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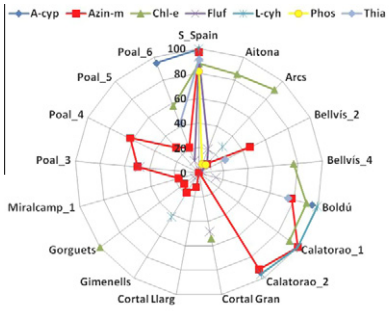
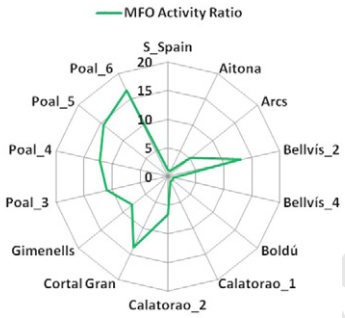
Graphical abstract

Assessment of insecticide resistance in eggs and neonate larvae of *Cydia pomonella* (Lepidoptera: Tortricidae) **pp xxx–xxx**

Marcela A. Rodríguez, Tânia Marques, Dolors Bosch, Jesús Avilla *



Insecticide efficacy on neonate larvae



MFO activity in neonate larvae

Highlights

▶ *Cydia pomonella* a key pest of pome fruits worldwide. ▶ Insecticide resistance and mechanisms checked on target stages (eggs and neonates). ▶ Resistance mechanisms in Spanish populations more diverse than in other countries. ▶ MFO is the most important enzymatic system involved. ▶ Diagnostic concentrations as tools for monitoring insecticide resistance in field.

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Assessment of insecticide resistance in eggs and neonate larvae of *Cydia pomonella* (Lepidoptera: Tortricidae)

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ABSTRACT

Spanish *Cydia pomonella* (L.) field populations have developed resistance to several insecticide groups. Diagnostic concentrations were established as the LC_{90} calculated on a susceptible strain (S_Spain) for five and seven insecticides and tested on eggs and neonate larvae field populations, respectively. The three most relevant enzymatic detoxification systems (mixed-function oxidases (MFO), glutathione *S*-tranferases (GST) and esterases (EST)) were studied for neonate larvae.

In eggs, 96% of the field populations showed a significantly lower efficacy when compared with the susceptible strain (S_Spain) and the most effective insecticides were fenoxycarb and thiacloprid. In neonate larvae, a significant loss of susceptibility to the insecticides was detected. Flufenoxuron, azinphos-methyl and phosmet showed the lowest efficacy, while lambda-cyhalothrin, alpha-cypermethrin and chlorpyrifos-ethyl showed the highest. Biochemical assays showed that the most important enzymatic system involved in insecticide detoxification was MFO, with highest enzymatic activity ratios (5.1–16.6 for neonates from nine field populations). An enhanced GST and EST activities was detected in one field population, with enzymatic activity ratios of threefold and fivefold for GST and EST, respectively, when compared with the susceptible strain. The insecticide bioassays showed that the LC_{90} used were effective as diagnostic concentrations. Measures of MFO activity alongside bioassays with insecticide diagnostic concentrations could be used as tools for monitoring insecticide resistance in neonate larvae of *C. pomonella*.

1. Introduction

The codling moth, *Cydia pomonella* (L.), is a key pest that affects fruit production of apples, pears and walnuts in almost all the areas where these crops are cultivated. Its resistance to synthetic insecticides (organophosphates, pyrethroids, abamectin, benzoylureas, benzhydrazides, neonicotinoids, and macrocyclic lactones) has been reported in several countries since the early 1990s [1–8].

Codling moth resistance is mainly due to the detoxification of the insecticides by the action of enzymes, and to the modification of the molecular target of an insecticide-specific group. The role of mixed-function oxidase (MFO) and glutathione *S*-transferase (GST) enzymatic complexes has been demonstrated in field populations from France, Italy, Armenia, Switzerland, Spain and Chile [5,7–12]. In addition, the role of the esterase (EST) enzymatic complex has been demonstrated in Spanish and Argentinean populations [12–14]. Two modifications of the insecticide molecular targets have been reported: a knockdown resistance mutation (*kdr*) in the sodium voltage-dependent channel is involved in the resistance to pyrethroids, and an acetylcholinesterase (AChE) mutation has been identified in a laboratory strain selected for resistance to azinphos-methyl and in field populations from the fruit production areas of Lleida (Spain) [7,15–17].

Two methodologies are used in the laboratory to detect and monitor insecticide resistance of codling moth: the application of diagnostic insecticide concentrations in bioassays [5,7,12,18–20], and the determination of the activity levels of MFO, GST and EST enzymatic complexes. Both have been tested on post-diapausing larvae and adults [5,7,8,12,13]. However, diapausing larvae may overestimate natural resistance because the target of insecticide applications is not this stage but eggs and neonate larvae [7]. Furthermore, neonate larvae provide more consistent results for evaluating the situation of resistance in the field [21].

The aim of the present study was to evaluate the insecticide resistance of eggs and neonate larvae of *C. pomonella* field populations from three areas of apple production in Spain. For this purpose the following objectives were established: (1) to estimate the efficacy of different insecticide groups on eggs and neonate larvae, using the LC_{90} of a susceptible strain (S_Spain) as a diagnostic concentration; (2) to estimate the activity of MFO, GST and EST

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enzymatic complexes in neonate larvae; and (3) to assess the usefulness of the methodology used in order to apply it as a tool for monitoring the resistance of *C. pomonella* in the field.

2. Material and methods

2.1. Orchards

The field populations were collected from Spanish apple orchards of three production areas, 2 in Catalonia (NE Spain) and 1 in Aragón (NE Spain). In Catalonia we used 14 orchards in Lleida (Aitona, Arcs, Barbens, Bellvís_2, Bellvís_4, Boldú, Escola, Gimenells, Palau, Poal_3, Poal_4, Poal_5, Poal_6, Miralcamp and Tarròs) and 3 orchards in Girona (Cortal Gran, Cortal Llarg and Gorguets); in Aragón we used 2 orchards in Zaragoza (Calatorao_1 and Calatorao_2).

Boldú was collected from an organic orchard (a mating disruption plus granulovirus (CpGv)–treated orchard), Gimenells was collected from an experimental orchard (a mating disruption plus insecticide–treated orchard), and Calatorao_1 was collected from an abandoned orchard. All the other field populations were collected from conventional, synthetic insecticide–treated orchards selected because codling moth populations were high and the percentage of injured apples during the season was high in spite of the control measures adopted.

