Detoxification and sublethal effects of neurotoxic insecticides in tortricid moths

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TESI DOCTORAL

Detoxification and sublethal effects of neurotoxic insecticides in tortricid moths.

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“A truly extraordinary variety of alternatives to the chemical control of insects is avalaible. Some are alredy in use and have achieved brilliant success. Others are in the stage of laboratory testing. Still others are little more than ideas in the minds of imaginative scientists, waiting for the opportunity to put them to the test.”

Silent Spring. Rachel Carson, 1962.

Ideas in the minds …

… waiting for the opportunity.

A mi madre,
y a la memoria de mi padre.

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ABSTRACT

Basic knowledge on lethal and sublethal insecticide effects on pest insects is particularly important for optimization and continuous improvement of IPM strategies, especially when insecticides are used as the main crop-protection strategy. The work that I carried out in my Ph.D. thesis provides thorough dose-mortality curves for three neurotoxic insecticides with different modes of action [chlorpyrifos (organophosphate, acetylcholinesterase inhibitor), λ-cyhalothrin (pyrethroid, sodium channel modulator), and thiacloprid (neonicotinoid, nicotinic acetylcholinesterase receptor agonist)] on three key fruit pest species [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller)]. Subsequently I analyzed the detoxification mechanisms of the three species against the three insecticides by studying the most common enzymatic detoxification groups [carboxylesterases (EST), glutathione-S-transferases (GST), and mixed-function oxidases (MFO)]. Finally, I explored if the sublethal doses of thiacloprid estimated in the first part of the thesis affect the sex-pheromone communication system of these species. A singular aspect of my thesis is that I focused on the adult stage, which is poorly represented in the toxicological scientific literature of Lepidoptera, probably because most insecticides are mainly designed to kill egg or larval stages. However, this choice ultimately led to some unexpected findings that I explain next.

On the first part of my thesis (Chapter 1) significant differences in mortality were found among insecticides and species. However, the most remarkable result was the significantly larger mortality of males than females to chlorpyrifos, the organophosphate insecticide. This result was unexpected because females are larger than males and therefore should be more resistant than them. This result led me to think that metabolic detoxification mechanisms could be involved in the different susceptibility of males and females to insecticides. However, when I explored this aspect using enzyme inhibitors (Chapter 2), the differences in enzymatic activity, although they

partially explained differences among species and insecticides, they did not explain those between sexes. The experiments, nevertheless, indicated that incubation time of the enzyme inhibitor, a factor often overlooked in these type of studies, could be important, and so I carried out a final experiment that showed that kinetic inhibition of one of the enzyme families played an important role in explaining sex differences.

In the second half of my thesis I used the dose-mortality curves to explore if sublethal doses of thiacloprid affect the sex-pheromone communication system of the three moth species. In Chapter 3, I explored the signaller (female calling behavior) and the signal (pheromone gland content) and found significant effects in the three species in one or both parameters, but with substantial differences among species. The most remarkable finding was that doses as low as $LC_{0.001}$, which would kill only 1 in 10^5 individuals, are sufficient to significantly impact the calling behaviour in at least one of the species. The effects on pheromone production were less substantial. In Chapter 4 I assessed if sublethal doses of thiacloprid affected males, which are the receiver in the pheromone communication system. When male behavioural response to the sex pheromone was recorded in a flight tunnel and the flight track was analysed with the triangle of velocities I found that, as with females, the lowest insecticide dose ($LC_{0.001}$) significantly impaired the ability of males to fly towards the pheromone source. When treated with insecticide fewer males took flight or reached the pheromone source, and they flew more slowly and with a lower thrust. When I explored if male's pheromone sensing was affected by recording antennal electrical responses (EAG) to biologically realistic pheromone doses, I found that not even the highest insecticide doses (LC_{20}) altered male EAG responses. Therefore, the abnormal behavioural response of insecticide-treated males to the sex pheromone is not due to an effect on pheromone sensing.

