Bulletin of Entomological Research (2020) **110**, 144–154
doi:10.1017/S0007485319000415

Enzymatic detoxification strategies for neurotoxic insecticides in adults of three tortricid pests

M.A. Navarro-Roldán[1]*, D. Bosch[2], C. Gemeno[1] and M. Siegwart[3]

[1]Department of Crop and Forest Sciences, University of Lleida (UdL), 25198-Lleida, Spain: [2]Department of Sustainable Crop Protection, Food and Agriculture Research Institute (IRTA) 25198-Lleida, Spain: [3]Agronomic National Research Institute (INRA), UR 1115 PSH, Plantes et Systèmes de culture Horticoles, 84914-Avignon, France

Abstract

We examined the role of the most important metabolic enzyme families in the detoxification of neurotoxic insecticides on adult males and females from susceptible populations of *Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller). The interaction between the enzyme families – carboxylesterases (EST), glutathione-S-transferases (GST), and polysubstrate monooxygenases (PSMO) – with the insecticides – chlorpyrifos, λ-cyhalothrin, and thiacloprid – was studied. Insect mortality arising from the insecticides, with the application of enzyme inhibitors – S,S,S-tributyl phosphorotrithioate (DEF), diethyl maleate (DEM), and piperonyl butoxide (PBO) – was first determined. The inhibitors' influence on EST, GST, and PSMO activity was quantified. EST and PSMO (the phase-I enzymatic activities) were involved in the insecticide detoxification in the three species for both sexes, highlighting the role of EST, whereas GST (phase-II enzymes) was involved only in *G. molesta* insecticide detoxification. *L. botrana* exhibited, in general, the highest level of enzymatic activity, with a significantly higher EST activity compared with the other species. It was the only species with differences in the response between sexes, with higher GST and PSMO activity in females than in males, which can be explained as the lower susceptibility of the females to the tested insecticides. A positive correlation between PSMO activity and the thiacloprid LD_{50}s in the different species-sex groups was observed explaining the species-specific differences in susceptibility to the product reported in a previous study.

Keywords: insecticide inhibitor, neurotoxic insecticide, detoxification, Tortricidae, adult, sex

(Accepted 28 May 2019; First published online 20 June 2019)

Introduction

All insects have detoxification abilities that vary among species and developmental stages (Yu & Hsu, 1993; Casida & Quistad, 2004). The ability to degrade toxic substances is essential to the survival of insect pests in the constantly changing chemical environment of the agroecosystem. Metabolism of toxic compounds involves two phases. Phase I is the addition of a polar group to the substrate or its cleavage. Phase II is the addition of sugar, amino acid, sulfate, or phosphate groups to the products resulting from phase I, to increase hydrophilicity so as to facilitate excretion by the insect (Bernard & Philogène, 1993). The three most important detoxification enzyme families in insects are polysubstrate monooxygenases (PSMO) [also called cytochrome P450 monooxygenases (P450)], carboxylesterases (EST), and

*Author for correspondence
Phone: +34 973 702531
Fax: +34 973 238264
E-mail: m.navarro.roldan@gmail.com

glutathione-S-transferases (GST) (Després *et al*., 2007). PSMO and EST are involved in phase I, whereas GST is involved in phase II (Bernard & Philogène, 1993). These enzyme families are subject to gene amplification, or overexpression, and variations in the coding sequence that can modify their detoxification capabilities (Li *et al*., 2007).

Metabolic enzymes changes have emphasized the importance of pest-detoxification capabilities in the development of pesticide-resistant populations (Scott, 1999; Enayati *et al*., 2005; Montella *et al*., 2012). Several *in vivo* methods using enzyme inhibitors to gauge insecticide susceptibility and biochemical *in vitro* methods to detect metabolic-detoxification enzyme changes are available to examine pesticide resistance (Brown & Brogdon, 1987). Several studies report that metabolic enzymes are involved in the detoxification of insecticides with different modes of action (MoA) in various lepidopteran pests (Biddinger *et al*., 1996; Ahmad & Hollingworth, 2004; Reyes *et al*., 2007; Siegwart *et al*., 2011), and in various insect developmental stages (Karoly *et al*., 1996; Yu *et al*., 2003; Rodríguez *et al*., 2010). Metabolic enzymes also help to elucidate the metabolic status of susceptible pest species (Usmani & Knowles, 2001; Guo *et al*., 2017; Vojoudi *et al*., 2017). Furthermore, the enzymes can be used to discover which metabolic groups are involved in resistance, comparing metabolic status among susceptible and resistant species (Sauphanor *et al*., 1997; Ahmad & Hollingworth, 2004; Reuveny & Cohen, 2004).

Chemicals that inhibit specific detoxification pathways are important in researching detoxification mechanisms, resistance, and MoA in insecticide studies (Metcalf, 1967; Snoeck *et al*., 2017). Chemical inhibition is particularly useful in this context due to the diverse range of chemical structures and metabolic routes of insecticides (Roberts & Huston, 1998). The most common inhibitors (also called synergists) for EST, GST, and PSMO enzymes are S,S,S-tributyl phosphorotrithioate (DEF), diethyl maleate (DEM), and piperonyl butoxide (PBO), respectively (Bernard & Philogène, 1993; Wu *et al*., 2007; Sial & Brunner, 2012; Snoeck *et al*., 2017).

Cydia pomonella (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller) are three key tortricid moth pest species which target Mediterranean fruit crops. They adversely affect mainly apples, peaches, and grapes, respectively, but have worldwide distribution hosts (Ioriatti *et al*., 2011; Kirk *et al*., 2013; Damos *et al*., 2015). Toxin susceptibility and metabolic enzyme activity commonly vary in different insect species (Yu, 2008). The specific enzymatic activity of an individual may be linked to its diet (Krieger *et al*., 1971; Rose, 1985); besides, the use of insecticides can select for different enzymatic detoxification mechanisms (Xue *et al*., 2010). Therefore, to study the basal levels of enzymatic detoxification activities in different species, susceptible strains reared for a long time in the laboratory, fed with the same artificial diet, can be very useful. In contrast, the use of field populations may imply the co-occurrence of different resistance mechanisms, complicating the interpretation of results (Soderlund & Bloomquist, 1990; Bosch *et al*., 2018).

Navarro-Roldán *et al*. (2017) found significant differences in adult toxicity levels among these species for three neurotoxic insecticides that have different MoAs [chlorpyrifos (organophosphate, acetyl cholinesterase inhibitor), λ-cyhalothrin (pyrethroid, sodium channel modulator), and thiacloprid (neonicotinoid, nicotinic acetyl cholinesterase receptor agonist)]. These three insecticides are usually recommended for the control of two of the three moth species (MAPAMA, 2017). In addition, females of all three species were less susceptible than males to thiacloprid (maximum 3.4-fold), whereas, they were more susceptible than males to chlorpyrifos (maximum 41.5-fold) (Navarro-Roldán *et al*., 2017). Higher female susceptibility to organophosphates had been reported previously in *G. molesta* (de Lame *et al*., 2001). Comparative research of the use of enzyme inhibitors and metabolic detoxification is necessary, therefore, to present a more complete picture of the detoxification abilities of these three species. Although adults are not the primary target of these insecticides, they are exposed to them in the field, and the adult stage facilitates the evaluation of differences in sex response.

The main objective of our research was to study the metabolic mechanisms involved in susceptible strains of *C. pomonella*, *G. molesta*, and *L. botrana* regarding their defense against different neurotoxic insecticides (without any enzymatic induction by insecticides). These insect species have previously presented dissimilar levels of susceptibility to identical insecticides. Two approaches were followed. Firstly, we examined whether the enzymatic inhibition using DEF, DEM, and PBO affected insecticide-induced mortality. Secondly, we quantified EST, GST, and PSMO baseline activity in untreated individuals and in those treated with enzyme inhibitors.

Materials and methods

Insects

Susceptible laboratory strains of *C. pomonella*, *G. molesta*, and *L. botrana* collected in Lleida (Spain), Piacenza (Italy), and La Rioja (Spain), respectively, were maintained under laboratory conditions for more than 5 years, without the introduction of wild individuals. Larvae were reared on an artificial diet (Ivaldi-Sender, 1974) in a growth chamber. Insects used in Lleida (mortality bioassays) were maintained at 25 ± 1°C. Those used in Avignon (quantification of enzymatic activity) were shipped from Lleida as larvae or pupae and were maintained at 27.5 ± 0.5°C, under a 16:8 h light:dark photoperiod. Pupae were separated by sex and checked daily for adult emergence, except for *C. pomonella*, which was separated at the adult stage, also on a daily basis. Adults were treated zero to 24 h post-emergence, during the first half of the photophase.