2.2. Insects

A laboratory susceptible *C. pomonella* strain (S_Spain) was used as the reference population. Previous results have shown that the response to insecticides of this strain is equal to that of other susceptible strains from France and Italy [13]. S_Spain has been maintained in our laboratory without exposure to insecticide for more than 17 years.

Larvae of three field populations (Poal_5, Poal_6, and Miralcamp) were collected from apples in the summer of 2006 and 2007. They were grouped by development stage and placed on a semi-artificial diet at 16:8 h light:dark, 22 ± 3 °C and 40 ± 5% relative humidity (RH), until adult emergence. The adults were placed in breeding cages to oviposit. The eggs and the larvae from these adults were used in the bioassays. If necessary in order to obtain a sufficient number of eggs, the embryonic development was delayed by placing the eggs at 4 °C for 24 h. The rest of the field populations were collected as diapausing larvae. The diapausing larvae were maintained at 12:12 h light:dark, and 6 ± 1 °C for at least 2 months. They were then transferred to the above-mentioned conditions, and the same procedure was applied.

2.3. Insecticide efficacy bioassays

Dose–response bioassays were conducted to determine the LC_{50} and LC_{90} values of the insecticides on eggs and neonate larvae of the susceptible strain (S_Spain). Eggs and neonate larvae from field populations were treated with the calculated LC_{90}.

2.3.1. Eggs

The ovicidal effect of five insecticides from four action groups (Table 1) was determined. They were selected among the most commonly used ovicides. The range of concentrations used in order to determine the dose–response lines are shown in Table 2. The bioassay was carried out by topical application of 0.1 µl of a solution of the insecticide technical product in acetone (for organic residue analysis, 99.4% purity, J.T. Beker, Serviquimia, Barcelona Spain) or tetrahydrofuran (stabilized with 0.025% BHT, J.T. Beker, Serviquimia, Barcelona, Spain) (Table 2) on less than 24-h-old eggs. A Hamilton flex syringe for precision flow coupled to a Harvard Pump 11 was used. A minimum of 60 eggs per concentration and per population were used (3 replicates of 20–30 eggs each). Each group of 20–30 treated eggs was placed into a 9 cm diameter plastic Petri dish with humid filter paper in order to prevent dryness, and maintained at 16:8 h light:dark, 22 ± 3 °C and 40 ± 5% RH. After 4 days, the eggs were checked in order to observe the development stage and humidity conditions. After 10 days of topical applications, the mortality was recorded. For the control, the same procedure was carried out with the solvent. Eggs from field populations were treated in the same way at the diagnostic concentration (LC_{90}, obtained in S_Spain).

2.3.2. Less than 24-h-old larvae (neonate larvae)

Commercial formulations of seven insecticides from four action groups (Table 1) were tested in less than 24-h-old (hereinafter neonate) larvae using 5–10 different concentrations (Table 2). They were selected among the most commonly used larvicides. A 2 µl/cm^2 of solution of the insecticide commercial formulation in water was applied on the surface of a 4 cm^2 semi-artificial diet piece, following the methodology developed by Bosch et al. [22]. The insecticide solution was homogeneously distributed with a humidified brush. After 2 h, one single neonate larva was deposited on the surface-treated diet and confined in a gelatine capsule, in order to oblige the contact and the feeding of the larva on the treated diet. One set of 16 neonate larvae per insecticide and concentration was placed into a plastic box in order to prevent dryness, and it was transferred to 16:8 h light:dark, 22 ± 3 °C and 40 ± 5% RH conditions. As a control, larvae were exposed to a water-treated diet. Two to five replicates were used per concentration and per population. The gelatine capsule was removed after 24 h, and mortality was assessed after 5 days. Each neonate larva was considered dead if it did not move when stimulated with a fine brush. Missing larvae were deducted from the initial number. The same procedure was used for neonate larvae from field populations using a diagnostic concentration (LC_{90}, obtained in S_Spain) for each commercial insecticide formulation.

2.4. Enzyme activity

The GST (glutathione *S*-transferase) and EST (esterase) activities were analyzed in vitro by homogenization on ice of 16–20 replications of 10 neonate larvae from each population in 100 µl of phosphate buffer (50 mM, pH 7.2) containing 0.4 mM final concentration of PMSF (phenylmethylsulfonylfluoride). These homogenates were centrifuged at 4 °C for 15 min at 15,000*g*. The supernatant of each sample was used as an enzymatic source [10]. GST activity was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate in COSTAR 96-well microplates with a UV transparent bottom. Each well was maintained on ice (4 °C) and supplied with 4 µl of larvae extract, 184 µl of sodium phosphate buffer (pH 7.2, 50 mM), 2 µl of reduced glutathione (0.1 M) and 10 µl of CDBN (30 mM). Right after the change in the optical density at 340 nm at time zero (*t*0) and after 1 min (*t*1), the absorbance at 30 °C was measured. Results were expressed in mM glutathione conjugated mg of protein^{-1} min^{-1} [10]. EST activity was measured using β-naphthyl acetate as substrate on 96-well transparent microplates. Each well was supplied with 90 µl of neonate larvae extract (equivalent to 0.9 µl of enzymatic extract per well) and 90 µl of sodium phosphate buffer (pH 6.5, 50 mM) containing β-naphthyl acetate (0.1 mM) per well. After 15 min of incubation at 30 °C, 20 µl of a staining reagent containing 3 g/L Fast Garnet and 35 g/L sodium dodecyl sulfate (SDS) was added to the solution. Absorbance of naphthol–Fast Garnet complex was measured after 15 min at room temperature and at 492 nm. The results were expressed in nmol of β-naphthol mg of protein^{-1} min^{-1} [10]. In both

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Table 1
Insecticides tested on *C. pomonella* eggs and neonate larvae.

