field populations varied from three to fourfold for female and male moths, respectively (table 5). Female and male moths in 23 and 22 field populations, respectively, had mean EST activities significantly lower than *Sv* strain (table 5). No field populations had mean EST activities significantly higher than *Sv* strain for either sex.

The linear correlations of enzyme activities of each of the three enzymes analyzed were positive and statistically significant across all moths (GST with MFO, $r = 0.72$, $P < 0.001$; GST with EST, $r = 0.79$, $P < 0.001$; and EST with MFO, $r = 0.81$, $P < 0.001$. Within each population sample, paired enzyme activities were significant correlated in 12 populations with three GST-MFO ($r = 0.37$–$0.50$), ten

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Table 4. Mean activities of mixed function oxidase (pg of 7OH formed/mg protein/min) and their resistance ratios (R/S) compared with the susceptible laboratory strain (*Sv*) for 29 field populations of male and female codling moth.

| Population | Orchard class | Male | | Female | |
|---|---|---|---|---|---|
| | | Mean (SE) activity | R/S[a] | Mean (SE) activity | R/S[a] |
| SA1 | – | 70.0 (28.3) | 2.7 | 291.9 (258.3) | 7.9 |
| NZ1 | Unsprayed | 59.3 (20.6)a | 2.3** | 30.7 (19.4) | 0.8 |
| NZ2 | – | 66.8 (29.7) | 2.5 | 138.6 (75.4) | 3.7 |
| Bu1 | Unsprayed | 498.5 (125.7) | 19.0*** | 663.4 (145.9) | 17.9*** |
| Bu2 | Sprayed | 95.9 (38.2) | 3.7* | 159.9 (70.3) | 4.3 |
| CR1 | Sprayed | 52.3 (17.0) | 2.0 | 11.4 (4.6) | 0.3 |
| CR2 | Sprayed | 18.8 (5.1)b | 0.7 | 21.8 (11.2)a | 0.6 |
| CR3 | Sprayed | 228.3 (87.6) | 8.7** | 267.9 (97.0) | 7.2*** |
| Fr1 | Sprayed | 1,468 (694) | 56.0*** | 489.6 (169.1) | 13.2*** |
| Fr2 | Unsprayed | 23.6 (4.9)b | 0.9 | 26.2 (23.4)a | 0.7* |
| Fr3 | Sprayed | 834.9 (184.3) | 31.9*** | 932.3 (234.6) | 25.2*** |
| Fr4 | Unsprayed | 8.0 (6.8) | 0.3*** | 0.1 (0.1) | < 0.01*** |
| Gr1 | Sprayed | 253.9 (91.1) | 9.7 | 174.0 (43.0) | 4.7 |
| Gr2 | Unsprayed | 10.3 (6.7) | 0.4* | 0.01 (0.01) | < 0.001** |
| Gr3 | Sprayed | 543.8 (202.8) | 20.8*** | 479.1 (106.8) | 12.9*** |
| Gr4 | Unsprayed | 424.6 (216.8) | 16.2 | 739.5 (331.3) | 19.9 |
| Sp1 | Sprayed | 1285 (268) | 49.0*** | 559.2 (145.5) | 15.1*** |
| US1 | Unsprayed | 38.8 (25.7)a | 1.5* | 3.9 (2.6)b | 0.1** |
| US2 | Unsprayed | 9.5 (4.3) | 0.4** | 17.3 (8.9) | 0.5 |
| US3 | Unsprayed | 41.5 (19.5) | 1.6 | 1.2 (0.7) | 0.03* |
| Ar1 | Unsprayed | 96.8 (35.3) | 3.7 | 168.7 (85.3) | 4.6 |
| Ar2 | Sprayed | 484.8 (120.0) | 18.5*** | 960.7 (321.9) | 26.0*** |
| Ch1 | – | 23.3 (5.0) | 0.9 | 14.0 (4.3) | 0.4 |
| Ch2 | Sprayed | 53.0 (14.6) | 2.0 | 13.1 (3.5) | 0.4 |
| Ch3 | Unsprayed | 31.8 (11.1) | 1.2 | 10.8 (3.8) | 0.3 |
| Ch4 | Sprayed | 15.9 (4.1) | 0.6 | 22.5 (6.6) | 0.6 |
| Ch5 | Unsprayed | 17.9 (4.3) | 0.7 | 8.4 (2.3) | 0.2 |
| Ur1 | Sprayed | 625.1 (169.7) | 23.9*** | 564.3 (323.3) | 15.3* |
| Ur2 | Sprayed | 8.5 (2.9)a | 0.3** | 0.1 (0.1)b | 0.1* |

Differences between field and laboratory populations were tested with the nonparametric, Wilcoxon rank sum test: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Row means followed by different letters were significantly different, $P < 0.05$, Wilcoxon rank sum test.
[a] R/S ratio equals the mean enzyme activity of the field population versus the susceptible laboratory strain (*Sv*).

