Universitat de Lleida

1 **Enzymatic activities role in neurotoxic insecticides detoxification and the importance of**

2 **time in the use of enzymatic activities inhibitors on male and female adults of tortricid**

3 **moth pests**

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13 **ABSTRACT**

14 The toxicities of three insecticides alone or in presence plus of three enzyme inhibitors and their

15 synergistic effect were studied on susceptible strains species of three economically important

16 tortricid moth pests species [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia*

17 *botrana* (Denis & Schiffermüller)]. Besides, activities of the three most important enzymatic

18 activities families [mixed-function oxidases (MFO), esterases (EST), and glutathione-S-

19 transferases (GST)], involved in metabolic detoxification of insecticides were measured with and

20 without enzymatic inhibition treatmenteffect. As enzyme inhibitors we used piperonyl butoxide

21 (PBO), S,S,S, tributyl phosphorotrithioate (DEF) and diethyl maleate (DEM), respectively to the

22 previously cited enzymes families. Our results shown that phase I enzymatic activities were

23 important in both sexes of three species, whereas phase II enzymes only were only important in

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G. molesta. As well, EST played a role in detoxification process of ~~chlorpyrifos and λ-cyhalothrin~~all insecticides tested. Thi~~ss is the~~ enzyme famil~~yy how~~ show the most differencies between species. ~~and~~ MFO are~~is~~ involved in~~nin~~ detoxification process of thiacloprid and activation of the organophosphate: chlorpyrifos in both sexes of three species. *L. botrana* has a particular profile compare to the two other species with enhanced activity of GST and MFO in males compare to females. ~~Species differences for EST and sex differences for MFO and GST activities in *L. botrana* were found in amounts of enzymatic activities measures~~. Inhibition tests followed by enzymatic measurement ~~shown that~~reveal significant inhibition ~~was only~~ observed for EST with DEF. However, ~~I~~an unexpected inhibition kinetic, although MFO and GST were not inhibited by PBO and DEM respectively at the tested time. Inhibition ~~differences across the time were~~ is observed with PBO in ~~males and females of two species, this cause could be one explanation for negative results in MFO inhibition~~ *G. molesta* ~~results~~and *L. Botrana*. In the first specie, a slight inhibition occurs from 12h after treatment, whereas a strong activation (10 times) appears for the second species.~~results. The implications of the observed metabolic mechanisms differences on previously reported susceptibility differences among these species and sex were discussed~~These results lead the question of using syngergist in agricultural strategy to control pest. T~~-~~his reveals part of the complexity of the mechanisms developed by pest for their protection against toxic.

KEY WORDS: insecticide inhibitors, neurotoxic insecticides, Tortricidae, adult insects, sex differences.

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Introduction

The metabolism or capacity of degrade toxic substances is essential~~so important~~ for the survival of a pest in a constant chemically changeable environment. All insects have detox capacity, ~~but probably It~~ ~~the amount~~ ~~can be expected to~~that vary among species and insect developmental stage (Yu and Hsu, 1993). Some~~,~~ ~~sor s~~timulation occur with~~es~~ host plants changes ~~by host plants~~ (Yang et al, 2001, Després et al, 2007), and other environmental stressors like insecticides (Poupardin et al, 2008) or~~,~~ herbicides (Yu, 2004)~~, etc~~. These constant insect adaptation to their environment is supported by the phenomenon of induction, ~~this phenomenon~~that consists on a detoxification activity enhanced (e.g., production of additional enzymes) by a chemical stimulus~~,~~ ~~those~~. Those variations in detox capacity are responsible, at least in part, for host plant selection and selective toxicity or resistance development of insecticides (Terriere, 1984). ~~Besides, in case of insecticides~~For example, ~~owing to the dose of them~~ we could find dose-dependent enzymatic activity -induction or inhibition by insecticide ~~owing to the dose of them, like occurs for some enzymatic activities~~in *Plutella xylostella* (Linnaeus) (Deng et al, 2016), and *Cydia pomonella* (Linnaeus) (Parra Morales et al, 2017), both treated with the organophosphate chlorpyrifos.

The three most important metabolic detoxification systems in insects ~~are~~involved cytochrome P450 monooxygenases (P450) [~~included in~~mixed-function oxidases (MFO) enzymes]~~.~~ carboxylesterases~~included], esterases~~ (EST), and glutathione-S-transferases (GST). These enzyme families~~, which~~could be subject to genomic changes that lead to gene amplification, overexpression, and coding sequence variation ~~in the groups of genes~~that modify their detoxification abilities ~~encoding these metabolic enzymes~~(Li et al, 2007). Metabolic transformation of the toxic compound, ~~normally~~could takes place in two phases, the former consist on the addition of a polar group to the substrate or the break of the molecule in two part, the latter involves the addition of sugars, aminoacids, sulphates or phosphate groups on substrate

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Besides, in case of insecticides, we could find enzymatic activity induction or inhibition owing to the dose

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resulted in the first phase, in case of this substrate was not enough hydrophilic to be excreted. MFO and EST enzymes are involved in phase I, GST in phase II (B-Bernard and Philogène, 1993).

Synergists used in agriculture, bind to these enzymes and interfere with general metabolic pathways of detoxification. The most presently used synergists are metabolic inhibitors, in consequence, the term synergist often implies this specific mode of action (B-Bernard and Philogène, 1993), which is the case of S,S,S, tributyl phosphorotrithioate (DEF), a total EST inhibitor, diethyl maleate (DEM), inhibitor of GST and piperonyl butoxide (PBO), a MFO inhibitor. In present study, we use equally the terms synergist and inhibitor, because an enzymatic inhibitor could be an insecticide synergist.

Synergists at some dosages are nontoxic components that lead to a significant increase of the activity of another substance. In concrete case of insecticides, synergists enhance their lethality, partially, because of the inhibition of detoxifying enzymes could reduce the defensive system of the insect (Ishaaya, 1993). The practical importance ofIn agriculture synergists are used for entomologist consist ofonf the more efficient, (i) to enhance the control of a insectspest by a mixture, the increase of(ii) to extend the activity spectrum of an insecticide, and or (iii) to restore the activity restoring of an insecticide against resistant strains of insects. Besides these considerations, synergist use and investigation support knowledge about detoxification mechanisms in insects, basic biochemical processes involved in insecticide resistance, and mode of action of insecticides (Metcalf, 1967).

Even though lot of reports in synergistic effect of insecticide toxicity were made, some aspects of insecticide synergism remain incompletely resolved. One case are synergist that could induce other enzymatic activities, e.g., in *Drosophila melanogaster* (Meigen), after 4 h of exposure to PBO, twelve P450 and five GST genes were induced and an increased production of GST

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~~enzymes~~that ~~by PBO exposure~~ could have the potential to increase insecticide tolerance ~~if these enzymes are capable of insecticide~~by metabol~~isation~~ism. (Willoughby et al, 2007). Other questioning aspects are the optimum pretreatment type and time between application of a synergist and the insecticide. The efficacy of synergist-insecticide application is partially dependent upon pretreatment time, e.g., the case of PBO-pyrethroid in *Helicoverpa armigera* (Hübner) (Young et al, 2005, 2006) and *Bemisia tabaci* (Gennadius) (Young et al, 2006); in PBO-carbamate in *Myzus persicae* (Sulzer) and *Aphis gossypii* (Glover) and in PBO-neonicotinoid in *B. tabaci* (Bingham et al, 2008).