Insecticide	RFC[a] (mg (a.i.)/L)	Chemical sub-group[b]	Formulation and active ingredient (a.i.) content (%)	Supplier	Solvent
Eggs					
Diflubenzuron	125	Benzoylurea	Technical product (90.0)	Syngenta, Spain	Tetrahydrofurane
Fenoxycarb	100	Fenoxycarb	Technical product (98.5)	Syngenta, Spain	Acetone
Flufenoxzuron	50–100	Benzoylurea	Technical product (99.5)	BASF, Spain	Acetone
Methoxyfenozide	96	Diacylhydrazine	Technical product (98.2)	Dow AgroSciences, Spain	Acetone
Thiacloprid	144	Neonicotinoid	Technical product (99.7)	Bayer CropScience, Spain	Acetone
Neonate larvae					
Alpha-cypermethrin	10–15	Pyrethroid	Dominex (10)	Agrodan, Spain	Water
Azinphos-methyl	400–500	Organophosphate	Gusation (20)	Aragonesas Agro, S.A., Spain	Water
Chlorpyrifos-ethyl	750–1000	Organophosphate	Cúspide (10)	Commercial Química Massó, Spain	Water
Flufenoxzuron	50–100	Benzoylurea	Cascade (10)	BASF, Spain	Water
Lambda-cyhalothrin	10–20	Pyrethroid	Karate (10)	Syngenta, Spain	Water
Phosmet	900	Organophosphate	Imidan (50)	Comercial Química Massó, Spain	Water
Thiacloprid	144	Neonicotinoid	Calypso (48)	Bayer CropScience, Spain	Water

[a] Recommended field concentration for *C. pomonella* control in Spain [40].
[b] IRAC [36].

Table 2
Toxicity of insecticides on *C. pomonella* eggs of the susceptible strain.

Insecticide	n[a]	Control mortality (%)	Concentration applied (mg (a.i.)/L)		LC_{50} (mg (a.i.)/L) (95% confidence intervals)	LC_{90} (mg (a.i.)/L) (95% confidence intervals)	HF[b]	Slope ± SE	SI[c]
			N°	Range					
Diflubenzuron	1200	15	7	2.3–144.0	0.2 (0.17–0.24)	0.9 (0.76–1.24)	0.77	1.91 ± 0.16	133
Fenoxycarb	1360	19	9	0.0002–0.05	0.002 (0.001–0.003)	0.04 (0.026–0.063)	0.81	0.99 ± 0.08	2590
Flufenoxuron	740	15	7	0.4–5.0	1.4 (1.07–1.64)	4.9 (3.95–6.52)	0.84	2.32 ± 0.27	15.4
Methoxyfenozide	680	14	7	0.008–6.0	0.1 (0.05–0.18)	1.8 (0.99–4.16)	1.94	1.04 ± 0.10	35.33
Thiacloprid	780	18	8	0.04–1.0	11.9 (6.99–17.00)	77.4 (56.21–119.16)	1.11	1.57 ± 0.19	1.86

[a] Sample size.
[b] Heterogeneity factor = χ^2/d.f.
[c] SI: security index = recommended field concentration/LC_{90}.

enzymatic systems (GST and EST), 12 wells of the enzymatic extracts were replaced by sodium phosphate buffer and used as controls. The protein content per enzymatic extract was obtained according to Bradford procedures [23], using bovine serum albumin as standard.

The MFO (mixed-function oxidases) activity was analyzed with an in vivo protocol. MFO activity was determined using 7-ethoxycoumarin-O-desethylation (ECOD) in a black, 96-well microplate. The neonate larvae were placed individually in a well with 100 µl 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 µl 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 microplate was 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 [10]. Before the incubation, 12 wells receiving glycine buffer were used as controls. The results of ECOD activity were expressed in pg of 7-OH (Hydroxycoumarine) larva^{-1} min^{-1}.All the enzymatic measurements were done using a VICTOR 3 Multilabel plate reader (PerkinElmer).

2.5. Data analysis

Probit analyses was carried out to determine the dose–response lines for eggs and neonate larvae in S_Spain, and the LC_{50}, LC_{90} (with 95% confidence limits) and heterogeneity factor were calculated (Polo Plus version 1.0, LeOra Software 2002–2009). For each insecticide, the security index (SI) was calculated by dividing the registered field concentration for *C. pomonella* control in Spain by the LC_{90} values obtained for S_Spain [4]. When a range of field concentrations was recommended, the highest one was used. The efficacy of each insecticide on field populations at the diagnostic concentration was calculated by Abbott's formula [24]. The efficacy of each insecticide on field populations was compared with its efficacy on S_Spain using a χ^2 test.

The MFO, GST and EST enzymatic activity were analyzed by an ANOVA followed by a Student–Newman–Keuls (SNK) test. The enzymatic activity ratio (EAR) was calculating by dividing the mean enzymatic activities of the field populations by those of S_Spain. The relative frequency of resistant individuals due to MFO (RMFO), EST (REST) and GST (RGST) activity within each population were calculated following Reyes et al. [7]. The highest activity value corresponding to 90% of S_Spain individuals was used as a threshold. For each population, the relative frequency of resistant individuals of the field populations was compared with that of S_Spain using a χ^2 test.

3. Results

3.1. Determination of the diagnostic concentrations

Tables 2 and 3 show the results of the probit analyses of the toxicity of the tested insecticides on codling moth eggs (Table 2) and neonate larvae (Table 3). The values of the heterogeneity factor (HF) were smaller than 1.35, except for methoxyfenozide on eggs

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Table 3
Toxicity of insecticides on neonate *C. pomonella* larvae of the susceptible strain.

Insecticide	n^a	Control mortality (%)	Concentration applied (mg (a.i.)/L)		LC_{50} (mg (a.i.)/L) (95% confidence intervals)	LC_{90} (mg (a.i.)/L) (95% confidence intervals)	HF[b]	Slope ± SE	SI[c]
			N°	Range					
Alpha-cypermethrin	445	10 (79)	10	0.28–3.0	0.63 (0.46–0.79)	2.41 (1.85–3.69)	1.19	2.22 ± 0.33	6.22
Azinphos-methyl	599	18 (110)	8	57.2–600.0	202.06 (181.90–223.19)	449.27 (390.92–542.33)	1.06	3.69 ± 0.34	1.11
Chlorpyrifos-ethyl	239	4 (47)	7	63.7–307.7	157.65 (132.07–186.19)	313.94 (252.46–463.49)	1.34	4.15 ± 0.55	3.19
Flufenoxuron	239	4 (46)	5	8.8–44.4	12.63 (10.85–14.26)	25.24 (21.82–31.17)	0.99	4.26 ± 0.55	3.96
Lambda-cyhalothrin	377	17 (93)	6	0.16–2.30	0.35 (0.29–0.42)	1.59 (1.23–2.29)	0.75	1.96 ± 0.21	12.58
Phosmet	660	18 (110)	8	65.8–750.1	421.84 (384.92–457.14)	813.78 (739.95–944.27)	1.04	4.49 ± 0.43	1.11
Thiacloprid	216	9 (32)	7	122.0–1000	314.92 (268.19–376.06)	875.71 (671.47–1309.50)	0.95	2.89 ± 0.35	0.16

[a] Sample size.
[b] Heterogeneity factor = χ^2/d.f.
[c] SI: security index = recommended field concentration/LC_{90}.

