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Title: **Susceptibility to Organophosphate Insecticides and Activity of Detoxifying Enzymes in Spanish Populations of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae)**

. DOI

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Running head: Rodríguez et al.: Spanish *Cydia pomonella* organophosphate susceptibility

1 **ABSTRACT**

2 The mechanisms associated with resistance of codling moth (*Cydia pomonella* (L.)) to
3 organophosphate insecticides in pome fruit orchards have been shown to depend on the
4 area. Our objectives were to evaluate the susceptibility of Spanish codling moth
5 populations to chlorpyrifos-ethyl, azinphos-methyl and phosalone, and the activity of
6 three enzymatic systems reported to be involved in resistance. Eleven field populations
7 and a susceptible strain used as a reference were tested using a bioassay consisting in
8 the topical application of a diagnostic concentration on post-diapausing larvae. The
9 enzymatic activity of mixed-function oxidases (MFO), glutathione-S-transferases (GST)
10 and esterases (EST) was measured in post-diapausing larvae and adults.

11 A significant decrease in the efficacy of the organophosphates was observed for all field
12 populations, though the decrease was smaller in the case of chlorpyrifos-ethyl. No
13 differences between sexes were detected. In post-diapausing larvae, the activity of the
14 three enzymatic systems was higher in all the field populations than in the susceptible
15 one. The possible implication of EST in codling moth insecticide resistance is reported
16 for the first time in European field populations. In adults, only MFO and GST were
17 implicated.

18 Codling moth resistance to organophosphates in Spanish populations must be taken
19 into account in the implementation of anti-resistance strategies.

20
21 **KEY WORDS** bioassay, *Cydia pomonella*, detoxifying enzymes, organophosphates,
22 resistance detection

Introduction

Codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is a key pest of pome fruits and walnuts that has traditionally been controlled with insecticides. The insecticides registered in Spain for codling moth control include different chemical groups (organophosphates, benzoylhydrazines, neonicotinoids, benzoylureas, carbamates, and pyrethroids), and different modes of action (acetylcholine esterase inhibitors, juvenile hormone mimics, inhibitors of chitin biosynthesis, ecdysone agonists/moulting disruptors, sodium channel modulators, nicotinic acetylcholine receptor agonists, and allosteric receptor activators). The process of re-registration of pesticides that is currently being carried out in the European Union (EU) has reduced the number of active substances available, but not the number of modes of action (EU Pesticides Database 2009, MARM 2009, IRAC 2007).

Organophosphates, especially azinphos-methyl, chlorpyrifos-ethyl and phosalone, have been widely used in Spain and in many other countries. At present azinphos-methyl and phosalone are prohibited and chlorpyrifos-ethyl is permitted in the EU (EU Pesticides Database 2009). Although effective for many years, a decrease in organophosphate efficacy has been reported in European populations from France, Italy, Switzerland, Spain, Bulgaria, and Armenia (Sauphanor et al. 1998a, Charmillot and Pasquier 2002, Charmillot et al. 2002, Charmillot et al. 2007, Reyes et al. 2007). However, it is the low effectiveness of azinphos-methyl, the insecticide most widely used against codling moth, that has been largely documented all over the world (in the USA by Varela et al. [1993], Knight et al. [1994], Chapman and Barrett [1997], and Dunley and Welter [2000]; in Chile by Reyes et al. [2004] and Fuentes-Contreras et al. [2007]; in South Africa by Giliomee and Riedl [1998]; in Australia by Thwaite et al. [1993]; in Israel

1 by Reuveny and Cohen [2004a]; and in France, Spain and Switzerland by Sauphanor et
2 al. [1998a], Bosch et al. [1999], Charmillot et al. [2007], and Reyes et al. [2007]).

3 Insect resistance is mainly due to the detoxification of the insecticides before they
4 reach their target site, with the participation of three enzymatic complexes: mixed-
5 function oxidases (MFO), glutathione-S-transferases (GST), and esterases (EST)(Bisset
6 2002). The reported metabolic resistance of *C. pomonella* to insecticides is mainly
7 based on the action of MFO and/or GST (Sauphanor et al. 1997, Bouvier et al. 2002,
8 Reyes et al. 2007, Ioriatti et al. 2007, Fuentes-Contreras et al. 2007). Other resistance
9 mechanisms that have been reported are a reduced catalytic activity of
10 acetylcholinesterase (AChE) in a laboratory strain resistant to azinphos-methyl
11 (Reuveny and Cohen 2004b), and an AChE mutation that gives it a reduced
12 susceptibility to organophosphates and carbaryl in Spanish field populations (Cassanelli
13 et al. 2006, Reyes et al. 2007).

14 The objectives of the present work were to determine the susceptibility of codling
15 moth Spanish field populations to the three most widely used organophosphates,
16 azinphos-methyl, phosalone and chlorpyrifos-ethyl, and to evaluate the detoxification
17 activity of the MFO, GST, and EST enzymatic complexes.

19 **Material and Methods**

20 **Codling Moth Populations and Insect Management.** The susceptible strain
21 (S_Spain) was used as the reference population. It was collected from an abandoned
22 apple orchard in Lleida (NE Spain) in 1992, and has been reared since then on a semi-
23 artificial diet based on dehydrated apple (Pons et al. 1994) in the laboratory of the UdL-
24 IRTA Centre for R+D.

1 Field codling moth populations were collected from apple orchards of the pome
2 fruit growing areas of Lleida and Girona (NE Spain). Except in one case,
3 organophosphates had been extensively used for several years, but damage at harvest
4 had occurred. The population named Gimenells was collected in an orchard under an
5 integrated pest management program with a low insecticide use.