GST-EST (r = 0.34–0.98) and two MFO-EST (r = 0.34–0.63) positive correlations. Only one population had more than one significant correlation: Ar2 (GST-MFO (r = 0.425; $P = 0.042$), GST-EST (r = 0.46; $P = 0.025$), and MFO-EST (r = 0.56; $P = 0.045$).

Significant differences in MFO activities were found between samples from sprayed (417 ± 108 pg of 7OH formed/mg protein/min) and unsprayed (125 ± 64) orchards ($P < 0.05$). GST activities for unsprayed (22,864 ± 2587 fluorescence units/mg protein) and sprayed (26,663 ± 4315) orchards were similar ($P = 0.89$). EST activities were also similar between the unsprayed, (201.3 ± 21.7 nm of β-napthol/mg protein/min) and sprayed 220.7 ± 19.7) orchards ($P = 0.43$).

The proportion of adults within each population having enzyme activities categorized as resistant varied from 0.0 to >90% (table 6). Insecticide-resistant strain, *Raz*, had a significant proportion of individuals with both GST and MFO resistance. Six field populations had significantly higher proportions of moths with GST resistance compared to the susceptible strains. These were from both sprayed and unsprayed orchards and only one was from Europe (the unsprayed, organic Gr4). Eleven populations had significantly high proportions of individuals with elevated MFO activity compared to the sensitive strains and all but two of these were from sprayed orchards. The two exceptions were Bu1 and Gr4, which were unsprayed. No populations had a significant proportion of moths with elevated EST activity, while 11 populations had significant proportions with low EST activity. Six populations among this later group were from unsprayed orchards. The only populations from sprayed orchards that contained significantly high proportions of individuals with low EST compared to the sensitive strains were from the Czech Republic and France (table 6).

For two of the three enzymatic systems, activities significantly differed between the European and non-European samples ($P < 0.001$, for each enzyme), with higher means values for MFO (443.04 (53.0) vs. 101.87 (52.7) pg of 7OH formed/mg protein/min) and GST (20,913.6 (1738.3) vs. 30,032.8 (1731.0) fluorescence units/mg protein) activities in European and non-European samples, respectively.

The *kdr* mutation was detected in 16 populations and from all countries except South Africa, Greece and Spain (table 7). The highest frequencies of *kdr* mutation were observed in populations from Argentina (31.5–63.6%) and France (12.5–82.5%). The proportion of the *kdr* mutation in the other countries never exceeded 20.0%. Significant heterozygote deficiency with regards to Hardy-Weinberg equilibrium were detected in the populations from four orchards (CR1, US1, Fr2 and Fr3; Fisher's exact tests, $0.0007 < P < 0.045$) out of the 16 polymorphic population samples where *kdr* was detected. The F290 mutation in the AChE conferring resistance to organophosphate insecticides

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Table 5. Mean activities of esterases (nm of B napthol/mg protein/min) and their resistance ratios (R/S) compared with a susceptible laboratory strain (*Sv*) for 29 field populations of male and female codling moth.