(HF = 1.94). The mortality in the controls was always lower than 20%.

The toxicity of the tested insecticides on codling moth varied greatly, with LC_{90} values ranging from 0.04 to 77.4 mg (a.i.)/L in eggs and from 1.6 to 875.7 mg (a.i.)/L in neonate larvae (Tables 2 and 3). On eggs, fenoxycarb was the most effective insecticide, with the LC_{50} and LC_{90} values lower than 0.01 mg (a.i.)/L and 0.1 mg (a.i.)/L, respectively (Table 2), while methoxyfenozide, diflubenzuron and flufenoxuron showed lower efficacy than fenoxycarb (Table 2). The neonicotinoid thiacloprid was the least effective insecticide (Table 2). However, the security index (SI) of the five insecticides tested was higher than 1 (Table 2). On neonate larvae, the insecticide with the lowest toxicity was thiacloprid, with values of 315 mg (a.i.)/L and 876 mg (a.i.)/L for LC_{50} and LC_{90}, respectively, and it was the only one that showed a SI smaller than 1 (Table 3). The two pyrethroids, alpha-cypermethrin (LC_{50} = 0.63 mg (a.i.)/L; LC_{90} = 2.41 mg (a.i.)/L) and lambda-cyhalothrin (LC_{50} = 0.35 mg (a.i.)/L; LC_{90} = 1.59 mg (a.i.)/L) were the most effective insecticides (Table 3). The other insecticides (phosmet, chlorpyrifos-ethyl, azinphos-methyl and flufenoxuron) showed intermediate efficacy (Table 3). For alpha-cypermethrin and lambda-cyhalothrin, the slopes of the dose–response lines were quite flat and ranged from 1.96 to 2.9, whereas for the 3 organophosphates and for flufenoxuron the dose–response lines were steeper, with slope values that ranged from 3.7 to 4.5 (Table 3).

3.2. Insecticide efficacy on field populations

The efficacy of the tested insecticides on the susceptible strain at the diagnostic concentrations applied ranged between 90.8% (diflubenzuron) and 98.8% (flufenoxuron) for eggs (Table 4), and between 82% (chlorpyrifos-ethyl) and 100% (lambda-cyhalothrin) for neonate larvae (Table 5).

The efficacy of all the tested insecticides on eggs of all the populations was significantly lower than their efficacy on eggs of the susceptible strain, except in the case of flufenoxuron on the Escola population (Table 4).

For neonate larvae, azinphos-methyl, phosmet (tested only on two field populations) and flufenoxuron were in general the least effective insecticides, showing a lower efficacy on the field populations than on S_Spain, except for the populations from the Zaragoza fruit-growing area (the abandoned orchard Calatorao_1, and Calatorao_2), while the pyrethroid alpha-cypermethrin (tested only on three field populations) was as effective on field populations as on S_Spain (Table 5).

Two populations from conventional orchards (Arcs and Poal_6) were resistant to the majority of the insecticides tested on them, except for chlorpyrifos-ethyl (Arcs) and alpha-cypermethrin (Poal_6) (Table 5). In two populations from conventional orchards (Arcs and Gorguets) azinphos-methyl showed low efficacy, while chlorpyrifos-ethyl did not. The most susceptible field populations were Boldú (organic orchard), Calatorao_1 (abandoned orchard) and Calatorao_2 (conventional orchard) (Table 5).

3.3. Detoxifying enzyme activity

Nine of the 13 field populations tested on neonate larvae showed an MFO activity significantly higher than that of S_Spain (dF = 13, 266; $F = 18.6$; $p \leqslant 0.0001$) (Table 6). The resistance ratios ranged from 5.1 to 16.6 times the MFO activity of the S_Spain strain (Table 6).

Table 4
Efficacy of insecticides at the diagnostic concentration on *C. pomonella* eggs of the susceptible strain, and of field populations.

Populations	Insecticide efficacy (%)[a]				
	Diflubenzuron (1 mg (a.i.)/L)	Fenoxycarb (0.04 mg (a.i.)/L)	Flufenoxuron (5 mg (a.i.)/L)	Methoxyfenozide (1.8 mg (a.i.)/L)	Thiacloprid (77 mg (a.i.)/L)
S_Spain	90.8	95.3	98.8	97.1	96.1
Barbens	10 (148.8)***				
Calatorao 1	70.3 (23.8)***	83.3 (7.5)**	37.4 (61.9)***	85.0 (9.9)**	86.7 (10.9)***
Calatorao 2	53.7 (42.1)***		68.5 (15.3)***		66.0 (37.1)***
Escola			81.6 (3.55)ns		
Gimenells	25.0 (105.8)***		68.8 (15.0)***		70.0 (31.5)***
Palau	0 (184.9)***	75.0 (16.3)***	72.2 (11.5)***	57.2 (45.2)****	74.2 (25.9)***
Poal_5	0(184.9)***		46.6 (45.4)***		89.4 (7.9)**
Poal_6	35.6 (81.4)***		26.6 (85.0)***		41.9 (77.6)***
Tarros	28.6 (97.0)***				

Mean of three replicates of 20–30 eggs per insecticide and population.
[a] The mortality obtained in eggs of the field populations was compared with the mortality obtained in the susceptible strain using the χ^2 test. Numbers in parentheses show χ^2 values (dF = 1; *p = 0.05; **p = 0.01 and ***p = 0.001. ns = not significant).

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Table 5
Efficacy of insecticides at the diagnostic concentration on *C. pomonella* neonate larvae of the susceptible strain, and of field populations.