6 Codling moth diapausing larvae were collected in corrugated cardboard bands
7 placed around the tree trunks in August and September 2003 (1 population, named
8 Poal_1), 2004 (7 populations, named Alcarràs_1, Alcarràs_2, Bellpuig, Menàrguens_1,
9 Lleida, Gimenells, and Poal_2), and 2005 (3 populations, named Bellvis_2, Poal_3, and
10 Girona). The collected larvae were kept for three months at 6 ± 1 °C, and a 12:12 h
11 (light: darkness) photoperiod, conditions that break the diapause. After this period, the
12 larvae were transferred to 22 ± 3 °C t, and a 16:8 h (light:darkness) photoperiod.
13 Twenty-four hours after the transfer, the larvae were used for the biochemical assays
14 and for the topical application bioassays described below. We will refer to them in the
15 text as post-diapausing larvae. For the biochemical assays in adults described below,
16 the post-diapausing larvae were kept in the abovementioned conditions until adult
17 emergence.

18 To obtain post-diapausing larvae from the susceptible strain, standard neonate
19 larvae were reared in plastic boxes (5 cm diameter x 3 cm height) for 40 days under
20 diapause-inducing conditions (25 ± 3 °C, and a 12:12 h [light:darkness] photoperiod).
21 The larvae were then removed from the diet, and were kept for three months under the
22 same conditions as the field larvae.

23 **Insecticide Efficacy.** Technical products of azinphos-methyl (93% of the active
24 ingredient, Bayer AG), phosalone (90% of the active ingredient, Rhône-Poulenc Agro

S.A), and chlorpyrifos-ethyl (97% of the active ingredient, Dow AgroSciences) were dissolved in acetone (for organic residue analysis, 99.4%). One diagnostic concentration was used per insecticide: 400 mg L⁻¹ of azinphos-methyl (Reyes et al. 2007), 3000 mg L⁻¹ of phosalone, and 1200 mg L⁻¹ of chlorpyrifos-ethyl (Pasquier and Charmillot 2003). These concentrations caused between 93 and 99.9% mortality in susceptible laboratory populations.

Male and female larvae were identified by distinguishing the testicular male spots located in the dorsal median region, and separated. A 1-μL drop of the insecticidal solutions, or acetone in the case of the controls, was applied with a Multipette Plus (Eppendorf) in the dorsal median region of the larva (Sauphanor et al. 2000, Pasquier and Charmillot 2003). Immediately after the treatment, 10 larvae of the same sex were transferred to 90 mm diameter Petri dishes containing 20 x 20 mm pieces of corrugated cardboard to allow the larvae to hide in them, and were kept at 22 ± 3 °C, and a 16:8 h (light:darkness) photoperiod. Petri dishes were checked daily until 15 days had elapsed since the last adult emergence, and the mortality was then recorded. Except in one case, a minimum of four replicates of 10 larvae each was carried out per concentration and larval sex (Table 1). The field populations tested on each insecticide depended on the number of post-diapausing larvae available per population.

Enzyme Activity Assays. Twenty post-diapausing larvae and 20 adults were used per population and enzymatic complex. Fluorescence assay for mixed-function oxidases (MFO) and absorbance assay for esterases (EST) were used in larvae and adults. The activity of glutathione-S-transferases (GST) was measured with fluorescence in adults and by absorbance in larvae. In all the cases, with the exception of Poal_1, a VICTOR³ multilabel plate counter (PerkinElmer) was used. For Poal_1 we

used an HTS 7000 microplate reader (PerkinElmer).

GST and EST Activity Measurements.

Codling Moth Extracts. Larval extracts were prepared by placing the entire larva in 1000 μ L phosphate buffer (50 mM, pH 7.2) with a concentration of 0.4 mM of PMSF (phenyl-methylsulfonyl fluoride) (Bouvier et al. 2002). Adult extracts were prepared from the dissected abdomen. Each abdomen was subject to homogenization in 150 μ L of the Hepes buffer (50 mM, pH 7.0) (Nauen and Stumpf 2002). All extracts were centrifuged for 15 minutes at 4 °C and 15.000 g using the supernatants as enzymatic sources (Bouvier et al. 2002; Reyes et al. 2004).

GST Procedure. The GST activity in larvae was determined in vitro using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate in transparent COSTAR microplates of 96 wells with UV absorbance. In each well, 4 μ L of the abovementioned larval extract (equivalent to 0.4 μ L insect per well), 185 μ L of reduced glutathione (GSH) (0.1 M) in phosphate buffer (50 mM, pH 7.2), and 10 μ L of CDNB (30 mM) were placed. In the controls, the extract was replaced by 4 μ L of the phosphate buffer (50 mM, pH 7.2). Right after this procedure, the changes in the optical density at 340 nm were measured by absorbance at 30 °C at time zero (t_0) and at time 1 min (t_1). The protein concentration was estimated with the Bradford method (Bradford 1976). Results were expressed in mM glutathione conjugate mg^{-1} protein min^{-1} (Bouvier et al. 2002).

In adults, monochlorobimane (MCB) was used as substrate in black microplates of 96 wells (Reyes et al. 2004). In each well, 30 μ L of the enzymatic substrate (equivalent to 0.2 μ L insect per well), 150 μ L of GSH 100 mM in Hepes buffer (50 mM, pH 7.0), and 20 μ L of MCB 30 mM were placed. In the control, the enzymatic extract was replaced by 30 μ L of 50 mM Hepes buffer. The microplate was incubated for 20

1 minutes at 22 °C and measured by fluorescence at 380 nm excitation and 465 nm
2 emission filters. Since GSH-bimane complex is not commercially available, the results
3 were expressed in GS-mCB fluorescence units insect⁻¹.