Insecticides and enzyme inhibitors

As insecticide active ingredients we used chlorpyrifos [TraceCERT® certified reference material, ≈100% (a.i.)], λ-cyhalothrin [PESTANAL® analytical standard, ≈100% (a.i.)], and thiacloprid [PESTANAL® analytical standard, ≈100% (a.i.)]. The enzyme-inhibitors were DEF [analytical standard, 97% (a.i.)], DEM [analytical standard, 97% (a.i.)], and PBO [technical grade, 90% (a.i.)] all of them distributed by Sigma-Aldrich, Madrid, Spain. All dilutions used in the bioassays were prepared from pure compounds using acetone as a solvent (CHROMASOLV® for HPLC, ≥99.9%, Sigma-Aldrich). Dilutions were stored in 2 or 4 ml acetone-rinsed glass vials at 7°C. The same stock of acetone used to prepare the dilutions was also used as the negative control.

Effects of enzyme inhibitors on insecticide-induced insect mortality

Insecticides were applied at the LC_{50} (Navarro-Roldán *et al*., 2017), and the inhibitors were applied at the highest

https://doi.org/10.1017/S0007485319000415 Published online by Cambridge University Press

Table 1. Concentration of enzyme inhibitor and insecticide used.

		Inhibitor (mg ml^{-1})[1]			Insecticide (ng µl^{-1})[2]		
Species	Sex	PBO	DEM	DEF	Chlorpyrifos	λ-Cyhalothrin	Thiacloprid
Cydia pomonella	Female	122.5	12.5	1.0	125.02	1.49	101.42
	Male	122.5	12.5	1.0	123.25	0.92	43.06
Grapholita molesta	Female	10.0	12.5	2.5	15.09	0.71	308.58
	Male	5.0	12.5	5.0	393.30	0.38	57.97
Lobesia botrana	Female	10.0	5.0	2.5	12.83	0.49	3859.50
	Male	5.0	5.0	2.5	7.91	0.24	1163.07

[1]Highest concentration of inhibitor which did not produce significantly larger mortalities than solvent ($N = 30$, *Fisher exact test*). A range of 3–15 concentrations per inhibitor, species and sex, were used.
[2]Insecticide LC_{50} (Navarro-Roldán, *et al*., 2017).

concentration that produced the same mortality as the solvent did (table 1) (the concentration was determined in preliminary tests). The treatments containing one insecticide mixed with one inhibitor used the same concentration as when they were applied alone. Between 60 and 115 individuals per treatment were used for the 96 treatment groups [solvent, enzyme-inhibitor, insecticide (LC_{50}), insecticide (LC_{50})+ enzyme-inhibitor, species, and sex]. Different treatments were tested each day depending on insect availability. Tests were performed in groups of at least 3–10 insects of the same treatment set, with repetitions for each treatment set varying from 6 to 13, until the desired sample size was achieved.

One or two adults were placed in a 10 ml test tube, anesthetized with a brief (10 s) flow of industrial grade CO_2 and placed upside down under a stereoscope. A 1 µl test solution was applied to the ventral thoracic region of each insect with a high-precision, positive displacement, repeatable-dispensing micropipette (Multipette® M4, Eppendorf, Germany). They were then transferred to a 150 ml polypropylene, non-sterile, clinical sample bottle (diameter, 57 mm; height, 73 mm). Three to ten individuals subjected to the same treatment were placed together in the same bottle. The lid of the bottle was punctured with ten holes (1 mm diameter) to allow gas exchange, and a 1.5 ml Eppendorf plastic vial, containing a 10% sugar solution and a cotton plug was placed on the bottom to supply nutrients during the observation period in the growth chamber.

Mortality was recorded 24 h after treatment. Adults observed with the naked eye were recorded as alive if they flew or walked apparently unaffected, or as moribund if they could barely walk or were lying on the bottom of the bottle but still moving. They were considered to be dead if they lay immobile on the bottom of the bottle. Mortality was estimated by adding together the number of moribund and dead insects.

Activity of detoxification enzyme families

Enzymatic activity was quantified by measuring the quantity of artificial substrate that converted to the product during the reaction time relative to the protein content of the sample extract. Whole abdomens of *G. molesta* and *L. botrana* were used, but in *C. pomonella* the anterior half of abdomen was used for the quantification of EST and GST activities, and the posterior half for PSMO. Abdomens were homogenized in the reaction solution (see below) in 1.5 ml Eppendorf tubes kept on ice. PSMO analysis was performed on the same day on fresh tissue, whereas GST and EST samples were frozen (−80°C) and analyzed once all samples were collected, to reduce sampling error (Reyes, 2007). The amount of product was determined by absorbance (EST and GST) and fluorescence (PSMO) using a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland). After enzyme quantification, the total protein content of each sample was measured using the Bradford colorimetric absorbance test, using bovine serum albumin to build the standard curve (Bradford, 1976). Before protein quantification, the samples were diluted to place them within the range of standard curves. *L. botrana* male samples were undiluted, *C. pomonella* female samples were diluted tenfold, and the rest were diluted fivefold.

Carboxylesterases

Abdomens were homogenized in 110 µl of Hepes buffer (50 mM, pH 7.0) and centrifuged at 10,000 g for 15 min at 4°C. The supernatants were stored before use at −80°C. To measure total non-specific EST activity, 1 µl of supernatant, or 1 µl of Hepes buffer (50 mM, pH 7.0) for blanks (three wells), were placed in a transparent micro-plate (96-wells, Sterilin®, Newport, UK) containing 194 µl of α-naphthyl acetate (α-NA) 30 µM substrate (Ulrich & Weber, 1972) in Hepes buffer (50 mM, pH 7.0). After a 20 min incubation period at 30°C in darkness, the reaction was stopped and the resulting solution colored by adding 55 µl of 0.2% Fast Garnet GBC, diluted in 2.5% sodium dodecyl sulfate. Absorbance of the reaction product (α-Naphtol) was measured at 590 nm. EST activity was expressed as nmol α-Naphtol min mg^{-1} of total protein using a standard curve of α-Naphtol (0–18 nmoles well^{-1}). Between 55 and 59 insects per species and sex were used.

Glutathione-S-transferase

The extracts were prepared as for EST and 2 µl of supernatant, or 2 µl of Hepes buffer (50 mM, pH 7.0) for blanks (three wells), were placed in a transparent micro-plate (96-wells, Sterilin®, Newport, UK) containing 198 µl of 4-dinitro-chlorobenzene (CDNB) 0.76 mM substrate (Nauen & Stumpf, 2002) plus 2.52 mM glutathione (GSH) in Hepes buffer (50 mM, pH 7). After a 2 min incubation period at 25°C the absorbance of the reaction product (CDNB-glutathione (GSH) conjugate) was measured at 340 nm in the kinetic mode (every 30 s for 3 min). Since the CDNB-glutathione

https://doi.org/10.1017/S0007485319000415 Published online by Cambridge University Press

conjugate was not commercially available we were unable to build a standard curve, so we used the molar extinction coefficient (9.6 $mM^{-1} \times cm^{-1}$) of CDNB-glutathione to convert absorbance of CDNB-glutathione in µmol. The final specific activity was expressed in µmol of CDNB-glutathione min mg^{-1} of total protein extracted. Between 55 and 60 insects per species and sex were used.

Polysubstrate monooxygenases

Abdomens were homogenized in an incubation solution containing 7-ethoxycoumarin O-deethylation (0.4 mM) substrate (Bouvier *et al*., 2002) in Hepes buffer (50 mM, pH 7.0). Three blanks containing 100 µl of incubation solution were used. After a 4 h incubation period at 30°C the reaction was stopped with 100 µl of glycine buffer (1.5 M, pH 10.3) and centrifuged at 10,000 g for 5 min at room temperature. The supernatant (approximately 200 µl) was placed in black micro plates (96-wells, Corming Costar®, New York, USA). Fluorescence of the enzymatic product [7-hydroxycoumarin (7-HC)] was quantified with 380 nm excitation and 465 nm emission filters. PSMO activity was expressed as pg of 7-HC min mg^{-1} of total protein by using a standard curve of 7-HC (0.5–4.5 nmoles $well^{-1}$). Between 57 and 60 insects per species and sex were used.