| Population | Orchard class | Male | | Female | |
|---|---|---|---|---|---|
| | | Mean (SE) activity | R/S[a] | Mean (SE) activity | R/S[a] |
| SA1 | – | 302.9 (94.6) | 0.8 | 336.6 (93.5) | 0.9 |
| NZ1 | Unsprayed | 185.8 (28.2) | 0.5*** | 149.6 (25.2) | 0.4*** |
| NZ2 | – | 229.2 (18.6) | 0.6*** | 200.6 (16.8) | 0.5*** |
| Bu1 | Unsprayed | 392.2 (47.5) | 1.1 | 363.2 (31.3) | 1.0 |
| Bu2 | Sprayed | 271.7 (58.2) | 0.7 | 286.8 (47.8) | 0.8 |
| CR1 | – | 144.8 (27.3) | 0.4*** | 143.0 (27.0) | 0.4*** |
| CR2 | Sprayed | 156.9 (15.9)a | 0.4*** | 119.4 (11.6)b | 0.3*** |
| CR3 | Sprayed | 187.5 (43.2) | 0.5*** | 109.0 (11.4) | 0.3*** |
| Fr1 | Sprayed | 173.4 (21.2)a | 0.5*** | 106.4 (13.1)b | 0.3*** |
| Fr2 | Unsprayed | 156.6 (16.7) | 0.4*** | 117.9 (17.5) | 0.3*** |
| Fr3 | Sprayed | 114.8 (17.9) | 0.3*** | 92.0 (6.8) | 0.2*** |
| Fr4 | Unsprayed | 138.4 (13.6) | 0.4*** | 127.2 (12.3) | 0.3*** |
| Gr1 | Sprayed | 277.2 (20.4) | 0.8** | 295.9 (32.1) | 0.8 |
| Gr2 | Unsprayed | 154.8 (17.6)a | 0.4*** | 94.1 (19.8)b | 0.3*** |
| Gr3 | Sprayed | 332.8 (24.2)a | 0.9 | 253.4 (18.1)b | 0.7** |
| Gr4 | Unsprayed | 330.6 (60.8) | 0.9 | 292.6 (80.3) | 0.8 |
| Sp1 | Sprayed | 302.6 (29.7) | 0.8 | 325.7 (43.2) | 0.9 |
| US1 | Unsprayed | 161.3 (14.6) | 0.4*** | 164.5 (12.0) | 0.4*** |
| US2 | Unsprayed | 211.3 (18.1) | 0.6*** | 176.2 (9.7) | 0.5*** |
| US3 | Unsprayed | 170.9 (19.9) | 0.5*** | 209.8 (24.8) | 0.6** |
| Ar1 | Unsprayed | 209.0 (23.9) | 0.6*** | 256.1 (18.4) | 0.7** |
| Ar2 | Sprayed | 251.7 (21.4) | 0.7** | 271.7 (27.1) | 0.7* |
| Ch1 | – | 251.2 (22.8) | 0.7** | 216.9 (20.0) | 0.6*** |
| Ch2 | Sprayed | 226.2 (29.3) | 0.6*** | 210.0 (17.6) | 0.6*** |
| Ch3 | Unsprayed | 161.5 (12.3) | 0.4*** | 157.9 (25.6) | 0.4*** |
| Ch4 | Sprayed | 230.2 (15.5) | 0.6*** | 198.1 (11.7) | 0.5*** |
| Ch5 | Unsprayed | 252.8 (26.7) | 0.7** | 191.9 (19.3) | 0.5*** |
| Ur1 | Sprayed | 267.7 (32.9) | 0.7* | 223.2 (32.8) | 0.6* |
| Ur2 | Sprayed | 229.2 (19.8) | 0.6** | 264.3 (36.6) | 0.7 |

Differences between field and laboratory populations were tested with the nonparametric, Wilcoxon rank sum test: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Row means followed by different letters were significantly different, $P < 0.05$, Wilcoxon rank sum test.
[a] R/S ratio equals the mean enzyme activity of the field population versus the laboratory insecticide-susceptible strain (*Sv*).

was only detected in the Spanish population (table 7). No departure to Hardy-Weinberg equilibrium was observed in this population (Fisher's exact test, $P = 0.401$).

## Discussion

Our study is the first attempt to investigate, simultaneously, all known resistance mechanisms in *C. pomonella* adults in populations collected from five continents. In addition, for the first time, all resistance mechanisms were analyzed in each male or female moth and enzyme activities were expressed per mg of protein. This allows precise measurement of the associations among resistance mechanisms within individual insects and between sexes irrespective of significant differences in their mean weight.

Unfortunately, sampling constraints impacted several aspects of this study. First, there were not enough insects obtained to include toxicological tests, but the analysis of resistance mechanisms could be a useful first approach. Then, an imbalanced experimental design with respect to the comparative numbers of sprayed and unsprayed orchards with chemical insecticides from each country, and the number of countries per continent limited our ability to more clearly evaluate the influence of management practices and geography. Some qualitative and quantitative measures of the degree of isolation of each orchard from surrounding populations of *C. pomonella*, especially for populations exposed to different pest management practices would be useful. For example, Bu1, Gr4 and Ar1 populations collected from unsprayed sites had significant proportions of individuals with elevated GST or MFO activity, and these orchards were situated within commercial apple production areas. Similarly, high proportions of the *kdr* allele were detected in several orchards (NZ1, Fr2, Fr4, US1, US3, Ar1), which were unsprayed with chemical insecticides. Among these orchards, the codling moth populations collected in Fr2 and US1 displayed heterozygote deficiency at the sodium channel locus. This suggests a selection of the *kdr* homozygote resistant genotypes that probably occurred in the conventional orchards surrounding these two sample sites. In addition, summarizing a more extended historical record of the spray practices made within each orchard may have been useful. The difficulty in obtaining a sample size large enough for meaningful statistical analysis was problematic for a few sites. The high intra-population variation in populations with low numbers of individuals (especially SA1 and Bu2) reduced the power of certain statistical tests.