4 *EST procedure.* Total esterase activity was measured in vitro using β -naphthyl
5 acetate as substrate on transparent microplates of 96 wells (methodologies adapted
6 from Bouvier et al. 2002 for larvae and from Reyes et al. 2004 for adults). For post-
7 diapausing larvae procedures, each well was supplied with 90 μ L of the larvae extract
8 (equivalent to 0.9 μ L insect in Poal_1, and 1.8 μ L insect per well for the rest of the
9 populations), and 90 μ L of sodium phosphate buffer (50 mM, pH 6.5) containing β -
10 naphthyl acetate (0.1 mM) per well. For adult procedures, each well was supplied with
11 0.1 mM of β -naphthyl in phosphate buffer (50 mM, pH 6.5), 0.5 μ L of the enzymatic
12 extract, and 89.5 μ L of Hepes buffer (50 mM, pH 7.0).

13 In both cases, in twelve wells the enzymatic extracts were replaced by the sodium
14 phosphate buffer, and were used as controls. After 15 min of incubation at 30°C, 20 μ L
15 of a staining reagent containing 3 g L⁻¹ Fast Garnet and 35 g L⁻¹ sodium dodecyl
16 sulphate (SDS) was added to the solution. Absorbance of naphthol fast-garnet complex
17 was measured after 15 min at room temperature and at 492 nm. The results were
18 expressed in nmol β -naphthol mg⁻¹ protein min⁻¹.

19 **MFO Activity Measurement.**

20 *Codling Moth Preparation.* The MFO activity was analyzed with an in vivo protocol
21 (Bouvier et al. 2002). All tissues were dissected in 6 g L⁻¹ sodium chloride on ice (\pm 4
22 °C). The post-diapausing larvae were cut into four fragments and each was deposited
23 individually in a well. For the analysis the sum of the result obtained in the four
24 fragments was considered as one individual. In the adults, the abdomens were

dissected and placed individually into the wells.

MFO Procedure. MFO activity was determined using 7-ethoxycoumarin-O-desethylation (ECOD) in a black microplate of 96 wells (Bouvier et al. 2002). Each fragment of post-diapausing larvae or adult abdomen was placed individually in a well with 100 μ L of sodium phosphate buffer (pH 7.2, 50 mM) and 7-ethoxycoumarin (0.4 mM). After 4 h of incubation at 30 °C, the reaction was stopped by adding 100 μ L of a glycine buffer (pH 10.4, 10^{-4} M)/ ethanol (v/v). In order to immerse the larvae fragment and clear the surface of the well, the microplates were centrifuged at 2000 g for 1 min after the incubation. The 7-hydroxycoumarine fluorescence was quantified with 380 nm excitation and 465 nm emission filters. Before the incubation, 12 wells used as controls received glycine buffer to stop the reaction. The results of ECOD activity were expressed in pg of 7-hydroxycoumarine (OH) formed larva⁻¹ min⁻¹.

Data analysis. The observed larval mortality was corrected with the mortality of the acetone-treated controls (Abbott 1925). The effect of the larval sex on the mortality per insecticide per population was tested with an ANOVA analysis, followed by a Student-Newman-Keuls (SNK) test ($P < 0.05$), using the statistical SAS program, version 8. Per insecticide, the total (female + male combined) mortality of the field population was compared with the total mortality of the susceptible one by a χ^2 test.

The activity of MFO, GST and EST was subjected to an ANOVA analysis, followed by a SNK test ($P < 0.05$). The frequency of resistant individuals (RMFO, REST and RGST) within each population was calculated, assuming that an individual is resistant when its enzymatic activity is higher than the enzymatic activity of 90% of the individuals from the susceptible strain (Reyes et al. 2007). For the activity of EST in adults, the frequency of individuals whose enzymatic activity was lower than the

enzymatic activity of 90% of the individuals from the susceptible strain was calculated and named R<EST (Reyes et al. 2007). The RMFO, REST, RGST, and R<EST of each field population were compared with the susceptible strain using a χ^2 test.

Results

Insecticide Efficacy. Sex did not affect larval mortality, except in the case of phosalone tested on the Menàrguens population. In this case, male mortality was slightly, but significantly, lower than female mortality ($F = 11.0$, $df = 1$, $P = 0.02$) (Table 1). The mortality of larvae from the susceptible strain was always $\geq 95\%$ (Table 1). The mortality of larvae from all field populations was significantly smaller than the mortality of S_Spain for the three insecticides tested ($P < 0.05$, Table 1). All field populations treated with azinphos-methyl (eleven) and phosalone (nine) showed a mortality lower than 50%. However, only in two of the six field populations treated with chlorpyrifos-ethyl was the mortality lower than this value (Table 1).

Enzymatic Activity in Larvae. Sex did not affect the enzymatic activity of EST, GST and MFO in codling moth post-diapausing larvae (Table 2). The EST and MFO enzymatic activity of male and female post-diapausing larvae from all field populations was significantly higher than that of the larvae from the susceptible strain (Table 2). Only females of Poal_1 and both sexes of Girona and Bellví_2 showed significant differences in GST activity from the susceptible strain (Table 2).

When data from both sexes were combined, there were significant differences in the MFO, EST, and GST activity ($F = 12.27$, $df = 5$, $P < 0.0001$; $F = 14.82$, $df = 5$, $P < 0.0001$; $F = 7.76$, $df = 5$, $P < 0.0001$, respectively). The larvae of all populations showed a significant increase in MFO and EST activity, while only four of them showed a

significant increase in GST activity compared with the susceptible strain (Table 3). The MFO activity of the field populations was 23.4 to 71.1 times higher than that of the laboratory susceptible strain; for EST, the ratio of the enzymatic activity ranged from 3.6 to 7.5, while the lowest enzymatic activity ratios were found for the GST activity, with values ranging from 1.3 to 2.8 (Table 3).