In a previous study (Navarro-Roldán et al. 2017) we report mortality of males and females adults of three tortricid moth species [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller)] treated with three neurotoxic insecticides having different modes of action [chlorpyrifos (organophosphate, acetylcholinesterase inhibitor), λ-cyhalothrin (pyrethroid, sodium channel modulator) and thiacloprid (neonicotinoid, nicotinic acetylcholinesterase receptor agonist)]. We found that females were less sensitive than males to thiacloprid, and higher female sensitivity to ~~organophosphate~~chlorpyrifos in all three species. This last result~~, which~~ was unexpected given that females are larger than males. Higher female sensitivity to organophosphates has been reported previously only in *G. molesta* (de Lame, 2001), but not (as far as we know) in other moth species.

Based on these previous findings, our main objective ~~is~~was to study the metabolic mechanism involved in~~of~~ pest toxicological defence. ~~of these insects~~ to the proposed insecticides, and ~~in a second way~~ to determine if, and which, degrading enzymes are involved in the lower male susceptibility to organophosphate ~~insecticides in the three tortricid species~~. For that, enzyme ~~inhibit~~synergism and toxicity by~~ors such as~~ DEF, DEM and PBO were tested ~~to determine the toxicity and synergism of the enzyme inhibitors numbered~~. In addition, enzymatic activities of

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EST, GST and MFO were measured, with and without in vivo enzyme-inhibitioned insects, to verify their possible roles in insecticide detoxification.

Materials and Methods

Insects. Susceptible laboratory strains of *C. pomonella*, *G. molesta* and *L. botrana* established from individuals collected in Lleida (Spain), Piacenza (Italy), and La Rioja (Spain), respectively, have been maintained under laboratory conditions for more than 5 years without introduction of wild individuals. Larvae were reared in artificial diet (Ivaldi-Sender 1974) in a rearing room. Insects used in Lleida were maintained at 25 ± 1 °C with a 16:8 hour light:dark photoperiod, (insects used in mortality bioassays with synergists), and in Avignon at 27.5 ± 0.5 °C with a 16:8 hour light:dark photoperiod. (insects used in all enzymatic activity testss). Pupae were separated by sex and checked daily for adult emergence, except for *C. pomonella* which was sexed at the adult stage, also in a daily basis.

Insecticides and 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.)) (all from Sigma-Aldrich, Spain). The inhibitors were S,S,S tributyl phosphorotrithioate (analytical standard, 97% (a.i.)), diethyl maleate (analytical standard, 97% (a.i.)), and piperonyl butoxide (technical grade, 90% (a.i.)). All the dilutions used in bioassays were prepared from pure compound using acetone (CHROMASOLV®, for HPLC, ≥99.9%. Sigma-Aldrich, Spain) as solvent. Dilutions were stored in 2- or 4-ml acetone-rinsed glass vials at 7ºC. The same stock of acetone used to prepare the dilutions was also used as the negative control treatment.

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Mortality Bioassays with Synergists. Newly emerged adults were separated from the pupal cages every day and received the insecticide/inhibitor treatments during the first half of the photophase at 0 to 24 hours post-emergence. Adults were placed individually or in pairs in 10-ml test tubes ~~and~~ where they received a brief (10 seconds) flow of industrial grade CO_2 ~~which quickly~~to be anesthetized ~~them~~. Immediately after being anesthetized they were placed upside down under ~~the field of view of~~ a stereo microscope. 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), and they were transferred immediately to a 150 ml polypropylene non-sterile clinical sample bottle (57 mm diameter x 73 mm-high). Individuals receiving the same treatment were placed in groups of 3 to 10 in the same bottle. The lid of the bottle was punctured to make 10 holes (1-mm-diameter each) to allow gas exchange, and a 1.5 ml eppendorf containing 10% sugar solution and a cotton plug was placed on the bottom to supply nutrients during the observation period. Bottles with ~~treatment~~treated~~ment~~ insects were placed in the rearing room.

Mortality was recorded at 24 h and 48 h post-treatment. Adults were observed with the naked eye and scored as alive if they flew or walked apparently unaffected, as moribund if they could barely walk or were laying on the bottom of the bottle but still moved, or as dead if they laid immobile on the bottom of the bottle. Mortality was estimated by adding the number of moribund and dead insects.

In Table S1 we show the insecticides and synergists concentrations used. For insecticide, we used the concentration corresponding to the LC_{50} according ~~with~~ results in Navarro-Roldan et al. (2017). S~~,s~~ynergists concentrations were estimated as the highest concentration that leading to~~no had~~ no mortality ~~significant differences~~ with the solvent in a ~~pool~~ range of three to fifteen

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concentrations per synergist, species and sex, with 30 insects per concentration used in synergist-solvent pairwise comparisons (*Fisher* exact test). After synergist concentration selection, between 60 and 115 insects per treatment were used. Treatment groups [solvent, synergist, insecticide (LC_{50}), and insecticide (LC_{50}) + synergist], for each combination of species, sex, insecticide and synergist were tested (96 treatment groups). Tests were performed on groups (i.e., repetitions) of at least 3 insects of the same treatment group, with different treatments tested each day depending on insect availability, until the desired sample size was achieved.

Enzymatic Activities. The general principle for the dosage of enzymatic activities is to measure during an enzymatic reaction the ~~quantity~~ speed of ~~the~~ ~~an enzymatic reaction~~ product release, reduced by ~~of an enzymatic reaction and to divide it by the~~ total protein content in~~of the~~ each protein extract~~sample~~. The amount of product is determined by a level of absorbance or fluorescence. MFO activity was measured on fresh tissue whereas GST and EST con be dose on frozen (-80°C) one. The posterior half ~~posterior parts~~ of adult abdomens were used for~~in~~ *C. pomonella* MFO dosage~~insects~~, and the whole abdomen ~~in insects~~ of *G. molesta* and *L. botrana*. A~~The a~~bdomens were directly placed in the reaction solution. Measurement of both, GST and EST activities were performed using the ~~second (~~anterior~~)~~ half of the abdomen of *C. pomonella* and the whole abdomen in the other two species, ~~those enzymatic activities necessitated~~after a preliminary phase of protein extraction. T~~he t~~otal protein content of each sample was measured with Bradford colorimetric test using bovine serum albumin to build the standard curve (Bradford, 1976). Fluorescence and absorbance were measured using a microplate reader (Infinite 200, Tecan, Männedorf, Switzerland). ~~Insect dissections~~All protein extracts were made in adult insects of 24-48 h age post-emergence.

Mixed-Function Oxidase (MFO). The MFO activity was determined using 7-ethoxycoumarin O-deethylation (ECOD) (Ulrich and Weber, 1972) adapted for in vivo analysis in microplates. From 57-60 adult abdomens per species and sex were dissected and directly homogenised in an incubation solution containing ~~100µl~~100 µl of Hepes buffer (50 mM, pH 7) ~~with~~ and 7-ethoxycoumarin (0.4 mM) on the ice. After 4h incubation at 30 °C, the reaction was stopped by adding 100 µL of 1.~~5M~~5 M glycine buffer (pH 10.3) and centrifuged at 10~~500~~000~~15000~~ ~~r.p.m~~g for 5 min at room temperature. Supernatants were individually placed in wells of black microplates (96-wells, Corming Costar®, New York, U.S). The 7-hydroxycoumarin (7-HC) fluorescence was quantified with 380 nm excitation and 465 nm emission filters. Three wells per microplates were left without samples but just the mix receiving glycine buffer previous to incubation to have a blank. Protein dosages were made on this reaction product after fluorescence measurement ~~and dilutions were required, a~~. Before protein dosage a 5 fold dilution was done ~~was made~~ for all samples except for ~~samples of~~ *C. pomonella* females that were 10 fold diluted and no dilution for *L. botrana* males. ~~that were not diluted, after that t~~As for GST and EST method, total proteins were measured using the Bradford method (Bradford, 1976). The MFO activity was expressed as pg of 7-HC/µg of total protein/min by using a standard curve of 7-~~Hydroxycoumarine~~ (HC) (0.5-4.5 nmoles/well) to convert it in fluorescence ~~in 7 HC quantity~~.