The results of my thesis have important implications in pest control. They suggest that residual insecticide doses could alter the outcome of semiochemical control methods because they alter the pheromone communication system of moths, which are often controlled with mating disruption. In addition, they reveal the biochemical mechanisms involved in insecticide detoxification in a phylogenetically close group of species. Future studies should explore if sublethal doses of insecticide affect semiochemical control under field conditions, and if they alter reproductive parameters. In addition, the role of detoxification should be further pursued to explain the striking sex differences in insecticide susceptibility. And finally, the neural mechanisms potentially involved in male and female impairment by sublethal doses of insecticide deserves further research.

RESUMEN

El conocimiento básico de los efectos letales y subletales que los insecticidas pueden generar en los insectos plaga resulta particularmente importante para la optimización y mejora de las estrategias de Control Integrado de Plagas (C.I.P), especialmente cuando el uso de insecticidas es la principal herramienta de control. El exhaustivo trabajo realizado a lo largo de la presente tesis da como resultado unas curvas de mortalidad dosis-respuesta para tres insecticidas neurotóxicos con diferentes modos de acción [clorpirifós (un organofosforado inhibidor de la acetilcolinesterasa (AChE)), λ-cihalothrín (un piretroide modulador de los canales de sodio), y tiacloprid (un neonicotinoide agonista de los receptores nicotínicos de la AChE)] sobre tres especies de polillas que son importantes plagas frutales [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), y *Lobesia botrana* (Denis & Schiffermüller)]. Acto seguido, se analizaron los mecanismos de detoxificación de las tres especies para los tres insecticidas mediante el estudio de las principales familias enzimáticas involucradas en la detoxificación [carboxilesterasas (EST), glutatión-S-transferasas (GST), y multifunción oxidasas (MFO)]. Finalmente, se exploró si dosis subletales del insecticida tiacloprid, estimadas en la primera parte de la tesis, tenían un efecto sobre el sistema de comunicación mediante feromonas sexuales en las tres especies. Un aspecto característico de esta tesis es que se centra en el insecto adulto, el cual está muy poco representado en la literatura científica del área de la toxicología en Lepidópteros, probablemente debido a que la gran mayoría de los insecticidas tienen a los huevos o a diferentes estadios de la larva como elemento diana. Sin embargo, el uso de adultos en este estudio nos reportará una serie de hallazgos inesperados que se explicarán a continuación.

En la primera parte de la tesis (Capítulo 1) se encontraron diferencias significativas en la mortalidad entre insecticidas y especies. No obstante, el resultado más destacable fue la diferencia entre sexos, sobretodo la menor susceptibilidad de los machos comparada con la de las

hembras para el insecticida organofosforado clorpirifós. Este resultado es inesperado debido a que las hembras son de mayor tamaño y suelen ser más tolerantes a insecticidas. Lo cual induce a pensar que los mecanismos de detoxificación metabólica pueden ser los responsables en las diferencias de susceptibilidad a insecticidas ente machos y hembras. Sin embargo, cuando se exploró este aspecto usando inhibidores enzimáticos (Capítulo 2), las diferencias en actividad enzimática no podían explicar las diferencias de susceptibilidad entre sexos, pese a que parcialmente sí pudieron explicar las diferencias entre insecticidas y especies. A pesar de todo, los experimentos demostraron que el tiempo transcurrido entre la aplicación del inhibidor y el análisis enzimático resultó ser de gran importancia, pese a ser un aspecto que normalmente es pasado por alto en este tipo de estudios. Con este ensayo se demostró, para uno de los grupos enzimáticos, que la cinética de inhibición puede jugar un papel importante en la explicación de las diferencias entre sexos.