The thresholds for determining the frequency of resistant individuals in post-diapausing larvae for MFO, EST and GST activity, respectively, were 438 nmol of β -naphthol mg protein⁻¹ minute⁻¹; 16.3 pg de 7OH larvae⁻¹ min⁻¹; and 12.7 mM of glutathione conjugated mg protein⁻¹ min⁻¹. The RMFO, REST, and RGST of all populations, except RGST in Gimenells, were significantly higher than the RMFO, REST, and RGST of the susceptible strain (Table 3). The RMFO of field populations ranged from 95 to 100%, the REST ranged from 50 to 100%, and the RGST ranged from 20 to 80% (Table 3).

Enzymatic Activity in Adults. When data from both sexes were combined, there were significant differences in the MFO, and GST activity ($F = 27.40$, $df = 8$, $P < 0.0001$; $F = 16.12$, $df = 8$, $P < 0.0001$, respectively), The adults of all populations showed a significant increase in MFO activity, while only 75% of the field populations showed a significant increase in GST activity compared with the susceptible strain. The ratio of the MFO and GST enzymatic activity ranged from 6.0 to 16.4, and from 1.9 to 4.8, respectively. No significant differences were observed in the EST activity ($F = 0.93$, $df = 8$, $P = 0.49$) (Table 3).

The thresholds for determining the frequency of resistant adults for MFO, EST and GST activity, respectively, were 546 nmol of β -naphthol mg⁻¹ protein min⁻¹; 17.5 pg 7OH formed adult⁻¹ min⁻¹; and 18078.0 GS-mCB fluorescence units insect⁻¹. The RMFO

of all field populations was significantly higher than the RMFO of the susceptible strain; the RGST of 5 out of 8 field populations was significantly higher than the RGST of the susceptible strain, while no any field population showed significant differences in the REST compared to that of the susceptible strain. The RMFO of field populations ranged from 85 to 100%, while the RGST ranged from 25 to 90% (Table 3). Any population showed a REST significantly different from that of the susceptible strain.

The threshold for determining the frequency of adults whose EST activity was lower than the enzymatic activity of 90% of the individuals from the susceptible strain ($R < EST$) was 244 nmol β -naphthol $mg\ protein^{-1}\ min^{-1}$. Two populations out of 8, Alcarràs_1 ($R < EST = 50\%$) and Poal_2 ($R < EST = 80\%$), showed a significantly higher frequency of $R < EST$ than the susceptible strain (Table 3).

Significant differences in enzymatic activity between sexes were observed in the field populations (Table 4). The MFO activity showed significant differences between sexes for four out of the eight tested populations, Alcarràs_1, Alcarràs_2, Gimènells, and Poal_1 ($F = 23.46$, $df = 1$, $P = 0.0001$; $F = 12.91$, $df = 1$, $P = 0.002$; $F = 4.46$, $df = 1$, $P = 0.04$ and $F = 5.19$, $df = 1$, $P = 0.03$, respectively), with significantly higher values for males in Alcarràs_1 and Poal_1, for females and in Alcarràs_2 and Gimènells (Table 4). The mean MFO activity per sex of all field populations was significantly higher than that of the susceptible strain, both in males ($F = 10.63$, $df = 8$, $P < 0.0001$) and in females ($F = 30.73$, $df = 8$, $P < 0.0001$) (Table 4). The GST activity showed significant differences between sexes in four out of the eight tested populations, Alcarràs_2, Menàrguens, Poal_1 and Poal_2 ($F = 6.12$, $df = 1$, $P = 0.02$; $F = 13.8$, $df = 1$, $P = 0.0016$; $F = 11.61$, $df = 1$, $P = 0.003$ and $F = 15.27$, $df = 1$, $P = 0.001$, respectively), and this activity was significantly higher in males than in females in all cases. The mean GST activity per sex

of the field populations was significantly higher than in the susceptible strain in two populations for males, and three for females (Table 4). The EST activity showed significant differences between sexes for the susceptible strain and one field population, Alcarràs_1 ($F = 5.38$, $df = 1$, $P = 0.03$ and $F = 5.23$, $df = 1$, $P = 0.03$, respectively), with a higher activity in females (Table 4). The mean EST activity per sex of the field populations was significantly lower than that of the susceptible strain in one population for males and in two for females (Table 4).

Comparison of Enzymatic Activity of Post-Diapausing Larvae and Adults. In the five field populations tested in post-diapausing larvae, the RMFO and RGST percentages showed similar values to those obtained in the eight field populations evaluated in adults. However, this was not the case with the REST values (Table 3). Only two field populations were tested in both post-diapausing larvae and adults, Gimènells and Poal_1, and showed differences in the percentage of REST between stages. Adults showed no significant variation in the EST activity, whereas post-diapausing larvae showed high levels of EST activity, with ratios of 3.6 and 3.9, respectively (Fig. 1, Table 3). No significant differences in the frequency of resistant individuals between diapausing larvae and adults in RMFO and RGST of Gimènells ($F = 1.03$, $df = 1$, $P = 0.31$; $F = 0.14$, $df = 1$, $P = 0.70$, respectively) and Poal_1 ($F = 2.11$, $df = 1$, $P = 0.14$; $F = 0.78$, $df = 1$, $P = 0.37$, respectively) were recorded (Fig.1). However, the percentage of REST frequency of resistant individuals in Gimènells ($F = 32.4$, $df = 1$, $P < 0.0001$) and Poal_1 ($F = 32.7$, $df = 1$, $P < 0.0001$) varied significantly according to the stage, with a big drop in adults and an increase in post-diapausing larvae.