Glutathione S-Transferase (GST). For protein extraction, anterior half abdomens in *C. pomonella* or the whole abdomen for *G. molesta* and *L. botrana* insects, were crushed in 110 µl of Hepes buffer (50 mM, pH 7) on the ice and the obtained homogenates were centrifuged at 10~~500~~000~~15000~~ ~~r.p.m~~g for 15 min at 4 °C. Supernatants were stored at -80 °C before use. When all extractions were finished, the supernatants were used as enzyme sources for reactions in a single test, per experiment, to limit handling errors (Bouvier *et al.*, 2002). GST activity was determined in transparent microplates (96-wells, Sterilin®, Newport, UK) using 2.4-dinitro-chlorobenzene (CDNB) as substrate (Nauen and Stumpf, 2002). The reaction mixture in one well consisted of 2

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μl of enzymatic extract, 198μl of a solution containing: 10 μl of 50 mM glutathione (GSH), 185

μl of Hepes buffer (50 mM, pH 7.0) and 3 μL of 50 mM CDNB. Three wells per microplate were

filled with 2 μl of Hepes buffer (50 mM, pH 7.0) instead of enzyme extract as blank. Absorbance

was measured, after 2 min of incubation at 25 °C, in kinetic mode every 30 seconds at 340 nm.

Since the CDNB-197glutathione-glutathione adduct conjugate was not commercially available,

we were unable to build a standard curve, so we used the molar extinction coefficient (9.6 mM^{-1}

x cm^{-1}) of CDNB-glutathione to convert absorbance in μmol of CDNB-glutathione. The final

specific activity was expressed in μmol of CDNB-glutathione/min/mg of total protein extracted.

Between 55-60 insects per species and sex were used.

Carboxylesterases (EST). The same protein extracts were used as for GST. Total non-specific

EST activity was measured with α-naphthyl acetate (α-NA) as substrate (Reyes, 2007). The

reaction mixture was 1 μl of protein extract and 194 μl of 30 μM α-NA in Hepes buffer (50 mM,

pH 7.0) in each microplate well. After 20 min of incubation at 30 °C in darkness, the reaction

was stopped and coloured by adding 55 μl of 0.2 % Fast Garnet GBC diluted in 2.5 % sodium

dodecyl sulphate solution. Absorbance was recorded at 590 nm, after incubation for 20 min in

darkness at room temperature. The standard curve with α-Naphtol (0-18 nmoles/well) was

elaborated to express activity in nmoles of product/min/mg of total proteins. Between 55-59

insects per species and sex were used.

Enzymatic Activities Inhibition and MFO Time Inhibition. After 0 to 24 hours post-

emergence adults received the inhibitor treatments and remained stored , both in same conditions

than t were explained in mortality bioassays. After 24h post-treatment. EST and GST extracts

were made fromor insect treated with DEF and DEM insect respectively treated respectively (17-

40 individuals per treatment), using the same methodology as explained for these enzymatic

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1 activities dosage. MFO extracts from PBO treated insects (16-25 individuals per treatment), were

2 made after 1h ~~post-treatment~~of exposure, except in the PBO inhibition time assay in which

3 extracts were made immediately post-treatment or with a time-elapsed of 0.5, 1, 2, 4, 12 or 16 h

4 post-treatment (10-35 individuals per treatment~~)~~.), plus one acetone control at 1h post-treatment.

5 *C. pomonella* insects were not used in this PBO inhibition time assay. MFO methodology was

6 the same as explained in enzymatic activities section.

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8 **Data Analysis.** All the statistical analyses were run in R software (R Core Team 2016). Analyses

9 were performed with generalized linear models (GLM), using Gaussian family functions for

10 continuous variables (enzymatic activities) and binomial family functions for binomial variables

11 (percentage of mortality). The glht() and/or the predictmeans() functions performed *Tukey´s*

12 multiple pairwise comparisons. Observed parameter means and their standard errors are shown

13 in tables and figures. Raw data and R scripts are available online (Repositoty UdL). Whenever

14 the term "significant" is used in the text regarding differences between treatments it indicates a

15 p-value < 0.05.

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17 <p align="center">**Results**</p>

18 **Bioassay ~~S~~withynergism of Enzyme Inhibitors ~~on the Susceptibility to Insecticides~~.** ~~P~~The

19 ~~p~~ercentage of mortality withfor enzyme inhibitors acting as insecticide synergists are shown in

20 Table 1, and their mortality ratios in Table S2. 35 significant differences of 54 total possibilities

21 were found i~~I~~n ~~comparisons made to assess the~~ synergistic effect of enzymatic inhibitor

22 regarding the mortality due to~~effect of~~ insecticide ~~alone~~ (LC_{50})~~, 35 significant differences were~~

23 ~~found, of 54 total possibilities~~. DEF, the EST inhibitor, is the synergist that provide more

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significant differences, only *L. botrana* females and *C. pomonella* males, both treated with

thiacloprid did not show significant mortality differences regarding to thiacloprid LC$_{50}$

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treatment. All these inhibition conduce to insecticide over-susceptibility, showing a correlation

between decreases of EST activities decrease and lessreduce protection against the three

insecticides tested. DEM only shown synergistic effect in *G. molesta* insects, except in females

treated with λ-cyhalothrin. PBO treatment shown the same pattern for all species and sexes

;PBO shown significant differences an enhanced mortality in all treatments when treated with

Thiacloprid ; a decrease of mortality occur with chlorpyrifos treatment ; and no mortality

modification with except in adults of *C. pomonella*, females of *L. botrana* and males of *G.*

molesta, all of them treated with λ-cyhalothrin. Particularly, all adults inhibited with PBO and

treated with chlorpyrifos shown a significant reduction of their mortality compared with

mortality provided by chlorpyrifos LC$_{50}$ treatments. lambda cyhalothrin treatment. Stronger

effects are observed after PBO treatment, for example *G. molesta* male became ten times fold

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more susceptible to chlorpyrifos and *C. pomonella* male became five times fold less susceptible

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to thiacloprid. *G. molesta* female and *L. botrana* males are two exception showing tiny synergic

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effect between PBO and lambda-cyalothrin (1.46 and 1.56 respectively). As bewas expected, the

synergists alone did not provide significant changes in mortality compared with solvents alone in

any of the possible combinations (species, sex and enzymatic inhibitor type).

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Enzymatic activities. Measurements of EST, GST and MFO on abdomens of susceptible males

and females from *C. pomonella*, *G. molesta* and *L. botrana* adult insects are shown in Figure 1.

Comparisons between groups (species and sex) shown that *L. botrana* females had the highest

levels of the three enzymatic activity groups, that were no different from *L. botrana* male in EST

and *G. molesta* in GST. Analysis of EST shown significant differences between species but no

between sexes in same species. In case of GST, differences between sexes were found for *L. botrana* insects, besides females of *C. pomonella* had significant less GST activity than the other two species, whereas males of *G. molesta* had high GST activity than *C. pomonella* males. MFO results shown that *C. pomonella* had the lowest male activity, *L. botrana* males had significant differences in MFO activity compared with *C. pomonella* insects, and *L. botrana* (as commented above) had the highest activity from all species-sex groups in MFO comparisons. Females of *L. botrana* have an activity 2.5 time higher than *C. pomonella* ones.