Effects of enzyme inhibitors on the activity of detoxification enzymes

To determine the effect of the enzyme inhibitors on enzymatic activity, adults were treated with inhibitors and after incubation, the enzymatic activities were measured as in the previous tests. The incubation period was 24 h for PSMO, EST, and GST (17–40 DEF and DEM-treated individuals). As PSMO-activity was not inhibited after 24 h (preliminary test, data not shown), a 1 h incubation period was assayed for PSMO (16–25 PBO-treated individuals). Time has been shown to play a role in enzymatic inhibition (Young *et al*., 2005; 2006; Bingham *et al*., 2008), therefore an additional test was performed to explore the effect of time after inhibitor application on PSMO activity in both *G. molesta* and *L. botrana* (*C. pomonella* was not available at the time of this test). The time intervals between inhibitor application and enzyme activity quantification (i.e., incubation) for this test were 0, 0.5, 1, 2, 4, 12, and 16 h, along with an acetone control at 1 h (10–35 individuals per treatment).

Data analysis

All the statistical analyses were performed using R software (R Core Team, 2016). We used generalized linear models with Gaussian family functions for continuous variables (enzymatic activities), or binomial family functions for binomial variables (mortality percentage). The glht() and/or the predict means() functions performed *Tukey's* multiple pairwise comparisons, using *multcomp* and *predictmeans* R-packages, respectively for specified functions. Raw data and R scripts are available online (http://hdl.handle.net/10459.1/60223). Whenever the term 'significant' is used in the text regarding differences among treatments, it indicates a *P*-value <0.05. Correlations among mortality caused by enzyme-inhibitors and insecticide LD_{50} were made.

Results

Effects of enzyme inhibitors on insect mortality caused by insecticides

Table 2 shows the effect of the enzyme inhibitors on insect mortality, caused by insecticides. As it was expected, the inhibitors alone did not significantly increase mortality compared with solvent alone. This confirmed that the inhibitor concentrations used were not toxic in themselves. DEF, the EST inhibitor, significantly increased insecticide mortality in all cases, except in *L. botrana* females and *C. pomonella* males treated with thiacloprid (*P*-value = 0.876 and 0.773, respectively). DEM, the GST inhibitor, significantly synergized the activity of the three insecticides in *G. molesta* (except in females treated with λ-cyhalothrin). PBO, the PSMO inhibitor, had the same effect on males and females for all three species: it significantly synergized with thiacloprid, decreased the effect of chlorpyrifos, and did not affect λ-cyhalothrin (except in *G. molesta* females and *L. botrana* males).

Of the nine correlations made for the combination sex-species, comparing insecticide LD_{50} with enzyme inhibition dose, only thiacloprid and PSMO activity showed a high coefficient of determination ($R^2 = 0.8989$).

Activity of detoxification enzyme families

EST, GST, and PSMO activities in abdominal extracts of susceptible male and female adults of *C. pomonella*, *G. molesta*, and *L. botrana* were shown in fig. 1. In general, *L. botrana* was the species that had higher enzymatic activity. If we compare the enzymatic activity among species and sexes, *L. botrana* females had, in general, higher enzymatic activity than the rest of the groups, being these differences significant only for PSMO. The only observed differences between sexes were higher GST and PSMO levels in *L. botrana* females. EST activity was significantly higher in *C. pomonella* than in *G. molesta*, whereas the opposite was true for GST activity. PSMO activity was significantly higher in *L. botrana* females than in any other group, up to 5.3 times higher than in *C. pomonella* females. In addition, PSMO activity in *L. botrana* and *G. molesta* males was significantly higher than in *C. pomonella* males.

Effects of enzyme inhibitors on the activity of detoxification enzymes

After the 24 h incubation period, DEF significantly inhibited EST activity in both sexes for all species, obtaining enzymatic activity inhibition ratios ranging between 2.78 (*G. molesta* female) and 15.75 (*L. botrana* males). Inhibition in males was higher. Unexpectedly, DEM increased GST activity in *G. molesta* males but did not produce any observable effects in both sexes of the other species. After the 1 h incubation period, PBO did not have any effect on PSMO activity (table 3). These results coincide with those obtained *a posteriori* in the study of the effect of time after inhibitor application on PSMO activity in both *G. molesta* and *L. botrana* (fig. 2). A significant reduction in PSMO activity was obtained only 4 h after the PBO treatment in *G. molesta* females compared with the 0 h treatment, but not after 1 h PBO treatment. In *G. molesta* males this activity was significantly reduced from 4 h onward (fig. 2). On the contrary, in *L. botrana*, PSMO activity increased significantly with time (i.e., induction). For both sexes, this effect started to change at 12 h after treatment but was higher for

https://doi.org/10.1017/S0007485319000415 Published online by Cambridge University Press

Table 2. Mortality (%) of adult males and females of *C. pomonella*, *G. molesta*, and *L. botrana* 24 h after treatment with solvent acetone or insecticide (chlorpyrifos, λ-cyhalothrin, or thiacloprid), with and without detoxification enzyme inhibitors (DEF, DEM, and PBO).

		Inhibitor						
Female species	Insecticide	No inhibitor	DEF	*P*-value	DEM	*P*-value	PBO	*P*-value
C. pomonella	No insecticide	1.67	1.67	1.000	1.67	1.000	11.67	0.215
	Chlorpyrifos	46.67	88.33	<0.001	58.33	0.547	11.67	<0.001
	λ-Cyhalothrin	33.33	65.00	0.003	28.33	0.927	16.67	0.148
	Thiacloprid	28.33	53.33	0.026	38.33	0.626	100.00	<0.001
G. molesta	No insecticide	0.00	3.33	1.000	11.67	1.000	3.33	1.000
	Chlorpyrifos	45.00	100.00	<0.001	95.00	<0.001	15.00	0.002
	λ-Cyhalothrin	56.67	83.33	0.007	76.67	0.076	82.86	0.005
	Thiacloprid	41.67	95.00	<0.001	80.00	<0.001	100.00	<0.001
L. botrana	No insecticide	1.74	6.67	0.371	1.67	1.000	0.00	1.000
	Chlorpyrifos	68.33	98.33	0.007	80.00	0.444	8.33	<0.001
	λ-Cyhalothrin	60.00	93.33	<0.001	45.00	0.330	76.67	0.165
	Thiacloprid	41.67	48.33	0.876	48.33	0.872	83.33	<0.001
		Inhibitor						
Male species	Insecticide	No inhibitor	DEF	*P*-value	DEM	*P*-value	PBO	*P*-value
C. pomonella	No insecticide	0.00	0.00	1.000	3.33	1.000	7.69	1.000
	Chlorpyrifos	40.00	80.00	<0.001	50.00	0.636	8.33	<0.001
	λ-Cyhalothrin	28.33	58.33	0.004	21.67	0.801	18.33	0.519
	Thiacloprid	18.33	25.00	0.773	23.33	0.887	100.00	<0.001
G. molesta	No insecticide	0.00	6.67	1.000	11.67	1.000	8.33	1.000
	Chlorpyrifos	48.33	100.00	<0.001	86.67	<0.001	5.00	<0.001
	λ-Cyhalothrin	55.00	98.33	<0.001	78.33	0.028	75.00	0.080
	Thiacloprid	40.00	91.67	<0.001	71.67	0.002	95.00	<0.001
L. botrana	No insecticide	0.00	14.29	1.000	0.00	1.000	5.71	1.000
	Chlorpyrifos	68.33	100.00	0.005	75.00	0.817	10.00	<0.001
	λ-Cyhalothrin	41.67	96.67	<0.001	45.00	0.978	65.00	0.040
	Thiacloprid	43.33	96.67	<0.001	48.33	0.933	98.33	<0.001

P-values indicate the difference between the treatment with enzyme inhibitor (columns 2, 4, and 6) and the treatments without enzyme inhibitor (column 1). ($N = 60–115$ individuals/treatment).

males acquiring a 9.5-fold increase in relation to females 16 h after treatment (fig. 2).