En la segunda parte de la tesis se emplearon las curvas de mortalidad dosis-respuesta para analizar si ciertas dosis subletales de tiacloprid tenían efecto sobre el sistema de comunicación mediante feromonas sexuales en las tres especies de polillas. En el Capítulo 3, se presta atención al emisor (comportamiento de llamada realizado por la hembra) y a la señal (contenido de feromona en la glándula), donde se encontraron efectos significativos para las tres especies en uno o ambos parámetros, pero con importantes diferencias entre especies. Resulta destacable que mínimas dosis como $LC_{0.001}$, que solo son capaces de matar 1 de cada 10^5 individuos, son capaces de afectar el comportamiento de llamada, en al menos una de las especies. Los resultados encontrados para la producción de feromona fueron menos llamativos. En el Capítulo 4 se evaluó el efecto de las dosis subletales de tiacloprid sobre el receptor (el macho), así como su respuesta. Cuando la respuesta comportamental del macho frente a estímulos de feromona fue grabada en túnel de vuelo y el trayecto de vuelo fue analizado usando el triángulo de velocidades, se encontró, como en el caso de las hembras, que la dosis inferior de insecticida ($LC_{0.001}$) afectaba de forma significativa la habilidad de los machos para volar hacia la fuente de feromona. Después de ser tratados con insecticida, menos machos eran capaces de iniciar vuelo o de alcanzar la fuente de feromona, y los que eran capaces volaban más despacio y con menor empuje. Por otro lado, el análisis de la percepción de estímulos de feromona sexual a nivel de antena en machos mediante el uso de electroantenogramas (EAG), reveló que las dosis de insecticida probadas no eran capaces de alterar las respuestas eléctricas. Por lo tanto, el comportamiento anómalo observado en los machos tratados con insecticida no es debido a un efecto sobre la percepción de la feromona.

Los resultados de esta tesis tienen importantes implicaciones en el control de plagas. Lo que sugiere que dosis residuales de insecticidas pueden alterar el resultado del control usando semioquímicos al afectar sobre el sistema de comunicación mediante feromonas sexuales de polillas, cuyo control está basado en técnicas como la interferencia del apareamiento o "mating disruption". Además de evidenciar los mecanismos bioquímicos involucrados en la detoxificación de insecticidas para un grupo de especies relacionadas filogenéticamente. Sin embargo, dejamos la puerta abierta a futuros estudios que permitan explorar si dosis subletales de insecticidas afectan al control de plagas mediante el uso de semioquímicos bajo condiciones de campo, así como los efectos que puedan generar sobre ciertos parámetros de la reproducción de estos insectos. Además del avance en el papel que desempeña la detoxificación metabólica en la posible explicación de las diferencias de susceptibilidad a insecticidas entre sexos. Así como el progreso en la investigación de los mecanismos neurológicos potencialmente involucrados en el deterioro de machos y hembras cuando están bajo los efectos de dosis subletales.

RESUM

El coneixement bàsic dels efectes letals i subletals que els insecticides poden generar en els insectes plaga resulta particularment important per l'optimització i millora de les estratègies de Control Integrat de Plagues (C.I.P), especialment quan l'ús d'insecticides és la principal eina de control. Al llarg de la present tesi s'ha realitzat una feina molt exhaustiva que dóna com a resultat unes corbes de mortalitat dosi-resposta per a tres insecticides neurotòxics amb diferents maneres d'acció [clorpirifòs (un organofosforat inhibidor de l'acetilcolinesterasa (AChE)), λ-cihalothrín (un piretroide modulador dels canals de sodi), i tiacloprid (un neonicotinoide agonista dels receptors nicotínics de l'AChE)] sobre tres espècies d'arnes que són importants plagues fruiteres [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), i *Lobesia botrana* (Denis & Schiffermüller)]. Tot seguit, es van analitzar els mecanismes de detoxificació de les tres espècies pels tres insecticides mitjançant l'estudi de les principals famílies enzimàtiques involucrades en la detoxificació [carboxil-esterases (EST), glutatió-S-transferases (GST), i multi-funció oxidases (MFO)]. Finalment, es va explorar si dosis subletals de l'insecticida tiacloprid, estimades en la primera part de la tesi, tenien un efecte sobre el sistema de comunicació mitjançant feromones sexuals en les tres espècies. Un aspecte característic d'aquesta tesi és que se centra en l'insecte adult, el qual està vagament representat dins la literatura científica de l'àrea de la toxicologia en Lepidòpters, probablement degut que la gran majoria dels insecticides tenen als ous o a diferents estadis larvaris com a element diana. No obstant, l'ús d'adults en aquest estudi ens reportarà una sèrie de descobriments insospitats que s'explicaran a continuació.