Inhibition of Enzymatic Activities. Measurements of three enzymatic activity groups after being inhibited are summarized in Table 2. Results for our laboratory test conditions shown significant differences in EST inhibition by DEF in all species-sex groups and significant differences in GST for *G. molesta* males. Enzymatic activity inhibition ratio (for significant differences commented), revealed that EST were inhibited in all cases by DEF, obtaining ratios ranging between 2.78 in *G. molesta* females and 15.75 in *L. botrana* males, whereasbut DEM increased the GST activity in *G. molesta* males with a ratio of 0.46. With the dosing method choose in this study Eenzymatic inhibitors did not appear to affect in rest of tested cases.

MFO Inhibition Throughout Time. Inhibition of MFO by PBO inhibitor was tested at different hours (Figure 2) in males and females of *G. molesta* andan *L. botrana*. A significant reduction of MFO activity was obtained at hour 4 hours after treatment compared with hour 0 in *G. molesta* females., which was not significant different with control at 1 h. In *G. molesta* males the reduction was maintained after 4 h and next (12 and 16 h). On the contrary, a significant enzymatic activity increase was observed in *L. botrana*. For females it appear after hour 4 hours,

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whereas and for males a significant 10 time increase effect for males was after hour was observed

16 2 hours after treatment (hour 0.5 had no significant differences).

Discussion

The first aim of this study was to determine the metabolic mechanisms involved in insecticide

detoxification. Synergism mortality bioassays results (Table 1) and mortality ratios (Table S2),

shown that EST played an important role in detoxification process of the three insecticides

tested. This result shows a non-specific action of these families of enzymes well known to have

transverse effects in other pest species. For example, ESTs are able of sequestering various

xenobiotic molecules thus preventing them from coming into contact with their molecular target

in *Myzus persicae*. This none-specific mechanism confers resistances to a broad spectrum of

insecticide (Devonshire, 1982). Finding this mechanism for all species and sexes suggests that it

is easily adopted. It would therefore be a cost-effective mechanism for the organism. It has

already been described that the increase in EST activity was due to gene amplifications

(Hemingway, J. 2000). Faucon observed in his work on the genomics of resistance to pyrethroids

in mosquitoes the importance of this evolutionary mechanism. Indeed he found that 41 genes

affected by gene amplifications were linked to deltamethrin resistance, so he hypothesizes that

this evolutionary mechanism is advantageous. By the way, the limit of this generalist mechanism

is clearly linked to the median level of resistance it confers in comparison with the MFO (Table

S2). This must be kept in mind that the strains tested in our studies are susceptible to

insecticides. In all proportion, observed phenomena may be exacerbated in resistant strains.

GST only seemed to be active in adult insects of *G. molesta*, in both sexes for the detoxification

of chlorpyrifos and thiacloprid, and in males detoxification of λ-cyhalothrin. According with our

enzymatic inhibition results, phase I enzymatic activities were important in both sexes of three

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species, whereas phase II enzymes only were important in *G. molesta.* This enzymatic family is known to be less often involved in the detoxification of insecticides. In a mini-review dealing with detoxification mechanisms of lepidopteran pests on 92 referenced cases, only 36% of the cases are attributed even partially to GSTs, compared with 63% for ESTs and 64% for MFOs (Navarro, in prep.).

A completely different profile appears with MFO families, with highly contrasted effects (Table S2). chlorpyrifos and λ-cyhalothrin and MFOThere are involved in a higher level than EST in detoxification process of thiacloprid and in activation of Chlorpyrifos in all species and sexes both sexes of three species. Their involvement in the detoxification of λ-cyhalothrin is less pronounced with small effects in *L. botrana* males and females of *G. molesta.* Again, these results are in perfect agreement with what is observed in other species. An entire paragraph is devoted to the bio-activation of organophosphates by MFOs in the review of M. Feyereisen (1999). Besides, EST were involved in detoxification of thiacloprid in both sexes of *G. molesta,* males of *L. botrana* and females of *C. pomonella,* meanwhile MFO could detoxify λ-cyhalothrin in females of *G. molesta* and males of *L. botrana.* Curiously, MFO were active in both sexes of three species with chlorpyrifos, but in these case MFO enzymes changing the nontoxic insecticide precursor in a toxic active ingredient, acting as an MFO action conduce to complex chemical reaction in which can lead to activation or detoxification (Levi, 1988)antagonist but our results show surprisingly only bio activation cases. Many examples describe in the literature, the capacity of this enzymatic family to detoxify thiacloprid, starting with honey bees (Iwasa, 2004). A recent review summarizes mechanisms involved in such resistance case on aphids, whiteflies, planthoppers, coleopteran, dipteran and lepidopteran species. They conclude that the major mechanisms are target site mutation and MFO detoxification (Bass, 2015). The innovative part of our results is the comparison between three species of Lepidoptera pests done on sexed individuals. At this level of analysis, this parallel demonstrates the none-specificity of the MFO

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between species but between insecticides. However, a closer analysis by observing the kinetics of inhibition by PBO gives a completely different picture of what is happening.

GST only seemed to be active in adult insects of *G. molesta*, in both sexes detoxification of chlorpyrifos and thiacloprid, and in males detoxification of λ-cyhalothrin. According with our enzymatic inhibition results, phase I enzymatic activities were important in both sexes of three species, whereas phase II enzymes only were important in *G. molesta*.

The possibilities of metabolic mechanisms involved in detoxification of insecticides are so variable, as we could see in Table S3, which list different examples of important worldwide pest with described cases of metabolic resistance to insecticides, including our study species and other Lepidopterans. *L. botrana* is poorly represented in these kind of metabolic studies because had few reported cases of resistance, as we know Civolani et al, 2014, described the only resistance case in this species. In Table S3, for susceptible strains we could see that, EST alone was the main mechanism of pyrethroids metabolism in larvae of *Cydia pomonella* (Sauphanor et al, 1997), female adults of *Agrotis ipsilon* (Hufnagle) and adults of *Helicoverpa zea* (Boddie) (Usmani and Knowles, 2001), and in organophosphates in adults of *C. pomonella* (Reuveny and Cohen, 2004), similar as we found for chlorpyrifos in *C. pomonella* and *L. botrana*, and for λ-cyhalothrin in *C. pomonella* and females of *L. botrana*. EST alone was important in resistance to organophosphates [*Choristoneura rosaceana* (Harris) (Pree et al, 2002); *C. pomonella* (Soleño et al, 2008); *G. molesta* (Usmani and Shearer, 2001; de Lame et al, 2001); *Platynota idaeusalis* (Walker) larvae (Biddinger et al, 1996) and adults (Karoly et al, 1996)], or pyrethroids [*Helicoverpa armigera* (Hübner) (Gunning et al, 1999; Young et al, 2005); *Spodoptera littoralis* (Boised) (Riskallah, 1983)]. Among On the margin of EST, the insensitivity to acetylcholinesterase (I.AChE) seems to be an important mechanism in tolerance to organophosphates their molecular target in adults of *C. pomonella* (Reuveny and Cohen, 2004;

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Cassanelli et al, 2006), female adults of *G. molesta* (de Lame, 2001), and larvae of *H. virescens* (Hamadain and Chambers, 2001), and to carbamates in adults of *G. molesta* (Kanga et al, 2001).