Discussion

The main aim of our study was to determine the metabolic mechanisms involved in insecticide detoxification in the adults of three susceptible tortricid moth pests that present varied susceptibility to the same insecticides, even at the sex level (Navarro-Roldán *et al*., 2017). Comparing the baselines of the three species, *L. botrana* had the highest level of enzymatic activity, highlighting intense EST activity (fig. 1). *L. botrana* was the only species with enzymatic activity differences between sexes, with higher GST and PSMO activity in females. There are few published studies about insecticide resistance in *L. botrana*. Civolani *et al*. (2014) is the only case on resistance in field populations (http://www.pesticideresistance.com); and the present work along with Hatipoglu *et al*. (2015) are the only studies on the enzymatic activity of *L. botrana*. Nevertheless, in this case, no distinction was made between sexes to compare with our results. In addition, different units and a non-specified kind of microplate reader were used. *G. molesta* had lower EST activity than the other species but higher GST activity than in *C. pomonella*. In these two species, there were no differences between sexes in any measured enzymatic activity. On the contrary, all the codling moth populations analyzed in Reyes *et al*. (2015), one susceptible and six field populations, showed higher GST activity in females. De Lame *et al*. (2001) found higher EST activity in *G. molesta* males but any susceptible population was analyzed. The fact that most of the tested populations were collected from the field could be the reason why the results were not similar to ours. Siegward *et al*. (2011) found higher PSMO activity in *G. molesta* females and Rodríguez *et al*. (2010) found no difference between the sexes in five field and one laboratory *C. pomonella* population. Nevertheless, in these two cases, the PSMO activity results were expressed per insect and not per unit weight of protein.

The significant reduction of EST activity when applying the synergist DEF, and the general increase in mortality of individuals simultaneously treated with DEF and insecticides, illustrate how EST plays a general role in detoxification by acting on all insecticides, for both sexes and all moth species examined. This non-specific action of the EST enzymatic family has been reported in other pest species. For example, EST is able to sequester several xenobiotic molecules in *Myzus persicae* (Sulzer), thus preventing contact of the insecticide with its molecular target, inducing resistance to a broad spectrum of insecticides (Devonshire & Moores, 1982). The broad action of EST suggests that it is a cost-effective mechanism, easily adopted by several insect species. Gene amplification is an important mechanism for EST regulation in insects (Hemingway, 2000). Its significance was demonstrated in *Aedes aegypti* L., in which up to 41 genes exhibiting gene amplification were linked to resistance to the pyrethroid deltamethrin (Faucon *et al*., 2015). However, one must remember that we used

https://doi.org/10.1017/S0007485319000415 Published online by Cambridge University Press

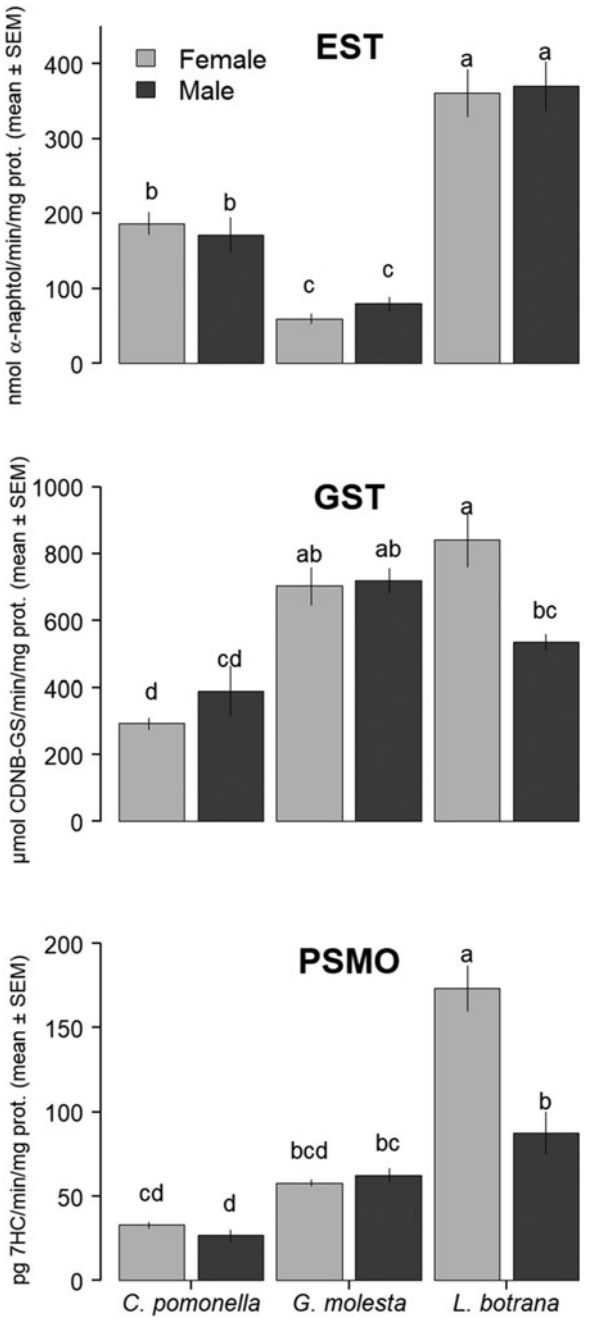

Fig. 1. EST, GST, and PSMO enzymatic activity in the abdomens of adult *C. pomonella*, *G. molesta*, and *L. botrana* from susceptible laboratory strains. Different letters indicate significant differences among bars for each enzyme family.

susceptible insect strains with a very basic enzymatic activity level; thus, enzyme activity may easily vary in populations subject to insecticide pressure.

Our mortality tests also showed how phase-I enzymatic activities (EST and PSMO) were involved in detoxification in all three species and in both sexes, whereas phase-II enzymes (GST) appeared to be less relevant in detoxification than the other two enzyme families, being important only in *G. molesta*.

Table 3. EST, GST, and PSMO activities on adult abdomens of susceptible male and female *C. pomonella*, *G. molesta*, and *L. botrana* adults, after application of DEF (24 h), DEM (24 h), and PBO (1 h), respectively.

			EST[2]			GST[2]			PSMO[2]		
Species	Sex	Inhibitor[1]	Mean ± SEM (*n*)	*P*-value	Ratio[3]	Mean ± SEM (*n*)	*P*-value	Ratio[3]	Mean ± SEM (*n*)	*P*-value	ratio[3]
C. pomonella	Female	(–)	566.38 ± 79.90 (39)	<0.001	7.76	2924.76 ± 1161.37 (40)	0.652	1.31	27.85 ± 2.37 (20)	0.880	1.02
	Female	(+)	72.95 ± 13.41 (24)			2240.50 ± 415.71 (24)			27.33 ± 2.70 (20)		
	Male	(–)	295.97 ± 58.25 (38)	0.002	4.37	1131.72 ± 112.39 (38)	0.104	0.71	19.42 ± 1.23 (20)	0.083	0.85
	Male	(+)	67.76 ± 19.94 (24)			1591.70 ± 313.08 (25)			22.78 ± 1.56 (20)		
G. molesta	Female	(–)	136.19 ± 23.13 (20)	<0.001	2.78	3174.73 ± 377.19 (20)	0.859	1.03	44.47 ± 3.29 (20)	0.728	0.97
	Female	(+)	49.01 ± 6.36 (20)			3095.28 ± 261.08 (20)			45.87 ± 2.54 (21)		
	Male	(–)	111.81 ± 25.35 (20)	0.004	3.63	1657.23 ± 482.40 (20)	0.006	0.46	56.68 ± 7.29 (16)	0.108	1.29
	Male	(+)	30.76 ± 7.89 (17)			3597.35 ± 531.96 (20)			43.84 ± 4.51 (20)		
L. botrana	Female	(–)	314.06 ± 69.92 (20)	<0.001	7.43	2477.93 ± 450.56 (20)	0.395	1.18	129.90 ± 16.31 (20)	0.909	1.02
	Female	(+)	42.26 ± 5.34 (20)			2093.99 ± 105.33 (20)			127.44 ± 14.85 (25)		
	Male	(–)	394.87 ± 43.11 (20)	<0.001	15.75	1683.82 ± 97.47 (20)	0.264	0.89	87.83 ± 20.11 (24)	0.663	0.86
	Male	(+)	25.07 ± 3.57 (18)			1900.70 ± 158.98 (25)			102.20 ± 28.10 (20)		

P-values indicate differences between inhibitor-treated and control for each group ($N = 16$–40 individuals/treatment).
[1](–) Control treatment (only with acetone), (+) Inhibitor treatment DEF, DEM, or PBO for EST, GST, and MFO, respectively.
[2]Enzymatic activities expressed in: EST (nmol α-naphtol min mg^{-1} protein), GST (μmol of CDNB-GS min mg^{-1} protein), and PSMO (pg of 7-HC min mg^{-1} protein).
[3]Ratio of the enzymatic activity inhibition.