Significant differences in the mean enzymatic activities were detected when the whole sample was analyzed. MFO and EST activities were higher in females, while GST activity was higher in males. Despite these differences, activity ratios were higher in females than in males for GST. This information suggests that, for this enzyme, the difference between sexes should not be involved in resistance. In

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Table 6. Frequency of codling moth adults within two laboratory strains and 29 field-collected populations characterized as resistant based on enzyme activities.

| Population | Orchard class | Moth frequency (%) | | | |
|---|---|---|---|---|---|
| | | GST[a] | MFO[b] | EST >[c] | EST <[d] |
| Sv | | 5.9 | 5.9 | 5.9 | 50.0 |
| *Raz* | | 59.3*** | 96.3*** | 25.9 | 37.0 |
| SA1 | – | 61.5** | 23.1 | 23.0 | 38.5 |
| NZ1 | Unsprayed | 18.2 | 9.1 | 0.0 | 54.5* |
| NZ2 | – | 7.5 | 15.0 | 0.0 | 35.0 |
| Bu1 | Unsprayed | 9.0 | 68.3*** | 34.1 | 17.1 |
| Bu2 | Sprayed | 0.0 | 50.0* | 16.7 | 16.7 |
| CR1 | – | 16.7 | 11.1 | 0.0 | 72.2*** |
| CR2 | Sprayed | 13.2 | 5.7 | 0.0 | 79.2*** |
| CR3 | Sprayed | 23.4 | 59.6*** | 4.3 | 80.9*** |
| Fr1 | Sprayed | 23.3 | 70.0*** | 0.0 | 76.7*** |
| Fr2 | Unsprayed | 6.7 | 3.3 | 0.0 | 80.0*** |
| Fr3 | Sprayed | 30.3 | 84.5*** | 0.0 | 90.9*** |
| Fr4 | Unsprayed | 0.0 | 3.3 | 0.0 | 80.0*** |
| Gr1 | Sprayed | 5.4 | 39.3** | 12.5 | 23.2 |
| Gr2 | Unsprayed | 0.0 | 0.0 | 0.0 | 81.3*** |
| Gr3 | Sprayed | 17.2 | 71.9*** | 18.8 | 17.2 |
| Gr4 | Unsprayed | 38.5* | 53.8** | 30.8 | 23.1 |
| Sp1 | Sprayed | 10.3 | 93.1*** | 17.2 | 10.3 |
| US1 | Unsprayed | 29.4 | 2.9 | 0.0 | 61.8** |
| US2 | Unsprayed | 42.4* | 3.0 | 0.0 | 45.5 |
| US3 | Unsprayed | 17.6 | 11.8 | 0.0 | 41.2 |
| Ar1 | Unsprayed | 30.6* | 27.8 | 4.8 | 33.3 |
| Ar2 | Sprayed | 82.6*** | 82.6*** | 4.3 | 13.0 |
| Ch1 | – | 17.5 | 0.0 | 0.0 | 30.0 |
| Ch2 | Sprayed | 23.9 | 8.9 | 2.2 | 43.5 |
| Ch3 | Unsprayed | 12.5 | 2.5 | 0.0 | 62.5** |
| Ch4 | Sprayed | 9.1 | 3.6 | 1.8 | 32.7 |
| Ch5 | Unsprayed | 5.0 | 0.0 | 5.0 | 42.5 |
| Ur1 | Sprayed | 61.1*** | 77.8*** | 5.6 | 27.8 |
| Ur2 | Sprayed | 10.5 | 0.0 | 0.0 | 26.3 |

Column percentages were compared with the corresponding susceptible strains using Fisher exact test: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

[a,b] Percentage of individuals expressing GST[a] or MFO[b] activity higher than the upper 95% confidence limit of the mean response of the *Sv* strain (42,589.2 fluorescence units/mg protein and 5.6 pg of 7OH formed/mg protein/min, respectively).

[c] Percentage of individuals expressing EST activity higher than the upper 95% confidence limit or less[d] than the lower 95% confidence limits of the mean response of the *Sv* strain (552.78 and 183.1 nm of β napthol/mg protein/min, respectively).

contrast, MFO activity values and ratios were higher in females than in males. Previous studies with *C. pomonella* have reported significant differences in the activities of detoxification enzymes (Fuentes-Contreras *et al.*, 2007) and different tolerance to insecticides (Varela *et al.*, 1993) between sexes within a population when the responses were expressed per whole insect. These differences in both studies were attributed to the larger size of female adults. Enzyme activities in our study were all reported per mg of protein, and few significant differences were found between sexes when each population was analyzed separately. Further investigations on these mechanisms are needed in order to validate these results.