We found that EST in combination with GST were mechanism that *G. molesta* used to detoxify chlorpyrifos and λ-cyhalothrin (in case of males), this metabolic combination in only used for susceptible *H. armigera* larvae in detoxifying Indoxacarb and Hexaflumuron (Vojoudi et al, 2017), and in resistance cases to the organophosphate azinphos-methyl in adults of *Epiphyas postvittana* (Walker) (Armstrong and Suckling, 1988, 1990) and larvae of *P. idaeusalis* (Karoly et al, 1996). When EST is combined with MFO, females of *G. molesta* and *C. pomonella* could detoxify λ-cyhalothrin and thiacloprid respectively, and both insecticides could be detoxified by males of *L. botrana*. EST plus MFO is used in metabolism of chlorantraniliprole by susceptible strains of *Plutella xylostella* (Wang et al, 2010), and of cypermethrin in larvae and adult males of *A. ipsilon*, and adult females of *Spodoptera frugiperda* (J.E. Smith) (Usmani and Knowles, 2001), and only in larvae of *H. zea* (Usmani and Knowles, 2001). Resistant strains of *C. pomonella* had this enzymatic combination to metabolize pyrethroids (Sauphanor et al, 1997), organophosphates (Reyes et al, 2011) and neonicotinoids like thiacloprid (Reyes et al, 2007; İşci and Ay, 2017), *H. armigera* in metabolize pyrethroids (Kranthi et al, 1997), and *S. littoralis* in carbamates (Yu et al, 2003).

Guo et al, (2017) found that the three metabolic groups of enzymatic activities were involved in detoxification of insecticides in susceptible adults of *G. molesta*, in our findings, the combination of the three groups was only used by these insects in metabolism of thiacloprid, similar case occurs with susceptible *C. rosaceana* in detoxification of chlorfenapyr and cypermethrin (Ahmad and Hollingworth, 2004). Whereas, ~~in resistance cases~~the three mechanisms were involved in detoxification of different insecticides in ~~C.~~resistant populations~~in~~ of *C. rosaceana* (Ahmad and Hollingworth, 2004), in *C. pomonella* (Voudouris et al, 2011), to organophosphates

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(Rodriguez et al, 2010; Reyes et al, 2015), or to IGRs (Reyes et al, 2011), in *G. molesta* to chlorpyrifos (Siegwart et al, 2011) and *S. frugiperda* (Yu et al, 2003).

In our results, we did not found that metabolic detoxification of insecticides were due to MFO or GST alone or combination of both, but other authors found it as a mechanisms of detoxification. In susceptible strains of *C. rosaceana*, MFO alone was involved in detoxification of Indoxacarb (Ahmad and Hollingworth, 2004) and Spinetoram (Sial and Brunner, 2011), and in larvae and adult males of *S. frugiperda* in metabolism of cypermethrin (Usmani and Knwoles, 2001). Besides, MFO generates resistance to Spinetoram in *C. rosaceana* (Sial and Brunner, 2011), in *C. pomonella* to tebufenocide (Ioriatti et al, 2007), to diflubenzuron and spinosad (Reyes et al, 2007), in *E. postvittana* to azinphos-methyl (Armstrong and Suckling, 1990), to methoxyfenozide in *S. littoralis* (Mosallanejad and Smagge, 2009), and to pyrethroids in *H. armigera* (Ahmad and McCaffery, 1991; Daly and Fisk, 1993), *H. virescens* (McCaffery et al, 1991; Martin et al, 1997), and *Ostrinia nubilalis* (Hübner) (Siegwart et al, 2017). GST alone in metabolism of azinphos-methyl in *C. rosaceana* susceptible strain (Ahmad and Hollingworth, 2004), and λ-cyhalothrin in adults of *C. pomonella* (Liu et al, 2014), and in resistance to pyrethroids in a resistant strain of *O. nubilalis* (Siegwart et al, 2017). And combination of both (MFO + GST) in metabolism of tebufenozide in susceptible and resistant *C. rosaceana* (Waldstein and Reissing, 2000) and diazinon in susceptible *P. xylostella* (Takeda et al, 2006), and in resistant *C. pomonella* to organophosphates (Reyes et al, 2007, 2009; Rodriguez et al, 2010).

Evidence of the agonist effect of MFO in chlorpyrifos that we found for our three species in male and female adults was found too for *Blattella germanica* (L.) (Valles et al, 1997), for susceptible larvae of *C. rosaceana* (Ahmad and Hollingworth, 2004), for adults of *Drosophila melanogaster* Meigen (Willoughby et al, 2007), and for larvae of *Amyelois transitella* (Walker) (Demkovich et

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al, 2015). In these cases, in absence of PBO, MFO made the bioactivation of P=S compounds into P=O analogs, which are the insecticide active form that binds more tightly to AChE, thus AChE is inhibited and cause a mortality increase in insects (Feyereisen, 1999; Yu 2015). However, in presence of PBO, the MFO are readily inhibited and the insecticide activation is reduced or suppressed, then the mortality of insects decreases (Metcalf 1967).

While the enzymatic activity inhibition tests allow knowing the enzymatic mechanisms involved in detoxification processes of a specific species (Bingham et al, 2008), the comparisons in enzymatic activities amount and metabolic studies using synergists, between susceptible and resistant strains in same species, could determine the enzymatic groups that lend the resistant condition (Scott, 1990). As we could see in Table S3, sometimes an enhancement of enzymatic activities that are metabolic active in susceptible strains are the cause of resistance, i.e., in metabolism of tebufenozide by larvae of *C. rosaceana* (Waldstein and Reissing, 2000), or to spinetoram (Sial and Brunner, 2011). But in other cases the resistant condition is owing to an enhancement of enzymatic activities that are not involved in detoxification mechanisms in the susceptible strain, i.e., in metabolism of deltamethrin in larvae of *C. pomonella* (Sauphanor et al, 1997), or chlorantraniliprole in larvae of *P. xylostella* (Wang et al, 2010). As well as the mechanism of resistance could be different between life stages (Armstrong and Suckling, 1990; Karoly et al, 1996; Yu et al, 2003; Rodriguez et al, 2010), or for the same species resistant to the same insecticides in different parts of the world (Reyes et al, 2007, 2009) or different geographic areas (Karuppaiah et al, 2017).

It is clear that the degree of synergism in a particular association of insecticide and synergist often varies from one insect species to the next (B-Bernard and Philogène, 1993). Fact that we could see in our enzymatic activity inhibition results among our tested species, which partially could explains the toxicity differences found among the combination between insecticide-species

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Comentado [MÁNR34]: we will reduce this part and delete the S3 table, because will be used in a short review

Comentado [MS35]: For me this part could be entirely suppress and directly use as a mini review, by adding a little more critical point of view.

Comentado [MS36]: For the mini-review too

(Navarro-Roldán et al, 2017), still we could not explain whole differences found between species and between sexes within species. Consequently, next step was to determine if the amounts of enzymatic activities are related to differences observed in toxicity assays.

At first sight seems that there is no correlation between toxicity results and the ~~amounts~~ level of enzymatic activities ~~measurement~~ (correlations not shown). Only thiacloprid and MFO activity correlation shows a good fit (Pearson coeff. ~~Figure~~ S3), which implies that MFO is the main mechanism in thiacloprid detoxification. Similar results were found in~~to~~ C. pomonella adults (Reyes et al, 2007), and in combination with EST in larvae (İşci and Ay, 2017). Besides, amounts of enzymatic activities (Figure 1), shows that the highest quantity of MFO was for L. botrana females, which was the most tolerant group for thiacloprid (Navarro-Roldán et al, 2017). Moreover, just we found sex differences in the quantification of GST and MFO activities for adults of L. botrana, having females higher amounts of both activities (Figure 1). Taking into account that GST are not a metabolic mechanism of detoxification for tested insecticides in this species (Table 1), only MFO could explains sex differences found for thiacloprid in L. botrana. This conclusion goes in the same way as the previous observation done on inhibitor on mortalities.