https://doi.org/10.1017/S0007485319000415 Published online by Cambridge University Press

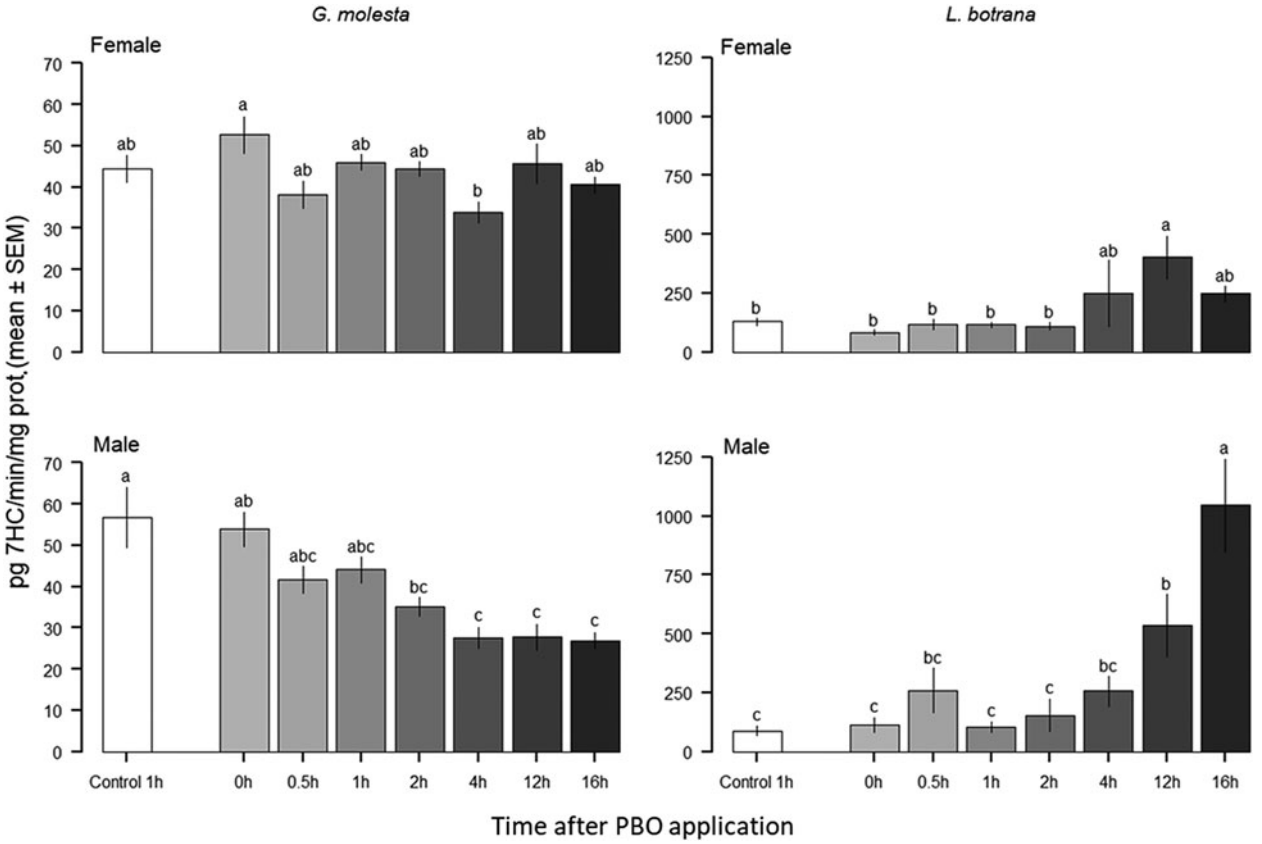

Fig. 2. Effects of time after application of PBO on PSMO enzymatic activity in the abdomens of adult *G. molesta* and *L. botrana* from susceptible laboratory strains. Different letters indicate significant differences among groups.

We reviewed 92 cases of detoxification mechanisms in Lepidoptera and found that GST was involved in only 36% of the cases; EST and PSMO were involved in a higher proportion, 63 and 64% of the cases, respectively (Navarro-Roldán, 2017). It was interesting that in our enzymatic activity test with inhibitor-treated insects, the activity of GST in *G. molesta* males was enhanced with the application of DEM. The opposite was expected, given that in the mortality bioassays, with insecticide-plus-enzyme inhibitors, the application of DEM increased susceptibility to all the tested insecticides, and especially on the males. The increase in detoxification activity in response to environmental stressors such as plant compounds, insecticides and herbicides, corresponds to an increase in enzyme production (induction) that is responsible, at least in part, for host-plant selection and selective toxicity or resistance development to insecticides (Terriere, 1984; Yang *et al.*, 2001; Yu, 2004; Després *et al.*, 2007; Poupardin *et al.*, 2008). Synergists are compounds that enhance the toxicity of an insecticide but are nontoxic on their own (Matsumura, 1985). However, these compounds do not always act as it is expected. For example, induction of enzymatic activities by the enzymatic inhibitor PBO was observed due to gene expression changes in *Drosophila melanogaster* (Meigen) (Willoughby *et al.*, 2007) and *Bemisia tabaci* (Zimmer *et al.*, 2017), but other synergist compounds have not yet been studied (Snoeck *et al.*, 2017). In addition, dose-dependent enzymatic activity induction (and inhibition) by insecticides has been observed in *Plutella xylostella* (L.) (Deng *et al.*, 2016), and *C. pomonella* (Parra-Morales *et al.*, 2017), both treated with the organophosphate chlorpyrifos.

Our results show that PBO produces a more diverse range of effects than the other two enzymatic synergists. PBO has a consistent and clear impact on the toxicity of thiacloprid and chlorpyrifos, resulting more variable with λ-cyhalothrin. In all species, for both sexes, the application of PBO leads to an increase in the toxicity of thiacloprid while the opposite occurs with chlorpyrifos. PBO is a well-known and widely used insecticide synergist, known to inhibit the activity of the insect cytochrome P450 detoxification system. PSMO reduction, caused by the action of the synergist, impacts insecticide efficacy. PSMO reduction decreases the activity of chlorpyrifos, due to a phenomenon known as bioactivation which has been described for organophosphates (Feyereisen, 1999) and detected in field populations with a high level of PSMO activity (Dunley & Welter, 2000; Bosch *et al.*, 2018). The complex chemical reactions of PSMO can lead to both insecticide bioactivation and detoxification (Levi *et al.*, 1988), as it was seen in honeybees (Iwasa *et al.*, 2004). We found that any PSMO-activity change was detected 1 h after the application of PBO in all of the observed insect species (table 3). This was in spite of the change in mortality produced by both insecticides, chlorpyrifos, and thiacloprid (table 2). PBO has also been reported to act as an inhibitor of EST in *Helicoverpa armigera* (Hübner) and *Bemisia tabaci* (Gennadius) (Young *et al.*,

https://doi.org/10.1017/S0007485319000415 Published online by Cambridge University Press

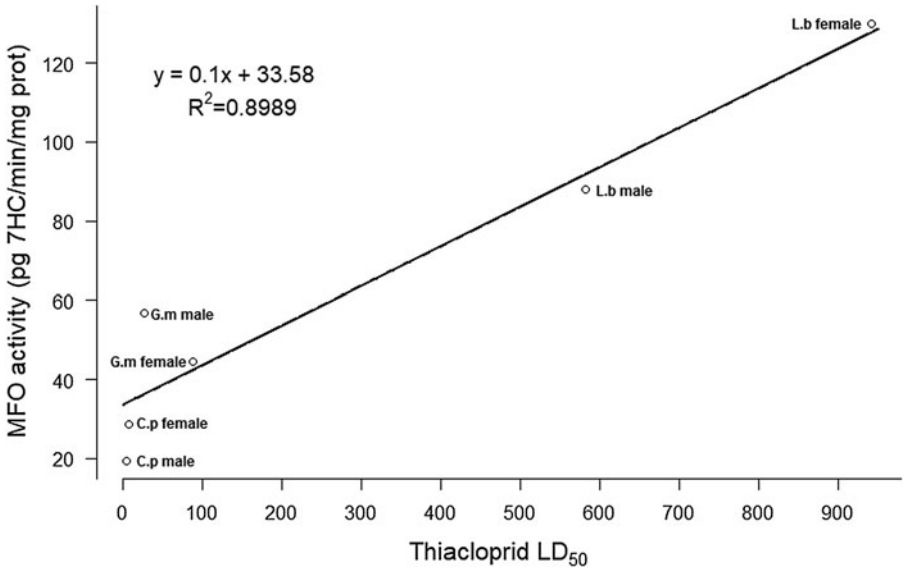

Fig. 3. Correlation between thiacloprid LD_{50} (ng of insecticide per mg of adult insect dry weight) (Navarro-Roldán *et al*., 2017) and PSMO activity of acetone treated individuals (table 3). C.p: *Cydia pomonella*; G.m: *Grapholita molesta*; L.b: *Lobesia botrana*.