Discussion

Insecticide Efficacy. Organophosphates are still widely used in codling moth control worldwide. In Spain, they may be used in conventional chemically conducted orchards, and as a support in integrated pest management orchards. The results of the topical application of diagnostic concentrations of azinphos-methyl, phosalone, and chlorpyrifos-ethyl in post-diapausing larvae showed a dramatic decrease in the susceptibility—mainly to azinphos-methyl and phosalone—of all the field populations studied. In years previous to the larvae collection the three organophosphates tested in this study had been used in the control programs of the orchards from which the populations came. The loss of insecticide efficacy may therefore be related to the treatment history of these orchards.

The marked reduction in the efficacy of azinphos-methyl and phosalone in codling moth control has been reported in several countries. The codling moth populations studied—30 from France, 11 from Italy, one from Armenia, four from Switzerland and one from Spain—showed a reduced mortality in response to phosalone, and many showed a low effectiveness of chlorpyrifos-ethyl and azinphos-methyl (Reyes et al. 2007). Similar results for azinphos-methyl have been observed in Swiss (Charmillot et al. 2007), North American (Knight et al. 1994), Argentinean (Soleño et al. 2003, 2004), and Chilean populations (Reyes et al. 2004, Fuentes-Contreras et al. 2007), for phosalone only in Swiss (Charmillot et al. 2002, 2007) and Czech populations (Stará et al. 2006), and for all three organophosphates in Armenian and Bulgarian populations (Charmillot et al. 2007).

The loss of susceptibility shown in this study was not general. Bellvíś_2, Poal_3, and Girona showed low susceptibility to azinphos-methyl and phosalone but responded

1 better to chlorpyrifos-ethyl. The same relation between effectiveness of chlorpyrifos-
2 ethyl and inefficacy of azinphos-methyl and phosalone has been seen in Swiss field
3 populations (Charmillot et al. 2002, 2007) and French populations (Reyes et al. 2007).
4 This background and the fact that our study was done on a non-target stage suggests
5 that the use of chlorpyrifos-ethyl in the field seems suitable for codling moth control, but
6 its control should be rational and supported by other alternatives in order to prevent the
7 appearance of resistance.

8 There is a tendency for females to have lower susceptibility to the insecticidal
9 applications (Varela et al. 1993, Sauphanor et al. 2000), but we observed that only in
10 one population were differences between sexes seen, showing a higher effectiveness of
11 phosalone against females. We must therefore rule out this tendency.

12 **Enzymatic Activity.** Several authors have found that the main biochemical
13 mechanisms of detoxification involved in insecticidal resistance in codling moth were
14 mixed-function oxidases (MFO) and glutathione S-transferases (GST) in larvae
15 (Sauphanor et al. 1997, Bouvier et al. 2002, Reyes et al. 2004, Fuentes-Contreras et al.
16 2007, Ioriatti et al. 2007, Reyes et al. 2007). Our results showed that these enzymes
17 were also involved in the detoxification process in adults. MFO showed a marked activity
18 in the studied populations, with enzymatic activity ratios similar to the ones obtained in
19 laboratory populations resistant to deltamethrin and diflubenzuron with much higher
20 frequencies of resistant individuals (Bouvier et al. 2002, Sauphanor et al. 1998b, 1999,
21 Boivin et al. 2002). The increase in the adult GST activity in six of the eight field
22 populations was also marked by the high activity ratios corresponding to those of
23 populations resistant to azinphos-methyl reported by Reyes et al. (2007). Similar results
24 were observed by the same authors in European field populations of *C. pomonella*. The

1 same effect was found in the MFO and in the GST activity on post-diapausing larvae,
2 which behaved as resistant populations, showing significant differences from the
3 susceptible strain.

4 With respect to these two enzymes in the detoxification of the evaluated
5 organophosphates by topical application, our results showed a decreased effect of
6 azinphos-methyl and phosalone in all field populations related to the high MFO and GST
7 activity levels. The MFO and GST activity has been significantly correlated with the low
8 susceptibility to azinphos-methyl in European populations. French and Spanish
9 populations have shown resistant ratios higher than those found in laboratory strains
10 (Reyes et al. 2007). Cross resistance between azinphos-methyl and phosalone in
11 codling moth populations has also been found (Sauphanor et al. 1999, Reyes et al.
12 2007).

13 Only two field populations showed low susceptibility to the chlorpyrifos-ethyl
14 bioassays. In the field populations of *C. pomonella* it has not yet been possible to
15 associate a mechanism that explained the loss of susceptibility of the populations to
16 chlorpyrifos-ethyl (Reyes et al. 2007), even though this organophosphate is within the
17 insecticides related to codling moth resistance (Sauphanor et al. 1999). This could mean
18 that another mechanism is involved in its detoxification.