En la primera part de la tesi (Capítol 1) es van trobar diferències significatives en la mortalitat entre insecticides i espècies. Tanmateix, el resultat més destacable va ser la diferència entre sexes, sobretot la menor susceptibilitat dels mascles comparada amb la de les femelles per l'insecticida organofosforat clorpirifòs. Aquest resultat és inesperat degut que les femelles són

més grans i acostumen a ser més tolerants a insecticides. La qual cosa indueix a pensar que els mecanismes de detoxificació metabòlica poden ser els responsables en les diferències de susceptibilitat a insecticides entre mascles i femelles. En conseqüència, quan es va explorar aquest aspecte utilitzant inhibidors enzimàtics (Capítol 2), les diferències en l'activitat enzimàtica no podien explicar les diferències de susceptibilitat entre sexes, tot i que parcialment es van poder explicar les diferències entre insecticides i espècies. No obstant, els experiments van demostrar que el temps transcorregut entre l'aplicació de l'inhibidor i l'anàlisi enzimàtic va resultar ser de gran importància, tot i ser un aspecte que normalment es passa per alt en aquest tipus d'estudi. Amb aquest assaig es va demostrar que la cinètica d'inhibició per un dels grups enzimàtics pot jugar un paper molt important en l'explicació de les diferències entre sexes.

Durant la segona part de la tesi es van utilitzar les corbes de mortalitat dosi-resposta per analitzar si certes dosis subletals de tiacloprid tenien efecte sobre el sistema de comunicació mitjançant feromones sexuals en les tres espècies d'arnes. En el Capítol 3, es focalitza en l'emissor (comportament de crida realitzat per la femella) i al senyal (contingut de feromona dins la glàndula), on es van descobrir efectes significatius per les tres espècies en un o ambdós paràmetres, però amb importants diferències entre espècies. Cal destacar que mínimes dosis com $LC_{0.001}$, que tan sols són capaces de matar 1 de cada 10^5 individus, poden afectar el comportament de crida, en almenys una de les espècies. Els resultats trobats per la producció de feromona varen ser menys significatius. Al Capítol 4 es va avaluar l'efecte de les dosis subletals de tiacloprid sobre el receptor (el mascle), així com la seva resposta. Quan el comportament de resposta del mascle enfront d'estímuls de feromona va ser gravada dins del túnel de vol, i el trajecte de vol va ser analitzat utilitzant el triangle de velocitats, es va descobrir, com en el cas de les femelles, que la dosis inferior d'insecticida ($LC_{0.001}$) afectava de forma significativa l'habilitat dels mascles per volar fins a la font de feromona. Després de ser tractats amb insecticida, menys mascles eren capaços d'iniciar el vol o bé, arribar a la font de feromona, i els que ho eren volaven més lentament i amb menys empenta. Per una altra banda, l'anàlisi de la percepció d'estímuls de feromona sexual a nivell d'antena en mascles, mitjançant l'ús d'electroantenogrames (EAG), va indicar que les dosis d'insecticida provades no eren capaces d'alterar les respostes elèctriques. Per aquest motiu, el comportament anòmal observat en els mascles tractats amb insecticides no és degut a un efecte sobre la percepció de la feromona.

Els resultats d'aquesta tesi tenen importants implicacions per al control de plagues. El que suggereix, és que dosis residuals d'insecticides poden alterar el resultat del control utilitzant semioquímics a l'afectar sobre el sistema de comunicació mitjançant feromones sexuals d'arnes, el control del qual es basa en tècniques com la interferència de l'aparellament o "mating

disruption". A més d'evidenciar els mecanismes bioquímics involucrats en la detoxificació d'insecticides per a un grup d'espècies relacionades filogenèticament. No obstant, deixem la porta oberta a futurs estudis que permetin explorar si dosis subletals d'insecticides afecten el control de plagues mitjançant l'ús de semioquímics sota condicions de camp, així com els efectes que puguin generar sobre certs paràmetres de la reproducció d'aquests insectes. A més a més, de l'avanç en el paper que exerceix la detoxificació metabòlica en la possible explicació de les diferències de susceptibilitat a insecticides entre sexes. Així com els avenços en la investigació en els mecanismes neurològics potencialment involucrats en el deteriorament de mascles i femelles quan es troben sota els efectes de dosis subletals.