2005; 2006). Hence, inhibition of EST by PBO can also occur and this should be examined in future work. Mortality was assessed 24 h after the application of the insecticide-plus-synergists and the enzymatic activity level measured 1 h after application of the synergist. Therefore, the effect of the inhibitor on the enzyme could have occurred at any time within this time interval. In a previous assay (data not shown), PBO treatment did not display a synergistic effect on PSMO enzymatic activity after 24 h. Due to this lack of inhibition, a test measuring the effect of time after PBO application on PSMO enzymatic activity was performed on *G. molesta* and *L. botrana* (fig. 2), the species available at the time of measurement. In *G. molesta*, we found a limited inhibition of 4 h after exposure. In *L. botrana* the PSMO activity increased for both sexes with a maximum of 9.5 times after 16 hours of PBO application for males. Hodgson & Levi (1998) found that, in mammals, PBO could function as a P450 inducer after a prolonged exposure to low doses, increasing the enzyme activity above the levels before the treatment. In addition, the PBO application is able to induce the expression of some detoxification gene families, as in the 32-fold one observed for the Cyp6A2 gene and for other PSMO genes by PBO in *D. melanogaster* (Willoughby *et al*., 2007). These results could explain the lack of inhibitor effect in our first enzyme-inhibition experiment. The dependence of the efficacy of a synergist-insecticide upon a pre-treatment time was observed with PBO on pyrethroids in *H. armigera* (Young *et al*., 2005; 2006) and *B. tabaci* (Young *et al*., 2006), PBO on carbamates in *M. persicae* and *Aphis gossypii* (Glover), and PBO on neonicotinoids in *B. tabaci* (Bingham *et al*., 2008). Interestingly, the changes in enzymatic activity over time revealed sex differences: Inhibition could be detected 4 h after the treatment on *G. molesta* males, but not on females. Likewise, the different enzymatic activities between *L. botrana* males and females may help in illuminating sex differences in insecticide susceptibility, as it was reported previously (Navarro-Roldán *et al*., 2017). Variations in enzymatic inhibition and induction observed in the kinetic experiment could help explain the differences in insecticide susceptibility between the sexes, as well as the lack of PMSO inhibition observed when enzyme quantification is made only once after inhibition. Nevertheless, further kinetic investigations involving the three species and the three enzyme inhibitors are needed to fully understand inhibitor-enzyme dynamics. As in other species, many 'metabolic enzyme-inducers' may be present and may influence the insect metabolic enzyme status in susceptible strains (Terriere, 1984; Yang *et al*., 2001; Yu, 2004; Després *et al*., 2007; Poupardin *et al*., 2008; Xie *et al*., 2011; Deng *et al*., 2016; Parra-Morales *et al*., 2017).

In a previous study, Navarro-Roldán *et al*. (2017) reported significant differences in mortality (LD_{50}) among insecticides (maximum 7800-fold), species (maximum 115-fold), and sexes (maximum 41.5-fold). In order to determine whether there is an association between enzymatic activity levels and mortality, we calculated the correlation between the LD_{50}s of the three species and the two sexes for each enzyme family and insecticide (Navarro-Roldán *et al*., 2017), along with the enzymatic activity reported herein. Of the nine regressions, only thiacloprid and PSMO activity have a high coefficient of determination (fig. 3, $R^2 = 0.8989$), showing that PSMO plays a role in thiacloprid detoxification in these species. The highest PSMO activity was observed for *L. botrana* females, the most tolerant group to thiacloprid (table 1, Navarro-Roldán *et al*., 2017). Nevertheless, other detoxification mechanisms must be involved since the increase of thiacloprid toxicity detected with the application of the synergist PBO was not due to the increase of PSMO activity levels. A significant role of the PSMO in the detoxification of thiacloprid has been reported in *C. pomonella* field adults (Reyes *et al*., 2007), and in combination with EST in larvae (İşci & Ay, 2017). The lack of correlation between enzymatic activity and mortality for the other insecticides and enzyme families may be due to lower insecticide selectivity and multiple target sites of the enzymatic groups resulting in mutual interactions (Ahmad & Hollingworth, 2004).

A remarkable finding from our previous study was lower susceptibility to the organophosphate insecticide chlorpyrifos

https://doi.org/10.1017/S0007485319000415 Published online by Cambridge University Press

in males of all three moth species compared to females, mainly in *G. molesta* (Navarro-Roldán *et al*., 2017). The expected pattern was higher susceptibility in males due to their smaller size. Shearer & Usmani, (2001) reported higher female susceptibility to organophosphates in *G. molesta*. In a follow-up study, de Lame *et al*. (2001) reported that the higher tolerance of males may be linked to a higher EST and acetylcholinesterase (AChE) activity which may counteract the higher male AChE sensitivity to organophosphates. Contrarily, we have not found significant differences in EST activity between sexes, although a similar procedure was used in both studies. Our European laboratory strain was reared on an artificial diet for more than five years without reintroduction of wild individuals, whereas the North American population (de Lame *et al*., 2001; Shearer & Usmani, 2001) consisted of colonies, aged less than 3 years, reared on green apples. Thus, it is expected that both colonies possess very dissimilar genomes that condition their respective detoxification enzyme systems. Even an insect's feeding pattern, which can differ markedly during its breeding cycle, may influence its immune system (Vogelweith *et al*., 2011). To further explain sex differences, de Lame *et al*. (2001) proposed a simultaneous involvement of other metabolic enzymatic families, like GST, or a lower bioactivation by PSMO of P = S compounds in the AChE inhibitory P = O analogs in males.

Conclusions

Differences in enzymatic activity quantification were found in the different studied species, being susceptible strains, reared for a long time in the laboratory and fed with the same artificial diet. *L. botrana* exhibited a general higher enzymatic activity, highlighting its high EST activity, and was the only species with differences in response between sexes for GST and PSMO activity. These enzymatic-activity differences could explain the lower susceptibility of females of this species to the insecticide products tested; however, they did not align with the sex differences reported in a previous study involving *C. pomonella* and *G. molesta* (Navarro-Roldán *et al*., 2017).

As was showed by the mortality tests, the phase-I enzymatic activities (EST and PSMO) were involved in the insecticide detoxification in the three species for both sexes, highlighting the role of EST, whereas GST, phase-II enzymes, appeared to play less of a role in insecticide detoxification, resulting involved only in *G. molesta* insecticide detoxification. In addition, the positive correlation observed between PSMO activity and the thiacloprid LD_{50} explained the species-specific differences in susceptibility to thiacloprid that were reported in our previous study (Navarro-Roldán *et al*., 2017).

The knowledge of the enzymatic activity level of susceptible populations of each species can be useful for susceptibility/resistant studies. The differentiation between sexes in *L. botrana* studies would be basic for a good characterization of the populations.

Acknowledgements

MAN-R was supported by a Ph.D. fellowship from the University of Lleida. This study was supported by the research Grant AGL2013-49164-C2-1- MINECO and AGL2016-77373-C2-2-R-MINECO, Spain. The authors wish to thank the CERCA Programme/Generalitat de Catalunya. Finally, our special appreciation goes to Jesús Avilla for his helpful comments on an earlier version of the manuscript, and to Sandrine Maugin for her laboratory collaboration.

References

Ahmad, M. & Hollingworth, R.M. (2004) Synergism of insecticides provides evidence of metabolic mechanisms of resistance in the oblique-banded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae). *Pest Management Science* **60**(5), 465–473.

Bernard, C.B. & Philogène, B.J. (1993) Insecticide synergists: role, importance, and perspectives. *Journal of Toxicology and Environmental Health-Part A* **38**(2), 199–223.