19 Our work has shown that in Spanish populations, in addition to MFO and GST,
20 EST are also involved in post-diapausing larvae, with a high activity level. It could be
21 that the action of EST are involved in chlorpyrifos-ethyl detoxification, as was shown in
22 another tortricid, *Choristoneura rosaceana* (Harris), in which EST were responsible for
23 azinphos-methyl detoxification (Smirle et al. 1998) and cross-resistance between these
24 two organophosphates was observed (Ahmad et al. 2002). Though resistance to

1 azinphos-methyl in codling moth was negatively correlated with the increase in EST
2 activity (Reyes et al. 2007), we have to relate this phenomenon to the strong
3 organophosphate selection, especially of azinphos-methyl. This insecticide has been
4 widely used for codling moth control and could be the main reason for Spanish field
5 population resistance. It may also explain the tendency of enhancement of the EST
6 activity found in populations of Argentina (Soleño et al. 2003, 2004), where it is the main
7 insecticide used for codling moth control. The high levels of EST enzymatic activity
8 found in post-diapausing larvae in all field populations tested were observed for the first
9 time for this species in Europe.

10 Enzymatic activity ratios in post-diapausing larvae and frequency of resistant individuals
11 in populations were related. In adults, the low frequency of resistant individuals resulted
12 in a low activity of EST. None of the eight populations went over 5% of resistant
13 individuals (REST). However, Alcarràs_1 and Poal_2 showed a significant $R < EST$
14 frequency increase compared with the reference strain (meaning that the individuals are
15 even more susceptible than the latter). The differentiation in the activity levels of EST
16 between these two stages is clearly observed in the results of Poal_1 and Giménells,
17 though the levels of MFO and GST activity in adults and post-diapausing larvae were
18 similar. One study mentioned that the bioassays on post-diapausing and neonate larvae
19 of Chilean populations could be related to the enzymatic bioassays on adults (Fuentes-
20 Contreras et al. 2007). In Spanish populations, the difference found in the EST activity
21 between adults and post-diapausing larvae showed that we can also apply the same
22 relationship. The differential expression of resistance mechanisms and level of
23 resistance between developmental stages have frequently been highlighted (Roush and
24 Luttrell 1989, Bouvier et al. 2002, Leonova and Slynko 2004). It has been also observed

1 that hydrolase-mediated resistance could be related to a decreased ability to hydrolyze
2 non specific substrates such as α -naphthyl acetate, leading to the mutant aliesterase
3 theory (Oppenoorth, 1985). It was thus hypothesized by Bush et al (1993) that the
4 reduced nonspecific esterase activity detected in OP-resistant adults of *C. pomonella*
5 from North Carolina could result from a modified esterase with lower specificity for
6 naphthyl acetate substrates. Our Spanish resistant populations could have a similar
7 modified esterase, over-expressed in the adult stage and leading to an increased
8 frequency of R<EST individuals, while another carboxylesterase with increased affinity
9 for these substrates could be more expressed in post-diapausing larvae than in the adult
10 stage.

11 The reduced participation of EST in the insecticide detoxification of codling moth has
12 been explained on the basis of the expression of a modified esterase with reduced
13 affinity to β -naphthyl acetate (Bush et al. 1993). However, this was the same substrate
14 that we used for the EST activity evaluation, so the little affinity reported (Bush et al.
15 1993) was not observed in our populations: instead, we observed a high affinity in post-
16 diapausing larvae. Another substrate, *p*-nitrophenyl acetate, has been used to measure
17 the total EST activity on a codling moth strain resistant to azinphos-methyl (Reuveny
18 and Cohen 2004b). However, not even using this other substrate was the EST
19 participation detected. We can therefore state that, unlike in other European
20 populations, in post-diapausing larvae of Spanish codling moth populations EST
21 participates in the organophosphate detoxification. Future studies should investigate the
22 mutation acting on the esterase expression in our populations. Other insect species
23 have shown a carboxyl esterase mutation that gives an increased hydrolysis activity on

organophosphates (Campbell et al. 1997, Devonshire and Field 1991, Beeman and Schmidt 1982, Townsend and Busvine 1969). In the Diptera *Lucilia cuprina* (Wiedemann) the substitution of an amino acid converts the carboxylesterase into a hydrolase that gives resistance to insecticides (Newcomn et al. 1997).

According to our study the difference in the enzymatic activity between sexes in adults showed no clear tendency that related resistance to sex. Therefore, these differences appear at random and are related to the resistant individuals analyzed per sample, which can be manipulated and are irrelevant in the evaluation of resistance.

As a conclusion, we can state that the high selection pressure of organophosphates in Spanish field populations has induced metabolic resistance. Thus, the effectiveness of these neurotoxic substances is reduced through the detoxification of the enzymatic systems (MFO and GST) in *C. pomonella* adults and post-diapausing larvae, and in the latter the action of EST is also involved as a new biochemical mechanism. Finally, we can point out that the topical application of discriminative doses permits and facilitates resistance detection in the field populations. Though post-diapausing larvae are not the target stage in codling moth control and the use of this stage could overestimate resistance (Reyes et al. 2007), we believe that it can provide information on a potential insecticide resistance development and be used to evaluate field resistance (Reyes et al. 2007). It should be noted that the problems of control in the Spanish populations studied were due to the high-frequency of resistant individuals. Furthermore, it is important to remember that the analyzed samples came from problematic orchards and are not representative of the majority of orchards, for which we would need more studies. Nevertheless, these results clearly show that any effort to diminish chemical application in areas of fruit production of Lleida and Girona should be

1 adapted to the control programs, especially if we consider the background of resistance
2 mechanisms present in codling moth populations in the orchards.

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20 **Tables**

21
22 Table 1. Corrected mortality (Abbott 1925) of *Cydia pomonella* male and female post-
23 diapausing larvae from the susceptible strain (S_Spain) and from 11 Spanish field
24 populations when topically exposed to diagnostic concentrations (DC) of three
25 organophosphates.