GENERAL INTRODUCTION

In Mediterranean areas, most of the countries have an economy that is dependent on agriculture. Europe is one of the world's largest and most productive suppliers of food, for example, almost two-thirds of the world's wine is produced in the European Economic Community (EEC), in countries like Italy, Spain, and France (Jenster and Jenster 1993). Spain is the major fruit and vegetables exporter of the European Union (EU) and one of the world´s top exporters together with China and the United States (USA). Close to 47 % of the Spanish fruit production is exported, reaching values of 10.000 million € and being the most important sector in agriculture (average of 2008-2013, Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente [MAPAMA] 2017). In our area, Catalonia, there are some important fruit crops like grapevines, peaches, apples and pears. In Table 1, we show the amount cultivated area relative to the total cultivated area of these crops in Spain.

Table 1. Total areas (Ha) destined to fruit crops (grapevines, peaches, apples, and pears) in Catalonia and Spain. (MAPAMA 2017).

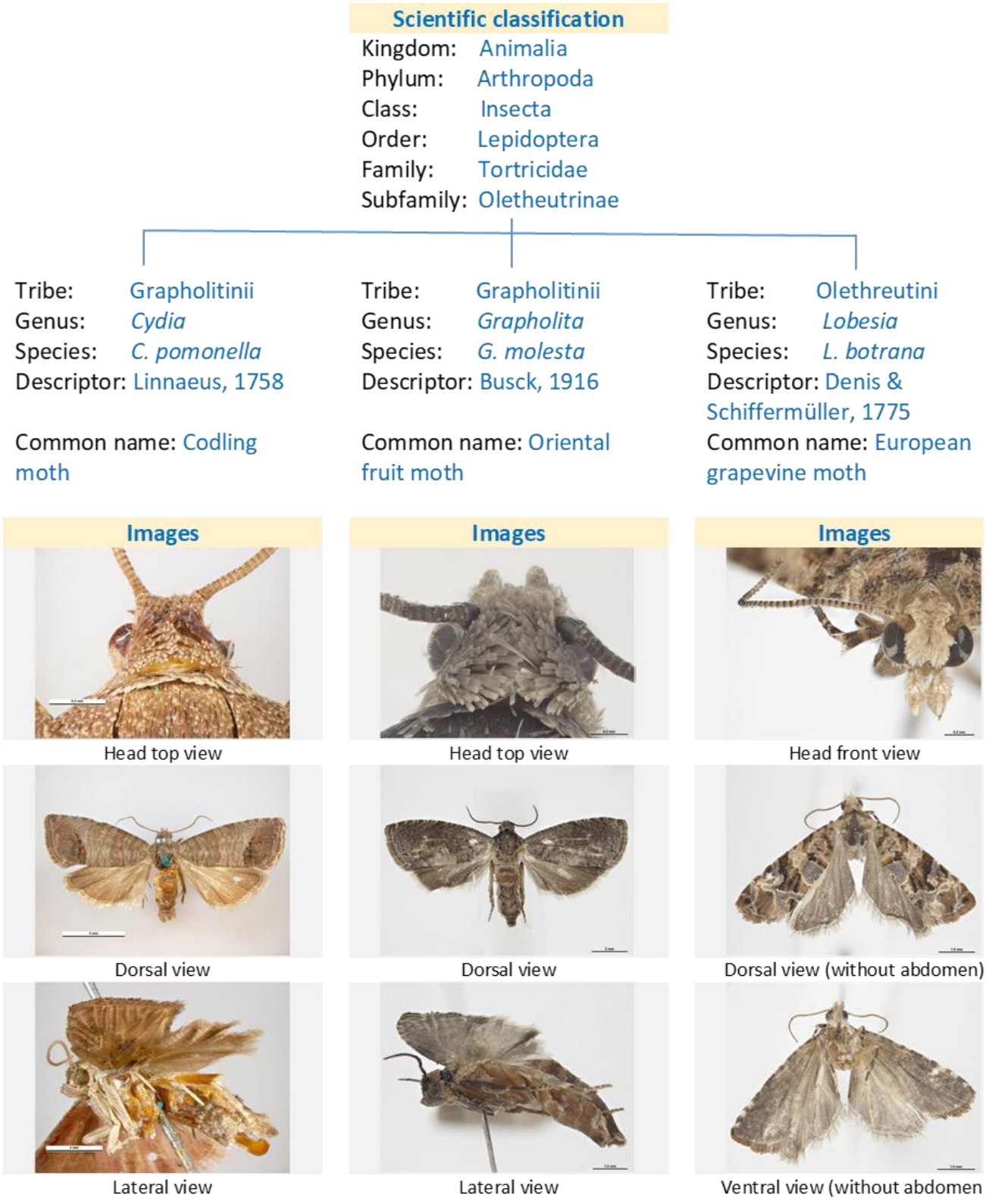
Fruit crop	Catalonia (Ha)	(%)	Spain (Ha)	Year
Grapevines	56,336	5.99	941,154	2015
Peaches	21,255	26.70	79,617	2012
Apples	10,924	33.67	32,441	2012
Pears	9,102	37.82	24,064	2012