Biddinger, D.J., Hull, L.A. & McPheron, B.A. (1996) Cross-resistance and synergism in azinphosmethyl resistant and susceptible strains of tufted apple bud moth (Lepidoptera: Tortricidae) to various insect growth regulators and abamectin. *Journal of Economic Entomology* **89**(2), 274–287.

Bingham, G., Gunning, R.V., Delogu, G., Borzatta, V., Field, L.M. & Moores, G.D. (2008) Temporal synergism can enhance carbamate and neonicotinoid insecticidal activity against resistant crop pests. *Pest Management Science* **64**(1), 81–85.

Bosch, D., Avilla, J., Musleh, S. & Rodríguez, M.A. (2018) Target-site mutations (*AChE* and *kdr*), and PSMO activity in codling moth (*Cydia pomonella* (L.) (Lepidoptera: Tortricidae)) populations from Spain. *Pesticide Biochemistry and Physiology* **146**: 52–62.

Bouvier, J.C., Boivin, T., Beslay, D. & Sauphanor, B. (2002) Age-dependent response to insecticides and enzymatic variation in susceptible and resistant codling moth larvae. *Archives of Insect Biochemistry and Physiology* **51**(2), 55–66.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** (1–2), 248–254.

Brown, T.M. & Brogdon, W.G. (1987) Improved detection of insecticide resistance through conventional and molecular techniques. *Annual Review of Entomology* **32**(1), 145–162.

Casida, J.E. & Quistad, G.B. (2004) Why insecticides are more toxic to insects than people: the unique toxicology of insects. *Journal of Pesticide Science* **29**, 81–86.

Civolani, S., Boselli, M., Butturini, A., Chicca, M., Fano, E.A. & Cassanelli., S. (2014) Assessment of insecticide resistance of *Lobesia botrana* (Lepidoptera: Tortricidae) in Emilia-Romagna Region. *Journal of Economic Entomology* **107**, 1245–1249.

Damos, P., Colomar, L.A.E. & Ioriatti, C. (2015) Integrated fruit production and pest management in Europe: the apple case study and how far we are from the original concept? *Insects* **6**(3), 626–657.

de Lame, F.M., Hong, J.J., Shearer, P.W. & Brattsten, L.B. (2001) Sex-related differences in the tolerance of oriental fruit moth (*Grapholita molesta*) to organophosphate insecticides. *Pest Management Science* **57**(9), 827–832.

Deng, Z.Z., Zhang, F., Wu, Z.L., Yu, Z.Y. & Wu, G. (2016) Chlorpyrifos-induced hormesis in insecticide-resistant and-susceptible *Plutella xylostella* under normal and high temperatures. *Bulletin of Entomological Research* **106**(03), 378–386.

Després, L., David, J.P. & Gallet, C. (2007) The evolutionary ecology of insect resistance to plant chemicals. *Trends in Ecology and Evolution* **22**(6), 298–307.

Devonshire, A.L. & Moores, G.D. (1982) A carboxylesterase with broad substrate specificity causes organophosphorus,

carbamate and pyrethroid resistance in peach-potato aphids (*Myzus persicae*). *Pesticide Biochemical Physiology* **18**, 235–246.

Dunley, J.E., & Welter, S.C. (2000) Correlated insecticide cross-resistance in azinphosmethyl resistant codling moth (Lepidoptera: Tortricidae). *Journal of economic entomology* **93**(3), 955–962.

Enayati, A.A., Ranson, H. & Hemingway, J. (2005) Insect glutathione transferases and insecticide resistance. *Insect Molecular Biology* **14**(1), 3–8.

Faucon, F., Dusfour, I., Gaude., T, Navratil, V., Boyer, F., Chandre, F., Sirisopa, P., Thanispong, K., Juntarajumnong, W., Poupardin, R., Chareonviriyaphap, T., Girod, R., Corbel, V., Reynaud, S. & David, J.P. (2015) Identifying genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing. *Genome Research* **25**, 1347–1359.

Feyereisen, R. (1999) Insect P450 enzymes. *Annual Review of Entomology* **44**(1), 507–533.

Guo, Y., Chai, Y., Zhang, L., Zhao, Z., Gao, L.L. & Ma, R. (2017) Transcriptome analysis and identification of major detoxification gene families and insecticide targets in *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae). *Journal of Insect Science* **17**(2), 43–57.

Hatipoglu, A., Durmusoglu, E. & Gürkan, O. (2015) Manisa ili bağ alanlarında Salkım güvesi [*Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae)] popülasyonlarının insektisit direncinin belirlenmesi. *Türkiye Entomoloji Dergisi-Turkish Journal of Entomology* **39**(1), 55–65.

Hemingway, J. (2000) The molecular basis of two contrasting metabolic mechanisms of insecticide resistance. *Insect Biochemistry and Molecular Biology* **30**(11), 1009–1015.

Hodgson, E & Levi, P.E. (1998), Interactions of piperonyl butoxide with cytochrome P450. pp. 41–54 *in* Jones, D.G. (Ed.), *Piperonyl Butoxide*. London, Academic Press.

Ioriatti, C., Anfora, G., Tasin, M., de Cristofaro, A., Witzgall, P. & Lucchi, A. (2011) Chemical ecology and management of *Lobesia botrana* (Lepidoptera: Tortricidae). *Journal of Economic Entomology* **104**(4), 1125–1137.

İşci, M. & Ay, R. (2017) Determination of resistance and resistance mechanisms to thiacloprid in *Cydia pomonella* L. (Lepidoptera: Tortricidae) populations collected from apple orchards in Isparta Province, Turkey. *Crop Protection* **91**, 82–88.

Ivaldi-Sender, C. (1974) Techniques simples pour elevage permanent de la tordeuse orientale, *Grapholita molesta* (Lep., Tortricidae), sur milieu artificiel. *Annales de Zoologie Ecologie Animale* **6**, 337–343.

Iwasa, T., Motoyama, N., Ambrose, J.T. & Roe, R.M. (2004) Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection* **23**, 371–378.

Karoly, E.D., Rose, R.L., Thompson, D.M., Hodgson, E., Rock, G.C. & Roe, R.M. (1996) Monooxygenase, esterase, and glutathione transferase activity associated with azinphosmethyl resistance in the tufted apple bud moth, *Platynota idaeusalis*. *Pesticide Biochemistry and Physiology* **55**(2), 109–121.

Kirk, H., Dorn, S. & Mazzi, D. (2013) Worldwide population genetic structure of the oriental fruit moth (*Grapholita molesta*), a globally invasive pest. *BMC Ecology* **13**(1), 12.

Krieger, R.I., Feeny, P.P. & Wilkinson, C.F. (1971) Detoxication enzymes in the guts of caterpillars: an evolutionary answer to plant defenses? *Science* **172**(3983), 579–581.

Levi, P.E., Hollingworth, R.M. & Hodgson, E. (1988) Differences in oxidative dearylation and desulfuration of fenitrothion by cytochrome P-450 isozymes and in the subsequent inhibition of monooxygenase activity. *Pesticide Biochemistry and Physiology* **32**(3), 224–231.

Li, X., Schuler, M.A. & Berenbaum, M.R. (2007) Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annual Review of Entomology* **52**, 231–253.

(MAPAMA). Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente. (2017) Registro de Productos Fitosanitarios. Available online at http://www.mapama.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/registro/menu.asp (Accessed April 2017).

Matsumura, F. (1985) *Metabolism of insecticides by animals and plants*. In Toxicology of Insecticides. Mastumura, F. (Ed.) pp. 203–298, Plenum Press, New York.

Metcalf, R.L. (1967) Mode of action of insecticide synergists. *Annual Review of Entomology* **12**(1), 229–256.

Montella, I.R., Schama, R. & Valle, D. (2012) The classification of esterases: an important gene family involved in insecticide resistance-A review. *Memorias do Instituto Oswaldo Cruz* **107** (4), 437–449.

Nauen, R. & Stumpf, N. (2002) Fluorometric microplate assay to measure glutathione S-transferase activity in insects and mites using monochlorobimane. *Analytical Biochemistry* **303** (2), 194–198.

Navarro-Roldán, M.A. (2017) Detoxification and sublethal effects of neurotoxic insecticides in Tortricid moths. PhD University of Lleida. **1**, 1–200. Available online at https://www.tesisenred.net/handle/10803/462995 (Accessed May 2019).

Navarro-Roldán, M.A., Avilla, J., Bosch, D., Valls, J. & Gemeno, C. (2017) Comparative effect of three neurotoxic insecticides with different modes of action on adult males and females of three tortricid moth pests. *Journal of Economic Entomology* doi: 10.1093/jee/tox113.