Insecticide	DC (mg L ⁻¹)	Population	n° of larvae	% Mortality ^a (n)		% Total mortality ^b	
				Female	Male	Mean	χ^2
Azinphos-methyl	400	S_Lleida	80	97 (40)	99 (40)	98	
		Alcarràs_1	80	43 (40)	40 (40)	42	74.7***
		Alcarràs_2	80	31 (40)	30 (40)	31	98.0***
		Bellpuig	80	46 (40)	48 (40)	47	65.2***
		Bellví_2	80	39 (40)	33 (40)	36	72.7***
		Escola	80	47 (40)	43 (40)	45	68.9***
		Girona	80	35 (40)	31 (40)	33	93.5***
		Gimenells	80	36 (40)	31 (40)	34	91.3***
		Meràrguens	80	23 (40)	21 (40)	22	120.3***
		Poal_1	80	7 (40)	9 (40)	8	162.6***
		Poal_2	80	11 (40)	11 (40)	11	152.6***
		Poal_3	80	36 (40)	33 (40)	34	91.3***
Chlorpyrifos-ethyl	1200	S_Lleida	80	96 (40)	97 (40)	97	
		Bellví_2	80	69 (40)	66 (40)	68	29.1***
		Escola	80	88 (40)	84 (40)	86	7.8**
		Gimenells	120	46 (60)	45 (60)	46	63.8***
		Girona	80	82 (40)	80 (40)	81	13.1***
		Poal_1	80	7 (40)	9 (40)	8	158.8***
		Poal_3	80	71 (40)	61 (40)	66	31.9***
Phosalone	3000	S_Lleida	160	95 (80)	96 (80)	95	
		Alcarràs_2	80	19 (40)	24 (40)	23	107.2***
		Bellví_2	80	27 (40)	26 (40)	26	99.6***
		Bellpuig	80	32 (40)	37 (40)	35	79.1***
		Escola	80	47 (40)	49 (40)	48	54.2***
		Gimenells	80	19 (40)	26 (40)	23	107.2***
		Menàrguens	76	28 (38) a	21 (38) b	25	102.1***
		Poal_1	120	21 (60)	22 (60)	22	109.8***
		Poal_2	80	11 (40)	10 (40)	11	141.6***
		Poal_3	80	41 (40)	38 (40)	40	68.9***

^a Different letters in the same line indicate significant differences ($P < 0.05$; Student-Newman-Keuls test).

Numbers in parentheses are the sample size.

^b The total mortality of each field population was compared with the total mortality of the susceptible strain (S_Spain) using a chi-square test (df =1; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

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1 Table 2. Mean \pm SEM of the MFO, GST and EST enzymatic activity in *Cydia pomonella* male and female post-diapausing
2 larvae from the susceptible strain (S_Spain) and from five field populations.

Population ^a	MFO activity ^b		GST activity ^b (mM glutathione		EST activity ^b (nmol β -naphthol	
	(pg 7OH insect ⁻¹ min ⁻¹)		conjugated mg of protein ⁻¹ min ⁻¹)		mg protein ⁻¹ min ⁻¹)	
	Male	Female	Male	Female	Male	Female
S_Spain	9.4 \pm 1.5a	7.4 \pm 1.7a	8.5 \pm 1.2a	6.3 \pm 1.3a	208 \pm 62a	207 \pm 44a
Bellvís_2	232 \pm 59b	160 \pm 26b	19.0 \pm 2.8b	22.4 \pm 2.7c	1900 \pm 322d	1205 \pm 158b
Gimenells	229 \pm 67b	184 \pm 70b	12.1 \pm 1.8ab	7.8 \pm 1.4a	547 \pm 124b	953 \pm 190b
Girona	333 \pm 99bc	264 \pm 103bc	18.4 \pm 4.5b	15.4 \pm 2.9bc	935 \pm 186c	766 \pm 75b
Poal_1	480 \pm 93bc	486 \pm 139c	15.1 \pm 2.2ab	16.5 \pm 1.5bc	821 \pm 106bc	784 \pm 130b
Poal_3	599 \pm 124c	595 \pm 124c	14.8 \pm 2.8ab	13.2 \pm 2.4ab	1129 \pm 130c	926 \pm 130b

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4 ^a n = 10 individuals per sex per population for GST and MFO; n = 20 individuals per sex per population for EST; n = 19 individuals for EST in
5 S_Spain.

6 ^b Values followed by the same letter in the same column are not significantly different ($P < 0.05$; Student-Newman-Keuls).

1 The enzymatic activity of MFO, GST and EST did not show significant differences between sexes for any laboratory or field populations ($P < 0.05$;
2 Student-Newman-Keuls).

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- 1 Table 3. Mean \pm SEM of the enzymatic activity, ratio of enzymatic activity and frequency of resistant individuals in larvae
2 and adults of *C. pomonella* field populations compared with the susceptible strain (S_Spain).

Population	MFO activity ^a		GST activity ^b		EST activity ^a		Enzymatic activity ratio ^c			Frequency of resistant individuals ^d (%)			
	n	Mean activity ^e	n	Mean activity ^e	n	Mean activity ^e	MFO	GST	EST	RMFO	RGST	REST	R<EST
<i>Post-diapausing larvae</i>													
S_Spain	20	8.4 \pm 1.1a	20	7.4 \pm 0.9a	39	208 \pm 28a	1	1	1	10	10	10	-
Bellvís_2	20	196.9 \pm 25.6b	20	20.7 \pm 1.9c	40	1553 \pm 192c	23.4	2.8	7.5	100***	70***	100***	-
Gimenells	20	207.4 \pm 47.4b	20	9.9 \pm 1.2ab	40	751 \pm 120b	24.6	1.3	3.6	95***	20 NS	50**	-
Girona	20	298.9 \pm 69.9b	20	16.8 \pm 2.6bc	40	851 \pm 99b	35.6	2.3	4.1	100***	45**	80***	-
Poal_1	20	483.3 \pm 81.3c	20	15.7 \pm 1.3bc	20	704 \pm 79b	57.5	1.8	3.9	100***	80***	90***	-
Poal_3	20	597.1 \pm 85.4c	20	14 \pm 1.8b	40	1028 \pm 92b	71.1	2.1	5.0	100***	45**	90***	-