In case of the other two insecticides, correlations probably did not work because the combinations and interactions of metabolic mechanisms involved in detoxification had more relative importance than the mechanisms studied by separate way, or as suggest Ahmad and Hollingworth (2004) and Kang et al, (2006), the slight unspecific or multiple target of enzyme inhibitors to a single enzymatic activity group, that could interfere on results.

~~Other~~ ~~questioning~~ ~~aspect could be if~~We wanted to verify the inhibition of enzymatic activities ~~were inhibited correctly~~by synergist~~, in.~~ ~~i~~In Table 2 we could see that EST were strongly inhibited after 24h of ~~by~~ DEF exposure, although we cannot see MFO and GST ~~were not~~

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inhibit~~ioned~~ by PBO and DEM respectively at the tested time~~.~~, I~~i~~n fact, we even observe the

opposite result with~~we could see~~ a significant GST induction in males of *G. molesta*. Induction

of enzymatic activities by an enzymatic inhibitor was observed too in *D. melanogaster*

(Willoughby et al, 2007). These unexpected results ~~lead to think that in our species the inhibition~~

~~occurs, but probably not at the~~raise the question of exposure time needed to cause enzymatic

inhibition. May be ~~when we made the enzymatic measure, moment when~~ the enzymatic

activities ~~could be~~were restored to their normal rates, when we measured it. ~~s~~Similar case was

observed ~~that~~ with EST recovering after inhibition by PBO during a~~n~~t 24h-period ~~for~~in *H.*

armigera (Young et al, 2005) or *Bemisia tabaci* Gennadius (Young et al, 2006).

~~As we could see in Figure 2, the~~Indeed, kinetic inhibition of MFO activity after one PBO

treatment (Figure 2) ~~was~~shows variations~~no~~not ~~inhibited by PBO equally~~ across the time. If in

G. molesta a limited inhibition is observed 4 h after exposure, the opposite effect occur in *L.*

botrana. Indeed, MFO activity increases up to ten times after 16 hours of PBO exposure in

males. We therefore imagine that we are experiencing a phenomenon of gene induction. The

arguments in favor of this statement are, the effect appears after a relatively long period of time

suggesting a biological rather than a chemical reaction chain, and, a study in 2007 shows an

induction of a Cyp6A2 gene, among other MFO genes, more than 32-fold by PBO in

Drososphila suzukii (Willoughgy, 2007). ~~, and t~~These results could explain the lack of~~negative~~

~~results in~~ inhibition ~~of~~by PBO ~~shown in Table 2~~primarily measure. Results on enzymatic

activity changes across the time (Figure 2) bring us to think that another possibility in sex

differences might be the different combinations of inhibition and/or induction of enzym~~es~~~~atie~~

~~activities~~ that we saw, like inhibition after 4h in *G. molesta* males and no remained effect in

females, as well as different enzymatic activity enhanced in males and females of *L. botrana*.~~.~~

Comentado [BD39]: What was shown with

Comentado [BD40]: ????

Comentado [MÁNR41]: indicar mas claro que existe una recuperación

Comentado [MS42]: OK with that but perhaps the reader will be a little confused because we always said that PBO is a MFo inhibitor, but not EST ones. Perhaps you can find another example with DEM on GST or PBO on MFO ?

Con formato: Fuente: Cursiva

Con formato: Fuente: Cursiva

Comentado [MS43]: Willoughby, L., Batterham, P. & Daborn, P. J. Piperonyl butoxide induces the expression of cytochrome P450 and glutathione S-transferase genes in Drosophila melanogaster. Pest Manag Sci 63, 803-808, doi:10.1002/ps.1391 (2007).

Con formato: Fuente: Cursiva

Comentado [BD44]: De 8 tests amb les dos sp només en dos casos no ha respost, els dos en lambda, tampoc podem dir que sigui un resultat molt negatiu.
Aquests resultats ens donen a entendre que 1 h després de l'aplicació del sinergista, momento en que es va fer l'estracte enzimàtic en el nostre estudi, el sistema enzimàtic de l'insecte no estaba inhibit pel sinergista

Comentado [BD45]: Effect of the inhibitor we saw, inhibition after 4 h in males of grafolita and no effect on females and increasing of the enzymatic activity in males and females of lobesia botrana

Con formato: Fuente: Cursiva

Con formato: Fuente: Cursiva

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Focusing on the bigger susceptibility sex differences to organophosphates, Shearer and Usmani (2001) and Navarro-Roldán et al, (2017), reported that adult males of *G. molesta* were more tolerant to this insecticide group than females. de Lame et al., (2001) indicate that the larger tolerance observed may be linked to larger acetylcholinesterase (AChE) and general EST levels in males than in females, but on the opposite we did not found significant differences in amounts of EST between sexes and, for that reason we could not conclude that EST were the explanation for our sex differences.

Nevertheless, we found that the three major enzymatic groups were involved on metabolism of chlorpyrifos, EST and GST as insecticide synergists metabolizer and MFO as insecticide agonist activator (Table 1). Besides Probably, for better understanding of these toxicity differences between sexes we had to study which enzymes would presented a higher activity in both sexes along the time after the insecticide treatment was applied, like in Parra Morales et al, (2017). Besides, in MFO inhibition by PBO we found a time effect (Figure 2) that could be the cause of susceptibility sex differences found in Navarro-Roldán et al, (2017). Figure 2 shows different effects of MFO after PBO treatment across time, in case of *G. molesta* we could see no significant inhibition of MFO (except at 4h) in females, and a significant inhibition at 4h and next onwards in males, whereas in *L. botrana* a case of enzyme induction was found in females (at 12h) and in males (at 12 and 16h). Remembering that MFO These results are chlorpyrifos agonist, only the significant bigger induction of MFO in *L. botrana* males could explain the major susceptibility to chlorpyrifos by males in *L. botrana* case. In *G. molesta* females levels very relevant for the use of MFO were maintained, this fact lead us synergists to hypothesize that there was a MFO inhibition like in males but in females there was a compensation and a little induction could occur, if this hypothesis is tested could explain the major susceptibility to chlorpyrifos in females study the enzymatic mechanisms of *G. molesta* resistance.

Yet, as we could achieve, the enzymatic inhibition depends on multiple factors like the insect species or treatment time, but others could be included like synergism cross inhibition, it means that more than one target for PBO, DEF or DEM might exist in the insects, besides the traditional target of the enzyme inhibitors (Wu et al. 2007). In addition, our recommendation in enzymatic inhibition assays is to cover a wide range of hours to know exactly how the inhibitor works on the enzymatic activity. Our finding of MFO induction by PBO in L. botrana raises the important question of the use of this synergists in viticulture. Development of more specific inhibitors targeting specialize MFO gene as CYP6 family could be the way to explore to replace this long way used synergist.

Further investigations about the role of other insecticide defence mechanisms in our susceptible species are needed to improve our conclusions, at least in detoxification of chlorpyrifos and λ-cyhalothrin. In addition, genetic studies are needed to validate the identification of the physiological factors involved in insecticide tolerance, similar that Armstrong and Suckling (1990) conclude for identifications in resistance cases.

Finally, careful considerations must be given to the pretreatment time, if enzymatic inhibitors want to be considered as insecticide pretreatments in practical applications in field conditions. In addition, our results in laboratory conditions could be very different in field, in which we have many "metabolic enzyme inducers" that could modify the amount of enzymatic activities that an insect could have without presence of such a "stressor" compounds (i.e., Yang et al, 2001; Yu, 2004; Després et al, 2007; Poupardin et al, 2008; Xie et al, 2011; Deng et al, 2016; Para Morales et al, 2017).