One of the most critical problems in these crops are the insect pests, causing yield losses and important reductions in economic benefits for the growers. The family *Tortricidae* accounts for 9,416 described species of moths (Pogue 2009) of which almost 700 are potential pests of agricultural fields (Zhang 1994). Some of these moth pests are well known in our fruit crops, like

Cydia pomonella (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller), cosmopolitan and poliphagous species (Pogue 2009) that are important pests of apple and pear, peach, and grapevines, respectively. These species have the potential to produce multiple generations by season under favourable conditions, and major damage by these pest is caused by larval feeding on fruits, but other host organs could be consumed by the larvae, like peach shoots by *G. molesta* (Myers *et al.* 2007), and flowers, fruit, leafs and shoots of grape vines by *L. botrana* (Lucchi *et al.* 2011). Figure 1 shows the taxonomic relationship of these species.

The importance of control pest is clear, but nowadays the indiscriminate use of insecticides as the only method of control pest is deprecated, owing to different risks associated to their widespread use, including pest resistance, target pest resurgence, secondary pest outbreaks, environmental contamination, and human health problems, among others (Devine and Furlong 2007). Concerns about these issues have increased the interest in the development of alternative options in pest control. Growers, as field managers, are the first ones to know the need to take care about our natural resources; in fact, their daily life depends on it. For that reason, they tend to avoid the negative effects of some agricultural practises, making a more sustainable and environmentally safe agriculture, always under the standards of the Common Agricultural Policy (CAP) and the Integrated Pest Management (IPM) programs. In the words of the Environmental Protection Agency (EPA), IPM refers to the strategy based on the use of a large variety of complementary control methods, such as biological and chemical ones, that allows controlling a pest, in addition to the reduction of insecticide use, and minimizing their negative impact on the environment (Environmental Protection Agency [EPA] 2017). Since their appearance, chemical pesticides are the main tool to control fruit and vegetable crops pests worldwide, among other technologies accepted in IPM, mainly due to their quick effect, low cost and relatively easy application (Waterfield and Zilberman 2012). Neurotoxic insecticides, which have adverse effects on the central or peripheral nervous system, account for 54 % of total insecticide sales worldwide (Sparks and Nauen 2015). They have 4 primary targets: acetylcholinesterase (AChE), voltage-gated sodium channels, AChE receptors, and γ-aminobutyric acid (GABAA) receptors (Casida 2009). Primary sales of neurotoxic insecticides are for neonicotinoids, pyrethroids and organophosphates. Insecticides groups are classified according to their mode of action (MoA) by the Insecticide Resistance Action Committee (IRAC) (Sparks and Nauen 2015). Neonicotinoids competitively modulate nicotinic acetylcholine receptors (nAChR) at the synapsis, changing their conformation and allowing a constant ionic flux. Pyrethroids block sodium channels invol-