Adults

S_Spain	20	12.2 ± 1.1a	20	8014 ± 1352a	20	344 ± 17a	1	1	1	10	10	10	10
Alcarràs_1	20	73.6 ± 7.5b	20	18688 ± 2291b	20	252 ± 20a	6.0	2.3	0.7	95***	45**	0 NS	50**
Alcarràs_2	20	110.8 ± 10.2bc	20	20833 ± 2171b	20	306 ± 21a	9.1	2.6	0.9	100***	60***	5 NS	20 NS
Bellpuig	20	74.5 ± 10.8b	20	13631 ± 2384ab	20	282 ± 16a	6.1	1.7	0.8	85***	25 NS	0 NS	35 NS
Escola	20	83.5 ± 7.2b	20	14971 ± 1964b	20	313 ± 19a	6.8	1.9	0.9	100***	50**	0 NS	20 NS
Gimenells	20	200.5 ± 16.1d	20	16326 ± 1562b	20	319 ± 20a	16.4	2.0	0.9	100***	25 NS	5 NS	15 NS
Menàrguens	20	79.1 ± 8.9b	20	14178 ± 1513ab	20	346 ± 43a	6.4	1.8	1.0	95***	25 NS	5 NS	25 NS
Poal_1	20	99.9 ± 7.3bc	20	35928 ± 3490c	20	276 ± 10a	8.2	4.5	0.8	100***	90***	0 NS	20 NS
Poal_2	20	120.1 ± 10.3c	20	38842 ± 3861c	20	225 ± 14a	9.8	4.8	1.2	100***	90***	0 NS	80***

^a EST activity measured in nmol β-naphthol mg protein⁻¹ min⁻¹; MFO activity measured in pg 7OH insect⁻¹ min⁻¹.

^b GST activity measured in GS-mCB fluorescence units insect⁻¹ in adults and in mM of glutathione conjugated mg⁻¹ of protein minute⁻¹ in diapausing larvae.

^c Enzymatic activity ratio = enzymatic activity of field population divided by enzymatic activity in the susceptible strain (S_Spain).

^d Frequency of resistant individuals was compared with the susceptible strain (S_Spain) using a chi-square test (df =1; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS, not significant).

- 1 ^e For each developmental stage, values of the enzymatic activity in the same column followed by the same letter are not significantly different ($P <$
- 2 0.05; Student-Newman-Keuls test)
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1 Table 4. Mean \pm SEM of the MFO, GST and EST enzymatic activity in *Cydia pomonella* adults of the susceptible strain
 2 (S_Spain) and eight field populations.

Population ^a	MFO activity ^b		GST activity ^b		EST activity ^b (nmol β -naphthol	
	(pg 7OH insect ⁻¹ min ⁻¹)		(GS-mCB fluorescence units insect ⁻¹)		mg protein ⁻¹ min ⁻¹)	
	Male	Female	Male	Female	Male	Female
S_Spain	12 \pm 1a	13 \pm 2a	7896 \pm 1485a	8132 \pm 2347a	307 \pm 14a	* 381 \pm 27a
Alcarràs_1	98 \pm 8bc *	49 \pm 5b	18891 \pm 3841ab	18486 \pm 2721abc	210 \pm 18b *	293 \pm 31ab
Alcarràs_2	82 \pm 10b *	140 \pm 11d	25603 \pm 3102b *	16064 \pm 2288abc	266 \pm 26ab	345 \pm 28ab
Bellpuig	72 \pm 11b	78 \pm 13bc	10083 \pm 1588a	17178 \pm 4323abc	306 \pm 22ab	258 \pm 22bc
Escola	92 \pm 7bc	75 \pm 7bc	17071 \pm 2253ab	12871 \pm 3199ab	290 \pm 27ab	336 \pm 27ab
Gimenells	169 \pm 16d *	232 \pm 21e	11083 \pm 1567a	21092 \pm 5607abc	285 \pm 27ab	353 \pm 26ab
Menàrguens	90 \pm 9bc	68 \pm 12bc	18527 \pm 1820ab *	9829 \pm 1470a	375 \pm 79ab	316 \pm 35ab
Poal_1	115 \pm 7bc *	85 \pm 8bc	45456 \pm 4559c *	26400 \pm 3240bc	274 \pm 10ab	278 \pm 18ab

Poal_2	139 ± 10cd	102 ± 10d	50245 ± 4953c	*	27440 ± 3086c	255 ± 73ab	195 ± 8c
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* Indicate significant differences between sexes in the same population after Student-Newman-Keuls (SNK) test at $P < 0.05$.

^a n = 10 individuals per sex and per population for GST, EST and MFO.

^b Values of the enzymatic activity in the same column followed by the same letter are not significantly different ($P < 0.05$; Student-Newman-Keuls test).

1 **Figures**

2 Figure 1. Frequency of post-diapausing larval and adult resistant individuals (RMFO, RGST, and REST) of *Cydia*
3 *pomonella* collected from two Spanish orchards (Poal_1 and Giménells). The asterisks show significant differences
4 between larvae and adults per enzymatic complex and field population (χ^2 test, *** $P = 0.001$; $df = 1$).