Populations	Insecticide[a] efficacy (%)[b]						
	A-cyp (2.4 mg (a.i.)/L)	Azin-m (450 mg (a.i.)/L)	Chl-e (315 mg (a.i.)/L)	Fluf (25 mg (a.i.)/L)	L-cyh (1.6 mg (a.i.)/L)	Phos (815 mg (a.i.)/L)	Thia (875 mg (a.i.)/L)
S_Spain	99.3	97.0	88.1	93.5	100	81.9	91.2
Aitona			85.4 (0.52)ns	20.2 (111.7)***		7.2 (113.2)***	
Arcs		9.4 (155.4)***	90.4 (0.2)ns	9.0 (144.6)***	28.3 (112.5)***	8.2 (109.9)***	
Bellvís_2		45.8 (63.8)***		2.5 (165.8)***			23.5 (91.8)***
Bellvís_4			76.7 (4.1)*				
Boldú	95.0 (2.7)ns	77.7 (16.5)***	90.2 (0.2)ns	14.3 (128.8)***	99.5 (1.0)ns		75.1 (9.1)**
Calatorao_1		100 (3.0)ns	91.5 (0.9)ns		100 (1.0)ns		
Calatorao_2	94.6 (3.7)ns	92.1 (2.4)ns			96 (4.1)*		
Cortal Gran		0 (188.3)***	54.0 (28.1)***	48.5 (49.7)***			
Cortal Llarg		12.2 (145.6)***					
Gimenells		19.2 (124.9)***		5.5 (154.8)***	41.7 (81.6)***		
Gorguets		15.1 (136.4)***	100 (12.7)ns				
Miralcamp_1		17.1 (130.5)***					
Poal_3		50.0 (56.7)***		48.0 (51.4)***			
Poal_4		62.0 (37.6)***					
Poal_5		27.3 (103.9)***					
Poal_6	94.8 (3.7)ns	21.6 (116.7)***	57.5 (22.8)***	10.3 (141.3)***			42.4 (53.8)***

Mean of 2–5 replicates of 16 larvae per insecticide and population.
[a] Insecticides (left to right): alpha-cypermethrin, azinphos-methyl, chlorpyrifos-ethyl, flufenoxuron, lambda-cyhalothrin, phosmet, and thiacloprid.
[b] The mortality obtained in neonate larvae of the field populations was compared with the mortality obtained in the susceptible strain using the χ^2 test. Numbers in parentheses showed χ^2 values (dF = 1; $^*p = 0.05$; $^{**}p = 0.01$ and $^{***}p = 0.001$. ns = not significant).

Table 6
Mean ± SEM of MFO, GST, and EST enzymatic activity, enzymatic activity ratio (EAR), and frequency of resistant individuals (RMFO, RGST, and REST) in neonate larvae of *C. pomonella* from the susceptible strain, and from field populations.

Populations	MFO (pg 7OH larva^{-1} min^{-1})			GST (mM glut. conj. mg protien^{-1} min^{-1})			EST (nmol β-naphtol mg protien^{-1} min^{-1})			Frequency of resistant Individuals (%)[a]		
	n	Mean activity[b]	EAR[c]	*n*	Mean activity[b]	EAR[c]	*n*	Mean activity[b]	EAR[c]	RMFO	RGST	REST
S_Spain	20	12 ± 0.26[a]	1	16	8.03 ± 2.4[a]	1	20	184.1 ± 46.2[a]	1	10	10	10
Aitona	20	48.5 ± 3.5 ab	1							100***		
Arcs	20	62.1 ± 7.1 bc	5.1	20	14.7 ± 6.0[a]	1	20	402.2 ± 84.0[a]	1	100***	20ns	30ns
Bellvís_2	20	157.0 ± 17.5 cd	13.1	16	24.4 ± 11.5[b]	3.0	20	935.0 ± 157.8[b]	5.1	100***	19ns	70***
Bellvís_4	20	18.1 ± 1.0 a	1							95***		
Boldú	20	17.8 ± 0.5 a	1	20	6.1 ± 1.8[a]	1	20	180.2 ± 33.1[a]	1	60***	10ns	5ns
Calatorao_1	20	37.9 ± 5.7 ab	1	16	8.5 ± 1.3[a]	1	20	251.4 ± 61.4[a]	1	100***	0ns	15ns
Calatorao_2	20	80.8 ± 12.7 bc	6.7							100***		
Cortal Gran	20	166.0 ± 14.1 cd	13.8				20	431.9 ± 64.3[a]	1	100***		40*
Gimenells	20	96.0 ± 18.9 c	8.0	20	9.2 ± 2.6[a]	1				100***	15ns	
Poal_3	20	131.0 ± 24 cd	10.9	17	12.3 ± 6.6[a]	1	20	403.4 ± 71.4[a]	1	100***	12ns	30ns
Poal_4	20	146.0 ± 14.1 cd	12.2							100***		
Poal_5	20	172.0 ± 20.3 cd	14.3	20	12.2 ± 3.6[a]	1	20	428.9 ± 133.7[a]	1	100***	15ns	20ns
Poal_6	20	199.0 ± 26.9 d	16.6	20	14.0 ± 3.3[a]	1	20	420.2 ± 89.3[a]	1	100***	40*	30ns

n, number of replicates.
[a] The differences in the frequency of resistant individuals in comparison with S_Spain were detected using the χ^2 test: dF = 1; ns, not significant; $^*p = 0.05$; $^{**}p = 0.01$; $^{***}p = 0.001$. Thresholds: 12.73 pg 7OH larva^{-1} min^{-1}, 19.12 mM of glutathione conjugated mg protein^{-1} min^{-1}, and 466 nmol of β-naphtol mg protein^{-1} min^{-1}, for RMFO, RGST and REST, respectively.
[b] Means followed by the same letter in the same column are not significantly different (Student–Newman–Keuls test, $p < 0.05$).
[c] EAR = enzymatic activity ratio = enzymatic activity of the field population divided by the enzymatic activity of the susceptible strain S_Spain, when the enzymatic activities were significantly different.