Among the three enzymes examined, only MFO activity was significantly different within a binomial classification of orchards based on recent spray history. This result confirms, at the world level, previous observations in French apple orchards, in which the proportions of resistant MFO phenotypes were positively correlated with the number of chemical insecticide treatments (Franck *et al.*, 2007). The GST and EST activities were not related with the number of chemical insecticide treatments in this study in spite of the importance of both detoxification mechanisms in the resistance to the azinphos-methyl and of GST in the resistance to emamectin (Reyes *et al.*, 2007). The spatial (immigration) and temporal (historical spray history) uncertainties association with our orchard classification system likely weaken its predictive capabilities. In contrast, significant correlation of enzyme activities with insecticide bioassay results is more direct and provides a best estimate of the impact of EST and GST expressions on insecticide resistance (Fuentes-Contreras *et al.*, 2007; Reyes *et al.*, 2007). Chile was the only country included in our study where *C. pomonella* populations collected from sprayed orchards did not exhibit elevated enzyme activities. In contrast, significant differences in the levels of all three enzymes were found previously among field populations from Chile when compared with a susceptible laboratory strain (Reyes *et al.*, 2004). A recent genetic study of *C. pomonella* populations across two major apple-growing regions in central Chile found low genetic differentiation among populations independent of orchard management practices (Fuentes-Contreras *et al.*, 2008). This lack of differentiation was attributed to the widespread occurrence of *C. pomonella* populations developing in unmanaged sites, such as host trees associated with homes surrounding orchards. Many Chilean commercial orchards receive large numbers of insecticide sprays, and recent regulatory efforts to remove extra-orchard sources of moths could seriously impact the evolution of resistance in this country.

Among the three detoxification enzymes, EST had the lowest variability in activity among the populations samples (resistance ratios <5), and no difference was detected between the two laboratory strains (table 2). As previously signaled, most populations expressed significantly lower EST activity than the laboratory strain. Previous studies with *C. pomonella* have reported lower (Bush *et al.*, 1993; Bouvier *et al.*, 2002; Reyes *et al.*, 2007) or higher activity than the reference strain (Sauphanor *et al.*, 2000; Soleño *et al.*, 2004) and no correlation (Fuentes-Contreras *et al.*, 2007) of EST activities with resistance to organophosphate insecticides. Further investigation is required to clarify the contribution of this enzymatic group to insecticide resistance in *C. pomonella*. Different authors have used different substrates to characterize EST activity in *C. pomonella* (Bush *et al.*, 1993; Reuveny & Cohen, 2004; Reyes *et al.*, 2007), and it is expected that all the EST are not involved in insecticide resistance. For that, using a single substrate to measure EST activity cannot be enough to conclude on its participation on insecticide resistance (Reyes *et al.*, unpublished data). Development of new analytical tools that can discriminate S and R forms of these enzymes, i.e. using other substrates or molecular tools, are needed (Taylor & Feyereisen, 1996; Field & Devonshire, 1998; Daborn *et al.*, 2002).

Different metabolic mechanisms prevail in the countries surveyed in the present study, although GST was widely distributed followed by MFO. Previous studies indicated that enhanced MFO and GST activity were the most frequently encountered resistance mechanisms in European populations of *C. pomonella* (Sauphanor *et al.*, 2000; Reyes

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Table 7. Genotype frequency (%) of codling moth adults within two laboratory strains and 29 field-collected populations for *kdr* and F290-AChE loci.