Figure 1. Taxonomy and diagnostic images of *C. pomonella*, *G molesta* and *L. botrana*. Images authorship (Plant Biosecurity Cooperative Research Centre [PBCRC] 2017)



ved in action potential generation and thus suppressed action potentials. Organophosphates act on acetylcholinesterase (AChE), the enzyme that degrades the neurotransmitter acetylcholine (ACh), so accumulations of ACh result in excessive stimulation of cholinergic receptors (Casida and Durkin 2009). These MoA are schematic represented at Figure 2.

To counteract the action of insecticides, insects have different detoxification mechanisms, which have evolved to neutralize natural toxins from secondary plant metabolites acquired through ingestion (Li *et al.* 2007). Metabolic transformation of a toxic compound could take place in two phases, the first one consists in the addition of a polar group to the substrate or the split of the molecule in two parts, by oxidation, hydrolysis and/or reduction. The second phase involves the addition of sugars, amino acids, sulphates or phosphate groups on the substrate that results from the first phase, in case this substrate was not hydrophilic enough to be excreted. In phase I the enzymatic groups involved are mixed-function oxidases (MFO) and carboxylesterases (EST), whereas glutathione-S-transferases (GST) are involved in phase II (B-Bernard and Philogène 1993). These three enzymatic groups are the most important metabolic detoxification systems in insects. The MFO, also known as microsomal cytochrome P450 monooxygenases, is one of the oldest and largest gene super-families. The details of catalytic events mediated by MFO are not fully understood, but mainly consist on oxidative reactions. Oxidation is also considered the most important reaction of phase I metabolic transformations. However, metabolic transformations of insecticides by MFO could result either in bio-activation or, more often, in detoxification (Feyereisen 1999). In organophosphorus insecticides, the activation of P=S to P=O by MFO results in a substantially increased activity of anticholinesterase agents, which translates into an increase of insecticide toxicity (Yu 2008). EST belong to the carboxylesterase gene family within the α/β hydrolase fold protein superfamily (Lenfant e*t al.* 2013), which includes proteases, lipases, dehalogenases, peroxidases and epoxide hydrolases, among others. The α/β hydrolase fold domain is found in a number of functionally different enzymes that are capable of hydrolysing a wide range of substrates (Montella *et al.* 2012). GST represent a complex group of proteins formed by two entirely distinct superfamilies that possess transferase activity. The catalytic reactions provided by these enzymes consist on the supply of electrons by the sulphur atom of glutathione, which provoke a nucleophilic attack on a second electrophilic substrate, such as endogenous natural substrates like epoxides, organic hydroperoxides, or activated alkenals resulting from oxidative metabolism (Sherratt and Hayes 2002).

Detoxification capacity varies among species and insect developmental stages (Yu and Hsu 1993). In a constantly changing environment, the insects needs to adapt to these alterations, en-