References Cited

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

Ahmad, M., & McCaffery, A. R. (1991). Elucidation of detoxication mechanisms involved in resistance to insecticides in the third instar larvae of a field-selected strain of *Helicoverpa armigera* with the use of synergists. Pesticide Biochemistry and Physiology, 41(1), 41-52.

Armstrong, K. F., & Suckling, D. M. (1988). Investigations into the biochemical basis of azinphosmethyl resistance in the light brown apple moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae). Pesticide biochemistry and physiology, 32(1), 62-73.

Armstrong, K. F., & Suckling, D. M. (1990). Correlation of azinphosmethyl resistance with detoxication enzyme activity in the light brown apple moth *Epiphyas postvittana* (Lepidoptera: Tortricidae). Pesticide Biochemistry and Physiology, 36(3), 281-289.

B-Bernard, C., & Philogène, B. J. (1993). Insecticide synergists: role, importance, and perspectives. Journal of Toxicology and Environmental Health, Part A Current Issues, 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:

24

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.

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.

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.

Cassanelli, S., Reyes, M., Rault, M., Manicardi, G. C., & Sauphanor, B. (2006). Acetylcholinesterase mutation in an insecticide-resistant population of the codling moth *Cydia pomonella* (L.). Insect biochemistry and molecular biology, 36(8), 642-653.

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(3), 1245-1249.

Daly, J. C., & Fisk, J. H. (1993). Expression of pyrethroid resistance in adult *Helicoverpa armigera* (Lepidoptera: Noctuidae) and selective mortality in field populations. Bulletin of entomological research, 83(01), 23-28.

de Lame, F.M., J.J. Hong, P.W. Shearer, and L.B. Brattsten. 2001. Sex-related differences in the tolerance of Oriental fruit moth (*Grapholita molesta*) to organophosphate insecticides. Pest Manag. Sci. 57(9):827-832.

1   Demkovich, M., Dana, C. E., Siegel, J. P., & Berenbaum, M. R. (2015). Effect of piperonyl

2   butoxide on the toxicity of four classes of insecticides to navel orangeworm (*Amyelois*

3   *transitella*)(Lepidoptera: Pyralidae). Journal of economic entomology, 108(6), 2753-

4   2760.

5   Deng, Z. Z., Zhang, F., Wu, Z. L., Yu, Z. Y., & Wu, G. (2016). Chlorpyrifos-induced hormesis

6   in insecticide-resistant and-susceptible *Plutella xylostella* under normal and high

7   temperatures. Bulletin of entomological research, 106(03), 378-386.

8   Després, L., David, J. P., & Gallet, C. (2007). The evolutionary ecology of insect resistance to

9   plant chemicals. Trends in Ecology & Evolution, 22(6), 298-307.

10   Feyereisen, R. (1999). Insect P450 enzymes. Annual review of entomology, 44(1), 507-533.

11   Gunning, R. V., Moores, G. D., & Devonshire, A. L. (1999). Esterase inhibitors synergise the

12   toxicity of pyrethroids in Australian *Helicoverpa armigera* (Hübner)(Lepidoptera:

13   Noctuidae). Pesticide Biochemistry and Physiology, 63(1), 50-62.

14   Guo, Y., Chai, Y., Zhang, L., Zhao, Z., Gao, L. L., & Ma, R. (2017). Transcriptome analysis and

15   identification of major detoxification gene families and insecticide targets in *Grapholita*

16   *molesta* (Busck)(Lepidoptera: Tortricidae). Journal of insect science (Online), 17(2).

17   Hamadain, E. I., & Chambers, H. W. (2001). Susceptibility and mechanisms underlying the

18   relative tolerance to five organophosphorus insecticides in tobacco budworms and corn

19   earworms. Pesticide Biochemistry and Physiology, 69(1), 35-47.

20   Ioriatti, C., Tasin, M., Charmillot, P. J., Reyes, M., & Sauphanor, B. (2007). Early detection of

21   resistance to tebufenozide in field populations of *Cydia pomonella* L.: methods and

22   mechanisms. Journal of applied entomology, 131(7), 453-459.

26

Ishaaya, I. (1993). Insect detoxifying enzymes: their importance in pesticide synergism and resistance. Archives of insect biochemistry and physiology, 22(1-2), 263-276.

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. Ann. Zoolog. Ecol. Anim. 6:337–343.

Kang, C. Y., Wu, G., & Miyata, T. (2006). Synergism of enzyme inhibitors and mechanisms of insecticide resistance in *Bemisia tabaci* (Gennadius)(Hom., Aleyrodidae). Journal of Applied Entomology, 130(6-7), 377-385.

Kanga, L. H. B., Pree, D. J., Plapp, F. W., & Van Lier, J. L. (2001). Sex-linked altered acetylcholinesterase resistance to carbamate insecticides in adults of the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae). Pesticide Biochemistry and Physiology, 71(1), 29-39.

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.

Karuppaiah, V., Srivastava, C., & Subramanian, S. (2017). Variation in insecticide detoxification enzymes activity in *Spodoptera litura* (Fabricius) of different geographic origin. Journal of Entomology and Zoology Studies, 5(3): 770-773

27

Kranthi, K. R., Armes, N. J., Rao, N. G., Raj, S., & Sundaramurthy, V. T. (1997). Seasonal dynamics of metabolic mechanisms mediating pyrethroid resistance in *Helicoverpa armigera* in central India. Pest Management Science, 50(2), 91-98.

Li, X., Schuler, M. A., & Berenbaum, M. R. (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. Annu. Rev. Entomol., 52, 231-253.

Liu, J., Yang, X., & Zhang, Y. (2014). Characterization of a lambda-cyhalothrin metabolizing glutathione S-transferase CpGSTd1 from *Cydia pomonella* (L.). Applied microbiology and biotechnology, 98(21), 8947-8962.

Martin, S. H., Ottea, J. A., Leonard, B. R., Graves, J. B., Burris, E., Micinski, S., & Church, G. E. (1997). Effects of selected synergists on insecticide toxicity tobacco budworm (Lepidoptera: Noctuidae) in laboratory and field studies. Journal of economic entomology, 90(3), 723-731.

McCaffery, A. R., Gladwell, R. T., El-Nayir, H., Walker, C. H., Perry, J. N., & Miles, M. J. (1991). Mechanisms of resistance to pyrethroids in laboratory and field strains of *Heliothis virescens*. Southwestern Entomologist Suppl, 15, 143-158.

Metcalf, R. L. (1967). Mode of action of insecticide synergists. Annual review of entomology, 12(1), 229-256.

Mosallanejad, H., & Smagghe, G. (2009). Biochemical mechanisms of methoxyfenozide resistance in the cotton leafworm *Spodoptera littoralis*. Pest management science, 65(7), 732-736.

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.

28

1 Navarro-Roldán, M.A., Avilla, J., Bosch, D., Valls, J., and Gemeno, C. (2017). Comparative

2   effect of three neurotoxic insecticides with different modes of action on adult males and

3   females of three tortricid moth pests. J Econ Entomol, tox113. doi: 10.1093/jee/tox113

4 Parra Morales, L. B., Alzogaray, R. A., Cichón, L., Garrido, S., Soleño, J., & Montagna, C. M.

5   (2017). Effects of chlorpyrifos on enzymatic systems of *Cydia pomonella* (Lepidoptera:

6   Tortricidae) adults. Insect science, 24, 455-466.

7 Poupardin, R., Reynaud, S., Strode, C., Ranson, H., Vontas, J., & David, J. P. (2008). Cross-

8   induction of detoxification genes by environmental xenobiotics and insecticides in the

9   mosquito *Aedes aegypti*: impact on larval tolerance to chemical insecticides. Insect

10   biochemistry and molecular biology, 38(5), 540-551.