| Population | Orchard class | *n* | *kdr* | | | F290-AChE | | |
|---|---|---|---|---|---|---|---|---|
| | | | SS | SR | RR | SS | SR | RR |
| *Sv* | | 30 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| *Raz* | | 30 | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 | 100.0 |
| SA1 | – | 13 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| NZ1 | Unsprayed | 22 | 77.3 | 22.7 | 0.0 | 100.0 | 0.0 | 0.0 |
| NZ2 | – | 38 | 94.7 | 5.3 | 0.0 | 100.0 | 0.0 | 0.0 |
| Bu1 | Unsprayed | 29 | 93.1 | 6.9 | 0.0 | 100.0 | 0.0 | 0.0 |
| Bu2 | Sprayed | 12 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| CR1 | – | 15 | 80.0 | 0.0 | 20.0 | 100.0 | 0.0 | 0.0 |
| CR2 | Sprayed | 15 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| CR3 | Sprayed | 18 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| Fr1 | Sprayed | 27 | 3.7 | 22.2 | 74.1 | 100.0 | 0.0 | 0.0 |
| Fr2 | Unsprayed | 27 | 81.5 | 3.7 | 14.8 | 100.0 | 0.0 | 0.0 |
| Fr3 | Sprayed | 30 | 70.0 | 13.3 | 16.7 | 100.0 | 0.0 | 0.0 |
| Fr4 | Unsprayed | 16 | 81.2 | 12.5 | 6.3 | 100.0 | 0.0 | 0.0 |
| Gr1 | Sprayed | 29 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| Gr2 | Unsprayed | 16 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| Gr3 | Sprayed | 32 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| Gr4 | Unsprayed | 13 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| Sp1 | Sprayed | 27 | 100.0 | 0.0 | 0.0 | 14.8 | 37.0 | 48.2 |
| US1 | Unsprayed | 29 | 82.8 | 17.2 | 0.0 | 100.0 | 0.0 | 0.0 |
| US2 | Unsprayed | 33 | 81.8 | 18.2 | 0.0 | 100.0 | 0.0 | 0.0 |
| US3 | Unsprayed | 17 | 70.6 | 29.4 | 0.0 | 100.0 | 0.0 | 0.0 |
| Ar1 | Unsprayed | 27 | 51.9 | 33.3 | 14.8 | 100.0 | 0.0 | 0.0 |
| Ar2 | Sprayed | 22 | 18.1 | 36.4 | 45.5 | 100.0 | 0.0 | 0.0 |
| Ch1 | – | 36 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| Ch2 | Sprayed | 35 | 97.1 | 2.9 | 0.0 | 100.0 | 0.0 | 0.0 |
| Ch3 | Unsprayed | 37 | 91.9 | 8.1 | 0.0 | 100.0 | 0.0 | 0.0 |
| Ch4 | Sprayed | 48 | 91.7 | 8.3 | 0.0 | 100.0 | 0.0 | 0.0 |
| Ch5 | Unsprayed | 35 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| Ur1 | Sprayed | 18 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| Ur2 | Sprayed | 19 | 97.3 | 2.7 | 0.0 | 100.0 | 0.0 | 0.0 |

*n*, number of moths examined.

*et al*., 2007). These two detoxifications mechanisms would also be implicated in insecticide resistance in codling moth populations from the New World. Note, however, that enhanced GST activity was rarer among the European populations compared to those from the New World (table 6). These data confirm, to some extent, previous results in the Chilean populations that showed that insecticide resistance was mainly related to an increase in GST activity in this country (Reyes *et al*., 2004; Fuentes-Contreras *et al*., 2007). Inversely, enhanced MFO activity is very frequent among the European populations as previously noted by Reyes *et al*. (2007). Interestingly, the two population samples in South America with the highest MFO activity (Ur1 and Ar2) also showed the highest GST activity. This suggests that enhanced MFO and GST activity could be combined to increase insecticide resistances.

The occurrence of the *kdr* mutation appeared to be widely distributed. The highest proportions of the *kdr* allele were observed in the population samples from France and Argentina (table 7). In all the countries, *kdr* occurs in samples collected in both sprayed and unsprayed orchards with chemical insecticides. The *kdr* mutation in *C. pomonella* was proved to have low fitness cost (Boivin *et al*., 2001), which may explain the high proportions of *kdr* observed at sample sites not treated with pyrethroids. Management of *C. pomonella* in Argentina relied heavily on the use of synthetic pyrethroids up through the 1990s. Field control failures with esfenvalerate were observed in several Argentine populations, and resistance to this compound was confirmed by bioassays (Cichón *et al*., 2003; Soleño *et al*., 2003). Similarly, resistance to deltamethrin was documented widely in apple production from southern France and attributed to both enhanced MFO activity and *kdr* mutation (Sauphanor *et al*., 1997; Bouvier *et al*., 2002; Brun-Barale *et al*., 2005).

The modified *AChE* detected in the *Raz* strain was only observed in the Spanish population. This mutation confers resistance to some organophosphate and carbamate insecticides (Cassanelli *et al*., 2006) but was not detected in samples (Fr1, Fr3, Gr3, Ar2, Ch2 and Ur2) from orchards heavily treated with these insecticides (6–7 applications) for years. These results suggest that other mechanisms, such as enhanced MFO activity, might be involved in the control failures with organophosphate insecticides reported from some of these orchards. An additional hypothesis is that other AChE mutations than those screened in the present study are involved in organophosphate resistances in the codling moth, as previously reported in *Drosophila* (Villatte *et al*., 2000).