The resistance threshold, established in order to estimate the relative frequency of resistant individuals (RMFO), was 12.73 pg 7OH larva^{-1} min^{-1}. All the field populations showed RMFO values significantly higher than the control ones, ranging from 60% to 100% (dF = 1; $F = 10.9$; $p = 0.0009$ and dF = 1; $F = 32.7$, $p \leqslant 0.0001$, respectively) (Table 6). However, in 4 cases (Aitona, Boldú, Bellvís_4 and Calatorao_1) there were no significant differences in their MFO activity compared with S_Spain (Table 6). The levels of the MFO activity in the resistant individuals of these field populations were mostly higher than the resistance threshold, but they did not reach the peaks found in other populations (e.g. Poal_6), as indicated by the ranges of MFO activity (Aitona: 18.52–75.8, Boldú: 11.1–17.8, Bellvís_4: 10.8–27.8 and Calatorao_1: 16.6–121.5 and Poal_6 19.6–449.2 pg 7OH larva^{-1} min^{-1}) (Fig. 1).

For GST activity, only Bellvís_2 showed higher activity levels than S_Spain (dF = 8, 164; $F = 1.99$; $p = 0.049$). The rest of the field populations showed no significant differences from S_Spain (dF = 8, 164; $F = 1.18$; $p = 0.31$) (Table 6). The resistant threshold based on S_Spain GST activity was established as 19.12 mM glutathione-conjugated mg protein^{-1} min^{-1}. The RGST that was observed in the field populations ranged from 0% to 40% (Table 6). For Bellvís_2, the single field population with GST activity levels of resistance (from 0 to 63 mM glutathione-conjugated mg protein^{-1} min^{-1}), showed a frequency of individual RGST resistance of only 19% (dF = 1; $F = 0.24$; $p = 0.63$), with no significant difference from S_Spain. In contrast, Poal_6 showed an RGST of 40% (dF = 1; $F = 4.8$; $p = 0.03$), but the mean GST activity levels (from 0 to 140 mM glutathione conjugated mg protein^{-1} min^{-1}) were not significantly different from that of S_Spain (Table 6, Fig. 2).

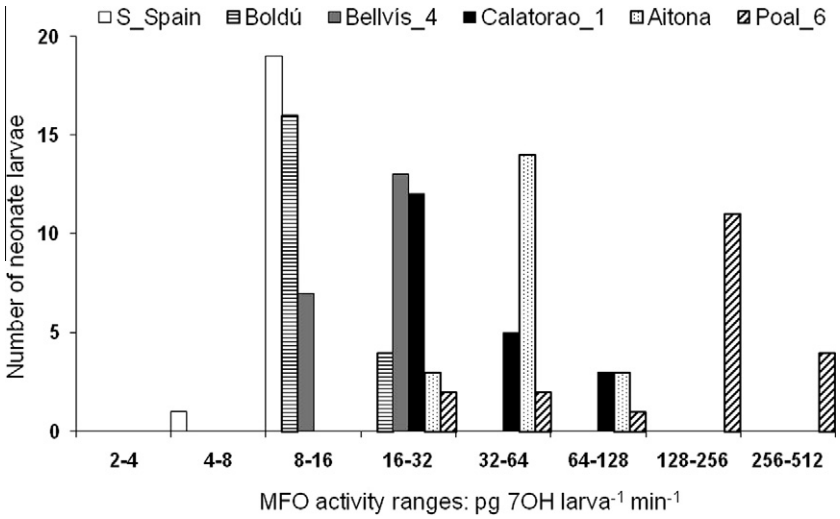

Fig. 1. Frequency distribution of mixed-function oxidase activity (MFO) in neonate (less than 24-h-old) *C. pomonella* larvae from the susceptible strain S_Spain and five field populations. The MFO activity of Aitona, Boldú, Calatorao_1 and Bellvís_4 was not significantly different from that of S_Spain, although more than 60% of the larvae of population were resistant (Table 6). Poal_6 is a typical example of a resistant population. Resistant threshold RMFO = 12.73 pg 7OH larva^{-1} min^{-1}.

As for EST activity, only Bellvís_2 showed significantly higher activity levels (dF = 8, 171; $F = 6.16$; $p \leqslant 0.0001$) than S_Spain (Table 6). The value of the resistance threshold was established as 466 nmol β-naphthol acetate mg protein^{-1} min^{-1}. According to this, two field populations showed high percentages of REST: Bellvís_2 (70%: dF = 1; $F = 15$; $p = 0.0001$) and Cortal Gran (40%: dF = 1; $F = 4.8$; $p = 0.03$) (Table 6). The increased frequency of resistant individuals at Cortal Gran is due to the EST activity levels of a few individuals that are above the threshold value, but show moderate enzymatic levels (Fig. 3).

Two field populations considered susceptible natural populations, Boldú (organic orchard) and Calatorao_1 (abandoned orchard), showed similar levels of activity to S_Spain in the three enzymatic systems evaluated (Table 6).

4. Discussion

4.1. Insecticide efficacy on eggs and neonate larvae

The dose–response lines obtained from S_Spain eggs and neonate larvae showed a good fit. Additionally, the variability in the control mortality was low, therefore securing a correct mortality in the samples subjected to the insecticide treatment. According to the above, we propose the calculated LC_{90} values on a susceptible strain S_Spain as a diagnostic concentration to detect resistance in immature target stages of *C. pomonella*. The susceptibility of S_Spain was previously compared with two susceptible strains from Europe (Italy and France). Eggs, neonate larvae, post-diapausing larvae and adults of the three strains showed equal susceptibility to insecticides, and also similar lethal concentrations for each insecticide used [25]. The use of LC_{90} as a diagnostic concentration to monitor the resistance to insecticides in field populations of *C. pomonella* has been suggested before [26]. The principal advantage of using these pre-established values in a laboratory susceptible strain is that the insect number is not a limiting factor to determining a concentration-mortality line. Therefore, the diagnostic concentration obtained (LC_{90}) minimizes the insect number per insecticide bioassay and optimizes the use of the individuals collected from different orchards. For example, in this study, the number of field populations per bioassay and per development stage depended on the initial number of larvae or eggs collected from the orchards. This led to initial proportions of individuals