Parra-Morales, L.B., Alzogaray, R.A., Cichón, L., Garrido, S., Soleño, J. & Montagna, C.M. (2017) Effects of chlorpyrifos on enzymatic systems of *Cydia pomonella* (Lepidoptera: Tortricidae) adults. *Insect Science* **24**, 455–466.

Poupardin, R., Reynaud, S., Strode, C., Ranson, H., Vontas, J. & David, J.P. (2008) Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito *Aedes aegypti*: impact on larval tolerance to chemical insecticides. *Insect Biochemistry and Molecular Biology* **38**(5), 540–551.

R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available online at http://www.R-project.org/ (accessed 1 September 2017).

Reuveny, H. & Cohen, E. (2004) Evaluation of mechanisms of azinphos-methyl resistance in the codling moth *Cydia pomonella* (L.). *Archives of Insect Biochemistry and Physiology* **57**(2), 92–100.

Reyes, M. (2007) La résistance aux insecticides chez le carpocapse des pommes: mécanismes, détection et variabilité géographique. PhD University of Avignon. Avignon.

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**(9), 890–902.

Reyes, M., Barros-Parada, W., Ramírez, C.C. & Fuentes-Contreras, E. (2015) Organophosphate resistance and its main mechanism in populations of codling moth

(Lepidoptera: Tortricidae) from Central Chile. *Journal of Economic Entomology* **108**(1), 277–285.

Roberts, T.R. & Hutson, D.H. (1998) Metabolic Pathways of Agrochemicals: Insecticides and Fungicides, (Vol. **1**). Cambridge, UK, Royal Society of Chemistry.

Rodríguez, M.A., Bosch, D., Sauphanor, B. & Avilla, J. (2010) Susceptibility to organophosphate insecticides and activity of detoxifying enzymes in Spanish populations of *Cydia pomonella* (Lepidoptera: Tortricidae). *Journal of Economic Entomology* **103**(2), 482–491.

Rose, H.A. (1985) The relationship between feeding specialization and host plants to aldrin epoxidase activities of midgut homogenates in larval Lepidoptera. *Ecological Entomology* **10** (4), 455–467.

Sauphanor, B., Cuany, A., Bouvier, J.C., Brosse, V., Amichot, M. & Bergé, J.B. (1997) Mechanism of resistance to deltamethrin in *Cydia pomonella* (L.) (Lepidoptera: Tortricidae). *Pesticide Biochemistry and Physiology* **58**(2), 109–117.

Scott, J.G. (1999) Cytochromes P450 and insecticide resistance. *Insect Biochemistry and Molecular Biology* **29**(9), 757–777.

Shearer, P.W. & Usmani, K.A. (2001) Sex-related response to organophosphorus and carbamate insecticides in adult oriental fruit moth, *Grapholita molesta*. *Pest Management Science* **7**(9), 822–826.

Sial, A.A. & Brunner, J.F. (2012) Selection for resistance, reversion towards susceptibility and synergism of chlorantraniliprole and spinetoram in obliquebanded leafroller, *Choristoneura rosaceana* (Lepidoptera: Tortricidae). *Pest Management Science* **68**, 462–468.

Siegwart, M., Monteiro, L.B., Maugin, S., Olivares, J., Malfitano-Carvalho, S. & Sauphanor, B. (2011) Tools for resistance monitoring in oriental fruit moth (Lepidoptera: Tortricidae) and first assessment in Brazilian populations. *Journal of Economic Entomology* **104**(2), 636–645.

Snoeck, S., Greenhalgh, R., Tirry, L., Clark, R.M., Van Leeuwen, T. & Dermauw, W. (2017) The effect of insecticide synergist treatment on genome-wide gene expression in a polyphagous pest. *Scientific Reports* **7,** 13440 Available online at http://www.nature.com/scientificreports.

Soderlund, D.M. & Bloomquist, J.R. (1990) Molecular mechanisms of insecticide resistance. pp. 58–95 *in Pesticide Resistance in Arthropods*, Roush, R.T. and Tabashnik, B.E. (Eds), New York, Chapman and Hall.

Terriere, L.C. (1984) Induction of detoxication enzymes in insects. *Annual Review of Entomology* **29**(1), 71–88.

Ullrich, V. & Weber, P. (1972) The O-dealkylation of 7-ethoxycoumarin by liver microsomes. A direct fluorometric test. *Hoppe-Seyler's Zeitschrift für physiologische Chemie* **353**(2), 1171–1177.

Usmani, K.A. & Knowles, C.O. (2001) Toxicity of pyrethroids and effect of synergists to larval and adult *Helicoverpa zea*, *Spodoptera frugiperda*, and *Agrotis ipsilon* (Lepidoptera: Noctuidae). *Journal of Economic Entomology* **94**(4), 868–873.

Vogelweith, F., Thiery, D., Quaglietti, B., Moret, Y. & Moreau, J. (2011). Host plant variation plastically impacts different traits of the immune system of a phytophagous insect. *Functional Ecology* **25**, 1241–1247.

Vojoudi, S., Saber, M., Gharekhani, G. & Esfandiari, E. (2017) Toxicity and sublethal effects of hexaflumuron and indoxacarb on the biological and biochemical parameters of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in Iran. *Crop Protection* **91**, 100–107.

Willoughby, L., Batterham, P. & Daborn, P.J. (2007) Piperonyl butoxide induces the expression of cytochrome P450 and glutathione S-transferase genes in *Drosophila melanogaster*. *Pest Management Science* **63**(8), 803–808.

Wu, G., Miyata, T., Kang, C.Y. & Xie, L.H. (2007) Insecticide toxicity and synergism by enzyme inhibitors in 18 species of pest insect and natural enemies in crucifer vegetable crops. *Pest Management Science* **63**, 500–510.

Xie, W., Wang, S., Wu, Q., Feng, Y., Pan, H., Jiao, X., Zhou, L., Yang, X., Fu, W., Teng, H., Xu, B. & Zhang, Y. (2011) Induction effects of host plants on insecticide susceptibility and detoxification enzymes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Pest Management Science* **67**, 87–93.

Xue, M., Pang, Y.H., Li, Q.L. & Liu, T.X. (2010) Effects of four host plants on susceptibility of *Spodoptera litura* (Lepidoptera: Noctuidae) larvae to five insecticides and activities of detoxification esterases. *Pest Management Science* **66**, 1273–1279.

Yang, X., Margolies, D.C., Zhu, K.Y. & Buschman, L.L. (2001) Host plant-induced changes in detoxification enzymes and susceptibility to pesticides in the two spotted spider mite (Acari: Tetranychidae). *Journal of Economic Entomology* **94**(2), 381–387.

Young, S.J., Gunning, R.V. & Moores, G.D. (2005) The effect of piperonyl butoxide on pyrethroid-resistance-associated esterases in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Pest Management Science* **61**(4), 397–401.

Young, S.J., Gunning, R.V. & Moores, G.D. (2006) Effect of pretreatment with piperonyl butoxide on pyrethroid efficacy against insecticide-resistant *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Bemisia tabaci* (Sternorrhyncha: Aleyrodidae). *Pest Management Science* **62**(2), 114–119.

Yu, S.J. (2004) Induction of detoxification enzymes by triazine herbicides in the fall armyworm, *Spodoptera frugiperda* (JE Smith). *Pesticide Biochemistry and Physiology* **80**(2), 113–122.

Yu, S.J. (2008) *The Toxicology and Biochemistry of Insecticides*, Boca Raton, FL, USA, CRC Press/Taylor and Francis.

Yu, S.J. & Hsu, E.L. (1993) Induction of detoxification enzymes in phytophagous insects: role of insecticide synergists, larval age, and species. *Archives of Insect Biochemistry and Physiology* **24**(1), 21–32.

Yu, S.J., Nguyen, S.N. & Abo-Elghar, G.E. (2003) Biochemical characteristics of insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (JE Smith). *Pesticide Biochemistry and Physiology* **77**(1), 1–11.

Zimmer, C.T., Panini, M., Singh, K.S., Randall, E.L., Field, L.M., Roditakis, E., Mazzoni, E. & Bass, C. (2017). Use of the synergist piperonyl butoxide can slow the development of alpha-cypermethrin resistance in the whitefly *Bemisia tabaci*. *Insect Molecular Biology* **26**(2), 152–163.