In summary, our study reports that a wide variation is present in both the enzymatic activities and the presence of genetic mutations associated with insecticide resistance in *C. pomonella* throughout its world-wide range. Elevated levels of MFO and the occurrence of the *kdr* mutation appeared to be the most frequent resistance mechanisms, but

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both elevated GST and lowered non-specific EST levels are often observed. The complexity of these relationships was not substantially clarified by examining the impact of recent spray history of orchards or their geography within or across the major apple production areas in the world. More likely, insecticide resistance in *C. pomonella* is affected by local conditions influencing selection pressure, the spatial distribution of managed and unmanaged hosts, and population movement between habitats. Several recent genetic studies of *C. pomonella* populations have shown that these factors can create widely different geographical patterns within France (Franck *et al*., 2007), Chile (Espinoza *et al*., 2007) and South Africa (Timm *et al*., 2006). However, within all geographical regions, effective efforts to combat resistance will require continuous monitoring of resistance mechanisms and the implementation of resistance management practices by both growers and the insecticide industry. Another point is that MFO was highly correlated with GST in some populations, which might denote a co-selection of these mechanisms. Further research is needed, however, to clarify the responsible factors involved.

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## References

**Analytical Software** (2003) Statistix 8 users manual. Analytical Software, Tallahassee, FL, USA.

**Audemard, H.** (1992) Population dynamics in codling moth. pp. 329–338 *in* Van der Geest, L.P.S. & Evenhuis H.H. (*Eds*) *Tortricid Pests*: *Their Biology, Natural Enemies and Control*. Amsterdam, Elsevier Science Publishers.

**Boivin, T., Chaber d'Hyères, C., Bouvier, J.C., Beslay, D. & Sauphanor, B.** (2001) Pleiotropy of insecticide resistance in the codling moth *Cydia pomonella*. *Entomologia experimentalis et applicata* **99**, 381–386.

**Boivin, T., Bouvier, J.C., Chadoeuf, J., Beslay, D. & Sauphanor, B.** (2003) Constraints on adaptative mutations in the codling moth *Cydia pomonella* (L.): measuring fitness trade-offs and natural selection. *Heredity* **90**, 107–113.

**Bouvier, J.C., Boivin, T., Beslay, D. & Sauphanor, B.** (2002) Age-dependent response to insecticide and enzymatic variation in susceptible and resistant codling moth larvae. *Archives of Insect Biochemistry and Physiology* **51**, 55–66.

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

**Brun-Barale, A., Bouvier, 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.

**Bush, M., Abdel-Aal, Y. & Rock, G.** (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.

**Cichón, L., Fernández, D., Soleño, J., Montagna, C., Anguiano, L. & Pechen de D'Angelo, A.** (2003) Ensayos de toxicidad en poblaciones de adultos de *Cydia pomonella* (Lepidoptera: Tortricidae) del Alto Valle (Argentina). p. 75 in *Resúmenes XXV Congreso Nacional de Entomología*, Chile.

**Croft, B.A.** (1982) Apple pests management. pp. 465–498 *in* Metcalf, L. & Luckman, W.H. (*Eds*) *Introduction to Pest Management* (2nd edn). John Wiley and Sons, New York.

**Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., Heckel, D., Batterham, P., Feyereisen, R., Wilson, T.G. & ffrench-Constant, R.** (2002) A single P450 allele associated with insecticide resistance in Drosophila. *Science* **297**, 2253–2256.

**De Sousa, G., Cuany, A., Brun, A., Amichot, M., Rahmani, R. & Berge, J.** (1995) A microfluorometric method for measuring Ethoxycoumarin-O-Deethylase activity on individual *Drosophila melanogaster* abdomens: interest for screening resistance in insect populations. *Analytical Biochemistry* **229**, 86–91.

**Espinoza, J.L., Fuentes-Contreras, E., Barros, W. & Ramírez, C.** (2007) Utilización de microsatellites para la determinación de la variabilidad genética de la polilla de la manzana *Cydia pomonella* L. (Lepidoptera: Tortricidae) en Chile central. *Agricultura Técnica* **67**(3), 244–252.

**Franck, P., Reyes, M., Olivares, J. & Sauphanor, B.** (2007) Genetic differentiation in the codling moth: comparison between microsatellite and insecticide resistant markers. *Molecular Ecology* **16**, 3554–3564.

**Field, L.M. & Devonshire, A.L.** (1998) Evidence that the E4 and FE4 esterase genes responsible for insecticide resistance in the aphid *Myzus persicae* (Sulzer) are part of a gene family. *Biochemical Journal* **330**, 169–173.

**Fuentes-Contreras, E., Reyes, M., Barros, W. & Sauphanor, B.** (2007) Evaluation of Azinphosmethyl Resistance and Activity of Detoxifying Enzymes in Codling Moth (Lepidoptera: Tortricidae) From Central Chile. *Journal of Economic Entomology* **100**(2), 551–556.