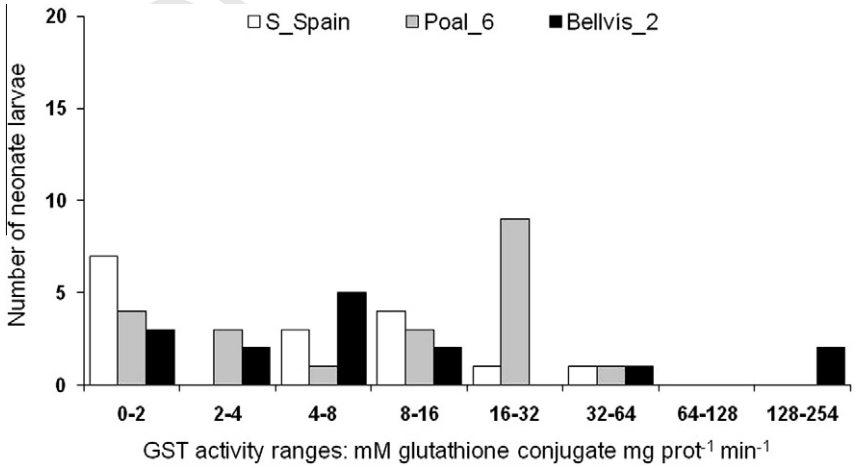

Fig. 2. Frequency distribution of glutatione *S*-transferase (GST) activity in neonate (less than 24-h-old) *C. pomonella* larvae from the susceptible strain S_Spain and two field populations. The GST activity of Bellvís_2 was significantly different from that of S_Spain, although only 19% of the larvae were resistant (Table 6). Poal_6 shows the opposite case: non-significant differences, but 40% of resistant larvae (Table 6). Resistant threshold RGST = 19.12 mM Glutathione Conj. mg protien^{-1} min^{-1}.

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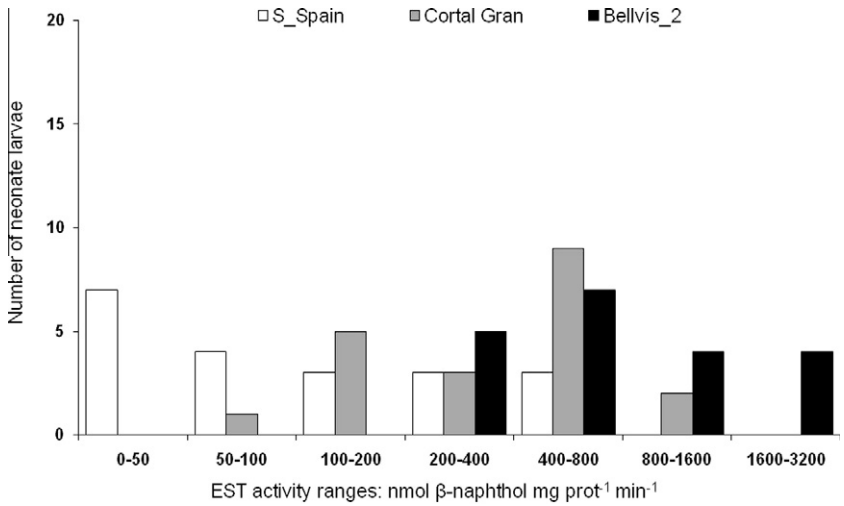

Fig. 3. Frequency distribution of esterase (EST) activity in neonate (less than 24-h-old) *C. pomonella* larvae from the susceptible strain S_Spain and two field populations. Although the REST of both populations was significantly greater than that of the control one, only Bellvís_2 showed a significantly higher EST activity than the control. Resistance threshold REST = 466 nmol of β-naphtol mg protien^{-1} min^{-1}.

per sex, copulation rate and fertility that were later obtained in our laboratory conditions. The establishment of a diagnostic concentration is essential in neonate larvae [21]. This was confirmed by our observations.

The differential susceptibility of S_Spain to the insecticides tested is also consistent with the literature. For example, Charmillot et al. [18] also reported fenoxycarb to be more toxic than methoxyfenozide, and, between benzoylureas, diflubenzuron to be more effective that flufenoxuron. The differences in the actual values of the LC's are due, among others factors, to the substrate were codling moth eggs are laid [27]. Exochorion permeability to insecticide penetration might play a certain role in insecticide toxicity, but there is no precise information in the case of codling moth.

In eggs our results showed that all except one field population showed less susceptibility than the reference strain S_Spain for all the insecticides tested. In neonate larvae we observed that some field populations showed high susceptibilities with the pyrethroids and the two organophosphates used (azinphos-methyl and chlorpyrifos-ethyl), although with azinphos-methyl high susceptibility was mainly detected in susceptible field populations.

In general, the insecticide effectiveness was lower in neonate larvae. The loss of susceptibility detected in the Spanish field populations may have been due to cross-resistance between organophosphates and other chemical groups, given the history of intensive organophosphate treatments, especially with azinphos methyl. This relationship between organophosphate-resistant selection and lower susceptibility to other insecticides—including those with a novel mode of action—has been demonstrated in the Tortricidae species *C. pomonella* for fenoxycarb, diflubenzuron, novaluron, teflubenzuron, pyriproxifen, methoxyfenozide, thiacloprid, spinosad, and phosmet [7,28–30]; and in *Choristoneura rosaceana* (Harris) and *Plantortrixs octo* (Welter) for methoxyfenozide and tebufenozide [31,32].

It is interesting that we recorded a higher efficacy with chlorpyriphos-ethyl than with azinphos-methyl for neonate larvae in all except one field population (Calatorao_1, from an abandoned orchard). This antagonism has been recorded previously in the monitoring of resistance of post-diapausing larvae of the codling moth in European field populations [7,8,20]. The increased use of azinphos-methyl in comparison with chlorpyriphos-ethyl may be the reason for this difference.

The field populations collected from the abandoned orchard (Calatorao_1) and the ecological orchard (Boldú) showed higher susceptibility to the insecticides tested and the immature stage studied. However, two populations collected from conventional orchards (Calatorao_2 for neonate larvae and Escola for eggs) also showed high susceptibility. Further research will aim to determine the relationship between the efficacy of the selected insecticides and orchard management.

Sauphanor et al. [4] in a *C. pomonella* laboratory resistant strain found higher effectiveness of flufenoxuron in neonate larvae than in eggs. In their study, two field populations showed a similar tendency towards this insecticide.