11 Pree, D. J., Whitty, K. J., Bittner, L. A., & Pogoda, M. K. (2003). Mechanisms of resistance to

12   organophosphorus insecticides in populations of the obliquebanded leafroller

13   *Choristoneura rosaceana* (Harris)(Lepidoptera: Tortricidae) from southern Ontario. Pest

14   management science, 59(1), 79-84.

15 Reuveny, H., & Cohen, E. (2004). Evaluation of mechanisms of azinphos-methyl resistance in

16   the codling moth *Cydia pomonella* (L.). Archives of insect biochemistry and physiology,

17   57(2), 92-100.

18 Reyes, M. (2007). La résistance aux insecticides chez le carpocapse des pommes: mécanismes,

19   détection et variabilité géographique. PhD, University of Avignon. Avignon.

20 Reyes, M., Barros-Parada, W., Ramírez, C. C., & Fuentes-Contreras, E. (2015).

21   Organophosphate resistance and its main mechanism in populations of codling moth

22   (Lepidoptera: Tortricidae) from central Chile. Journal of economic entomology, 108(1),

23   277-285.

1 Reyes, M., Franck, P., Charmillot, P. J., Ioriatti, C., Olivares, J., Pasqualini, E., & Sauphanor, B.

2   (2007). Diversity of insecticide resistance mechanisms and spectrum in European

3   populations of the codling moth, *Cydia pomonella*. Pest management science, 63(9), 890-

4   902.

5 Reyes, M., Franck, P., Olivares, J., Margaritopoulos, J., Knight, A., & Sauphanor, B. (2009).

6   Worldwide variability of insecticide resistance mechanisms in the codling moth, *Cydia*

7   *pomonella L.* (Lepidoptera: Tortricidae). Bulletin of entomological research, 99(04), 359-

8   369.

9 Riskallah, M. R. (1983). Esterases and resistance to synthetic pyrethroids in the Egyptian cotton

10   leafworm. Pesticide Biochemistry and Physiology, 19(2), 184-189.

11 Rodríguez, M. A., Bosch, D., Sauphanor, B., & Avilla, J. (2010). Susceptibility to

12   organophosphate insecticides and activity of detoxifying enzymes in Spanish populations

13   of *Cydia pomonella* (Lepidoptera: Tortricidae). Journal of economic entomology, 103(2),

14   482-491.

15 Sauphanor, B., Cuany, A., Bouvier, J. C., Brosse, V., Amichot, M., & Bergé, J. B. (1997).

16   Mechanism of Resistance to Deltamethrin in *Cydia pomonella* (L.)(Lepidoptera:

17   Tortricidae). Pesticide biochemistry and physiology, 58(2), 109-117.

18 Scott, J. G. (1990). Investigating mechanisms of insecticide resistance: methods, strategies, and

19   pitfalls. In Pesticide resistance in arthropods (pp. 39-57). Springer US.

20 Shearer, P. W., & Usmani, K. A. (2001). Sex-related response to organophosphorus and

21   carbamate insecticides in adult Oriental fruit moth, *Grapholita molesta*. Pest management

22   science, 57(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(3), 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.

Siegwart, M., Thibord, J. B., Olivares, J., Hirn, C., Elias, J., Maugin, S., & Lavigne, C. (2017). Biochemical and Molecular Mechanisms Associated With the Resistance of the European Corn Borer (Lepidoptera: Crambidae) to Lambda-Cyhalothrin and First Monitoring Tool. Journal of Economic Entomology, tow267.

Soleño, J., Anguiano, L., de D'Angelo, A. P., Cichón, L., Fernández, D., & Montagna, C. (2008). Toxicological and biochemical response to azinphos-methyl in *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) among orchards from the Argentinian Patagonia. Pest management science, 64(9), 964-970.

Takeda, T., Nakamatsu, Y., & Tanaka, T. (2006). Parasitization by *Cotesia plutellae* enhances detoxifying enzyme activity in *Plutella xylostella*. Pesticide biochemistry and physiology, 86(1), 15-22.

Terriere, L. C. (1984). Induction of detoxication enzymes in insects. Annual review of entomology, 29(1), 71-88.

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.

Usmani, K. A., & Shearer, P. W. (2001). Susceptibility of male oriental fruit moth (Lepidoptera: Tortricidae) populations from New Jersey apple orchards to azinphosmethyl. Journal of economic entomology, 94(1), 233-239.

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.

Valles, S. M., Koehler, P. G., & Brenner, R. J. (1997). Antagonism of fipronil toxicity by piperonyl butoxide and S, S, S-tributyl phosphorotrithioate in the German cockroach (Dictyoptera: Blattellidae). Journal of Economic Entomology, 90(5), 1254-1258.

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.

Voudouris, C. C., Sauphanor, B., Franck, P., Reyes, M., Mamuris, Z., Tsitsipis, J. A., ... & Margaritopoulos, J. T. (2011). Insecticide resistance status of the codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) from Greece. Pesticide Biochemistry and Physiology, 100(3), 229-238.

Waldstein, D. E., & Reissig, W. H. (2000). Synergism of tebufenozide in resistant and susceptible strains of obliquebanded leafroller (Lepidoptera: Tortricidae) and resistance to new insecticides. Journal of economic entomology, 93(6), 1768-1772.

Wang, X., Li, X., Shen, A., & Wu, Y. (2010). Baseline susceptibility of the diamondback moth (Lepidoptera: Plutellidae) to chlorantraniliprole in China. Journal of Economic Entomology, 103(3), 843-848.

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(5), 500-510.

Xie, W., Wang, S., Wu, Q., Feng, Y., Pan, H., Jiao, X., Zhou, L., Yang, X., Fu, W., Teng, H., Xu, B. and Zhang, Y. (2011), Induction effects of host plants on insecticide susceptibility and detoxification enzymes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). Pest. Manag. Sci., 67: 87–93. doi:10.1002/ps.2037

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 twospotted 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.

1   Yu, S. J. (2004). Induction of detoxification enzymes by triazine herbicides in the fall

2   armyworm, *Spodoptera frugiperda* (JE Smith). Pesticide Biochemistry and Physiology,

3   80(2), 113-122.

4   Yu, S.J. (2015). Mode of action of insecticides. In The toxicology and biochemistry of

5   insecticides (133-174). Boca Ratón, FL: CRC press.

6   Yu, S. J., & Hsu, E. L. (1993). Induction of detoxification enzymes in phytophagous insects: role

7   of insecticide synergists, larval age, and species. Archives of Insect Biochemistry and

8   Physiology, 24(1), 21-32.

9   Yu, S. J., Nguyen, S. N., & Abo-Elghar, G. E. (2003). Biochemical characteristics of insecticide

10   resistance in the fall armyworm, *Spodoptera frugiperda* (JE Smith). Pesticide

11   Biochemistry and Physiology, 77(1), 1-11.

12

13   **Figure captions**

14

15   **Figure 1.** Enzymatic activities of EST, GST and MFO on adult abdomens of susceptible male

16   and female individuals from C. pomonella, G. molesta and L. botrana species. Different letters

17   indicate significant differences among species-sex insects groups for each enzymatic activity

18   ($P<0.05$, after glm).

19

20   **Figure 2.** MFO enzymatic activity on adult abdomens of susceptible male and female

21   individuals from G. molesta and L. botrana after different hours of inhibition with PBO. ($P<0.05$,

22   after glm).