**Fuentes-Contreras, E., Espinoza, J.L., Lavandero, B. & Ramírez, C.** (2008) Population Genetic Structure of Codling Moth (Lepidoptera: Tortricidae) from Apple Orchards in Central Chile. *Journal of Economic Entomology* **101**(1), 190–198.

**Guennelon, G., Audemard, H., Fremond, J.C. & El Idrissi Ammari, M.A.** (1981) Progrès réalisés dans l'élevage de la Carpocapse *(Laspeyresia pomonella*) sur milieu artificiel. *Agronomie* **1**, 59–64.

**Kaminbelsky, N. & Wool, D.** (1993) Ecological aspects of digestive enzyme covariation in almond aspects of digestive enzyme covariation in Almond moth larvae, *Ephestia cautella* (Walker) (Lepidoptera, Phyticidae). *Journal of Stored Products* **29**(4), 323–332.

**May, R.R. & Dobson, A.P.** (1986) Population dynamics and the rate of evolution of insecticide resistance. pp. 170–193 *in* National Academy of Sciences (*Eds*) *Pesticide Resistance: Strategies and Tactics for Management*. Washington, DC, National Academy Press.

**Nauen, R. & Stumpf, N.** (2002) Fluorometric microplate assay to measure glutathione-S-transferase activity in insects and mites using monochlorobimane. *Analytical Biochemistry* **303**, 194–198.

**Reyes, M., Bouvier, J.C., Boivin, T., Sauphanor, B. & Fuentes-Contreras, E.** (2004) Susceptibilidad a insecticidas y actividad enzimática en *Cydia pomonella* L. (Lepidoptera: Tortricidae) proveniente de tres huertos de manzano de la región del Maule, Chile. *Agricultura Técnica* **64**(3), 229–237.

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

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

**Rousset, F.** (2008) Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. *Molecular Ecology Resources* **8**, 103–106.

**Sauphanor, B., Cuany, A., Bouvier, J.C., Brose, V. & Berge, J.B.** (1997) Mechanism of resistance to deltamethrin in field populations of *Cydia pomonella* L. (Lepidoptera: Tortricidae). *Pesticide Biochemistry and Physiology* **58**, 109–117.

**Sauphanor, B., Brosse, V., Monier, C. & Bouvier, J.C.** (1998) Differencial ovicidal and larvicidal resistance to benzoylureas in the codling moth, *Cydia pomonella*. *Entomologia Experimentalis et Applicatta* **88**, 247–253.

**Sauphanor, B., Bouvier, J.C., Beslay, D., Bosch, D. & Avilla, J.** (2000) Mechanisms of azinphos-methyl resistance in a strain of *Cydia pomonella* from southern Europe. *21st International Congress of Entomology*, 20–26 August, Iguassu, Brazil.

**Soleño, J., Montagna, C., Anguiano, L., Fernández, D. & Pechén de D'Angelo, A.** (2003) Toxicidad de esfenvalerato y Metil azinfos en poblaciones de larvas diapausantes de *Cydia pomonella* (Lepidoptera: Tortricidae) del alto valle de Río Negro y Neuquén. p. 75 in *Resúmenes XXV Congreso Nacional de Entomología*, Chile.

**Soleño, J., Anguiano, L., Pechén de D'Angelo, A. & Montagna, C.** (2004) Tolerancia a Metilazinfos en una población de larvas diapausantes de *Cydia pomonella* en el alto valle de Río Negro y Neuquén. p. 8 in *Resúmenes XXVI Congreso Nacional de Entomología*, Chile.

**Taylor, F. & Feyereinsen, R.** (1996) Molecular biology and evolution of resistance to toxicants. *Molecular Biology and Evolution* **13**, 719–734.

**Thwaite, W.G., Williams, D.G. & Hately, A.M.** (1993) Extent and significance of azinphos-methyl resistance in codling moth in Australia. *Pest Control and Sustainable Agriculture* **93**, 166–168.

**Timm, A.E., Geertsema, H. & Warnich, L.** (2006) Gene flow among *Cydia pomonella* (Lepidoptera: Tortricidae) geographic and host populations in South Africa. *Journal of Economic Entomology* **99**, 341–348.

**Ulrich, V. & Weber, P.** (1972) The O-dealkylation of 7-ethoxycoumarine by liver microsomes: a direct fluorometric test. *Hope-Serler's Z Physiological Chemistry* **353**, 1171–1177.

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

**Villatte, F., Ziliani, P., Marcel, V., Menozzi, P. & Fournier, D.** (2000) High number of mutations in insect acetylcholinesterase may provide insecticide resistance. *Pesticide Biochemistry and Physiology* **67**, 95–102.

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

**Walsh, P.S., Metzger, D.A. & Higuchi, R.** (1991) Chelex (R) 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* **10**, 507.