In our study the highest diagnostic concentration was obtained for thiacloprid (LC_{90} = 77.4 mg L^{-1} and 875.7 mg L^{-1} for eggs and neonate larvae, respectively). Stará and Kocourek [26] found a lower thiacloprid concentration in neonate larvae than the one we obtained (1.83 mg L^{-1}). In contrast, the diagnostic concentration we obtained with fenoxycarb was 160 times lower than the one obtained by Sauphanor et al. [4] in neonate larvae of codling moth. We believe that these differences between diagnostic concentrations (LC_{90}) are due to the methodologies used. Stará and Kocourek [26] used different methods to calculate the insecticide concentration per cm^2 and the mortality recorded, and Sauphanor et al. [4] tested the insecticide by placing it in contact with the eggs, while our treatments were done by topical application. A similar study done in eggs showed that the LC_{50} obtained by dipping apple fruit in methoxyfenozide was 15 times lower than the LC_{50} used in our study [33]. In another study of topical application bioassays of fenoxycarb in post-diapausing larvae, the LC_{90} obtained was only 10 times higher than the one obtained in our study in eggs [6]. We can attribute this LC_{90} ratio to the different development stage used.

Sauphanor et al. [34] recommended the test on non-target instars as a more convenient tool in a routine monitoring for early detection of resistance because the number of insect from field populations that can be analyzed was a limiting factor in target instars, and resulted in a poor estimation of the resistance of the initial populations. Recently, Reyes and Sauphanor [21] recommended a standard method for monitoring in neonate larvae similar to the method used in this study for field populations, applying diagnostic concentrations in laboratory susceptible and resistant strains of *C. pomonella* and one field population from France.

In our case, both bioassays performed for eggs and neonate larvae provided similar information concerning the susceptibility

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levels to insecticide compounds in field populations for the codling moth target-instars when compared with a susceptible strain. The comparison between field populations or resistant phenotypes and a susceptible strain confirms the data obtained on insecticide resistance, independently of the methodology used. However, like Reyes and Sauphanor [21], we think that a standard insecticide bioassays methodology will help to better understand resistance in *C. pomonella*.

The instruments used in the egg bioassays (a Harvard Pump 11 and a Hamilton Syringe) and the ingredients of the semi-artificial diet are commercially available, and are also easy to manipulate and manufacture [35]. Therefore, the methodology used follows the regulations proposed by the Insecticide Resistance Action Committee (IRAC) for a suitable resistance monitoring test [36].

4.2. Enzymatic activity in neonate larvae and insecticide effectiveness

The number of insects is an important factor in the assessment of resistance. For the enzymatic assays of the 17 initial neonate larvae field populations, we could only test 13 for MFO activity and 7 in the three enzymatic complexes. The reason for this is that the field populations used for MFO activity were measured by in vivo assays performed on one neonate larvae per measure, following the procedures described by Bouvier et al. [10] In contrast, in the biochemical assays done by these and other authors for EST and GST, a pool of the 10 individuals for each extract and reading was used. Due to the above, we needed 200 individuals per field population to measure EST and GST activities, so we could only work with eight field populations for each enzymatic complex. In the future efforts should be made to reduce the number of neonate larvae needed for these protocols in order to obtain more information on the participation of these enzymatic systems in the insecticide detoxification of Spanish field populations of codling moth.

The determination of enzymatic activities in all three enzymes (MFO, GST and EST) in neonate larvae is very important because it provides the most consistent results with the field situation [21]. Moreover, the development stages may show differences in their susceptibility to insecticides. Rodríguez et al. [8] observed higher EST activity in larvae than in adults of *C. pomonella*, and the same observation was made by Usmani and Knowles [37] in the noctuids *Agrotis ipsilon* (Hufn.) and *Spodoptera frugiperda* (Smith).

According to our results, the lack of insecticide efficacy obtained can be attributed to detoxification activity of MFO. The present results were in accordance with those of Reyes and Sauphanor [21], who observed higher MFO activity in neonate larvae and no involvement of GST and EST activities. Although only nine of the 13 field populations showed higher levels of MFO activity, they all showed a high frequency of resistant individuals. This suggests that insecticide bioassays combined with MFO measures that use a low number of individuals could be used as tools for monitoring and detecting resistance in the field.

A common route of insecticide metabolism in *C. pomonella* is conjugation by GST and oxidation by MFO. Both systems, or only one of the two, have been involved in insecticide detoxification in larvae of European populations and in adults of field populations from Armenia, France, Italy, Switzerland, Spain and Chile [5,7,9–12,38]. Only one field population (Bellvís_2) showed high mean enzymatic activity levels for MFO, GST and EST. This is the first record of the enhanced EST levels in neonate larvae of the codling moth. The role of the esterases in the metabolism of insecticides in *C. pomonella* has recently been confirmed for post-diapausing larvae in Spanish and Argentinean field populations [8,13,14]. Smirle et al. [39] found that EST activity related to organophosphates detoxification was involved in *C. rosaceana*. In view of our results, GST and EST are not involved as a generalized insecticide-resistant mechanism, so they cannot be considered as a monitoring tool for codling moth neonate larvae.

Our studies also showed that a high frequency of resistant individuals (RMFO, RGST, and REST) was not always directly related to the mean enzymatic activity in a field population. Examples are the results found in Bellvís_2 and Poal_6 for the GST activity; in Cortal Gran for the EST activity; and in Aitona, Bellvís_4, Boldú and Calatorao_1 for the MFO activity (Figs. 1–3). The frequency of resistant individuals has been used by other authors in order to determine the susceptible-resistant composition of the field populations of *C. pomonella* [5,8,12,13]. However, it is also important to establish the enzymatic activity ranges and to record the number of individuals that are in each one [9,10]. The distribution of individuals among the different enzymatic activity ranges proved to be a good method for showing anomalies. According to the examples mentioned above, it provided a better understanding of the dynamics of resistance vs. susceptibility of the samples. A greater understanding of the different parameters encountered in the enzymatic assays will help to optimize the program practices for Integrated Resistance Management in *C. pomonella*.

In Spanish field populations the genetic diversity of *C. pomonella* for insecticide resistance varies greatly due to the involvement of MFO, GST and EST, and also of the insensitive AChE mutation [7,8,16,17]. Therefore, all the knowledge obtained about heritable individual capacities in field populations will be of great help in decision making and in the development of tools to control resistance in *C. pomonella*, an insect with many adaptive advantages.

In conclusion, first, there is a great decrease in susceptibility to insecticides used against eggs and larvae from Spanish field populations of *C. pomonella*. Second, although it has been found that the three enzyme systems studied are involved in insecticide detoxification by neonate larvae of *C. pomonella*, the main mechanism of resistance is MFO. Finally, proposed bioassays in immature stages of *C. pomonella* and the assessment of MFO activities are a good system for monitoring insecticide resistance in field populations.

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