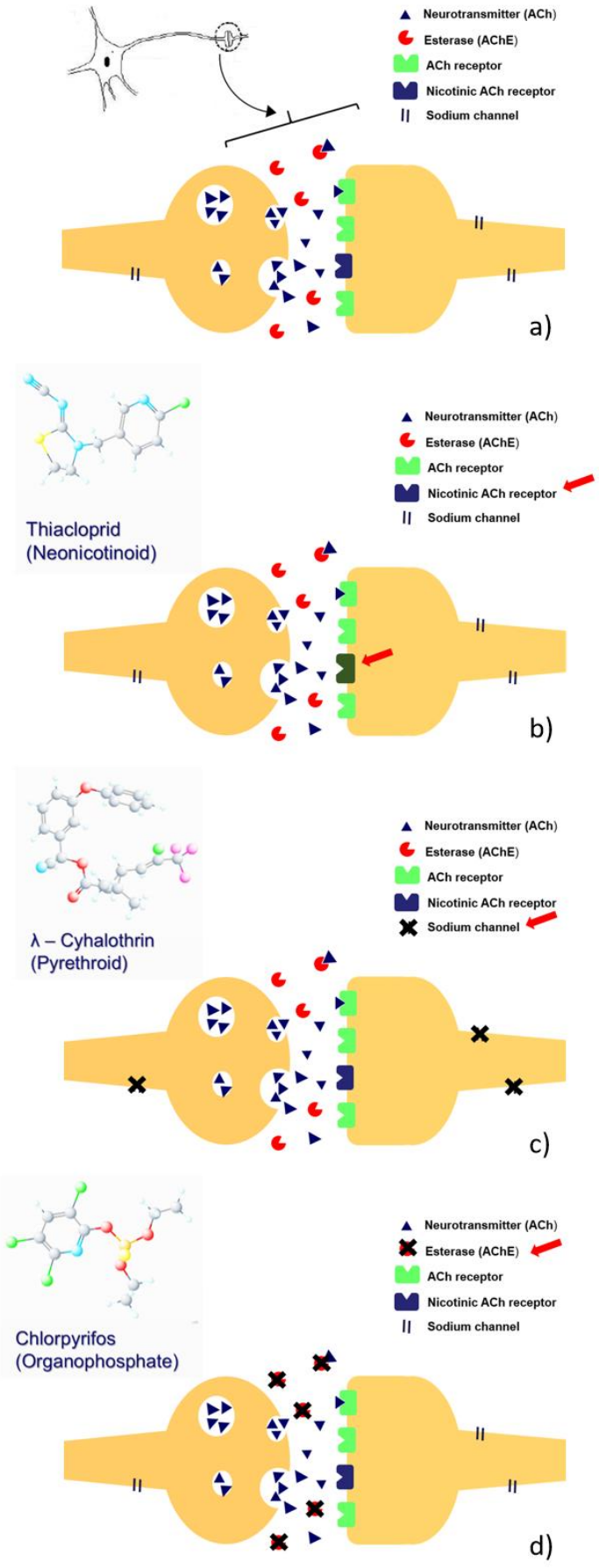


Figure 2. Schematic representation of the mode of action of different insecticide groups according with IRAC classification and based on Casida and Durkin (2009). Representation of normal synapsis and their components (a); effects of neonicotinoids like thiacloprid on synapsis (b); effects of pyrethroids like λ-cyhalothrin on synapsis (c); and effects of organophosphates like chlorpyrifos on synapsis (d).

hancing their detoxification activity systems by producing additional quantities of enzymes. This normally happens after the influence of exogenous chemical stimuli, phenomenon termed "induction" (Terriere 1984). Induction of metabolic detoxification enzymes could be triggered by changes in host plant (Yang *et al.* 2001, Després *et al.* 2007) or other environmental stressors like insecticides (Poupardin *et al.* 2008) or herbicides (Yu 2004). The enhancement in xenobiotic metabolism involves amplification, overexpression, and coding-sequence variation in the three major groups of genes encoding metabolic enzymes (Li *et al.* 2007). These changes in detox capacity are responsible, at least in part, for host plant selection and selective toxicity or resistance development against insecticides (Terriere 1984). To restore the activity of insecticides against resistant insects in agriculture, inhibitors of these metabolic enzymes can be used. Enzymatic inhibitors bind to the enzymes and interfere with general metabolic pathways of detoxification. Enzymatic inhibitors, also called insecticide synergists for the synergistic effect they cause on insecticide effectiveness, have been used commercially for about 50 years. The most widely used are S,S,S, tributyl phosphorotrithioate (DEF), diethyl maleate (DEM), and piperonyl butoxide (PBO), as EST, GST and MFO inhibitors respectively (B-Bernard and Philogène 1993).

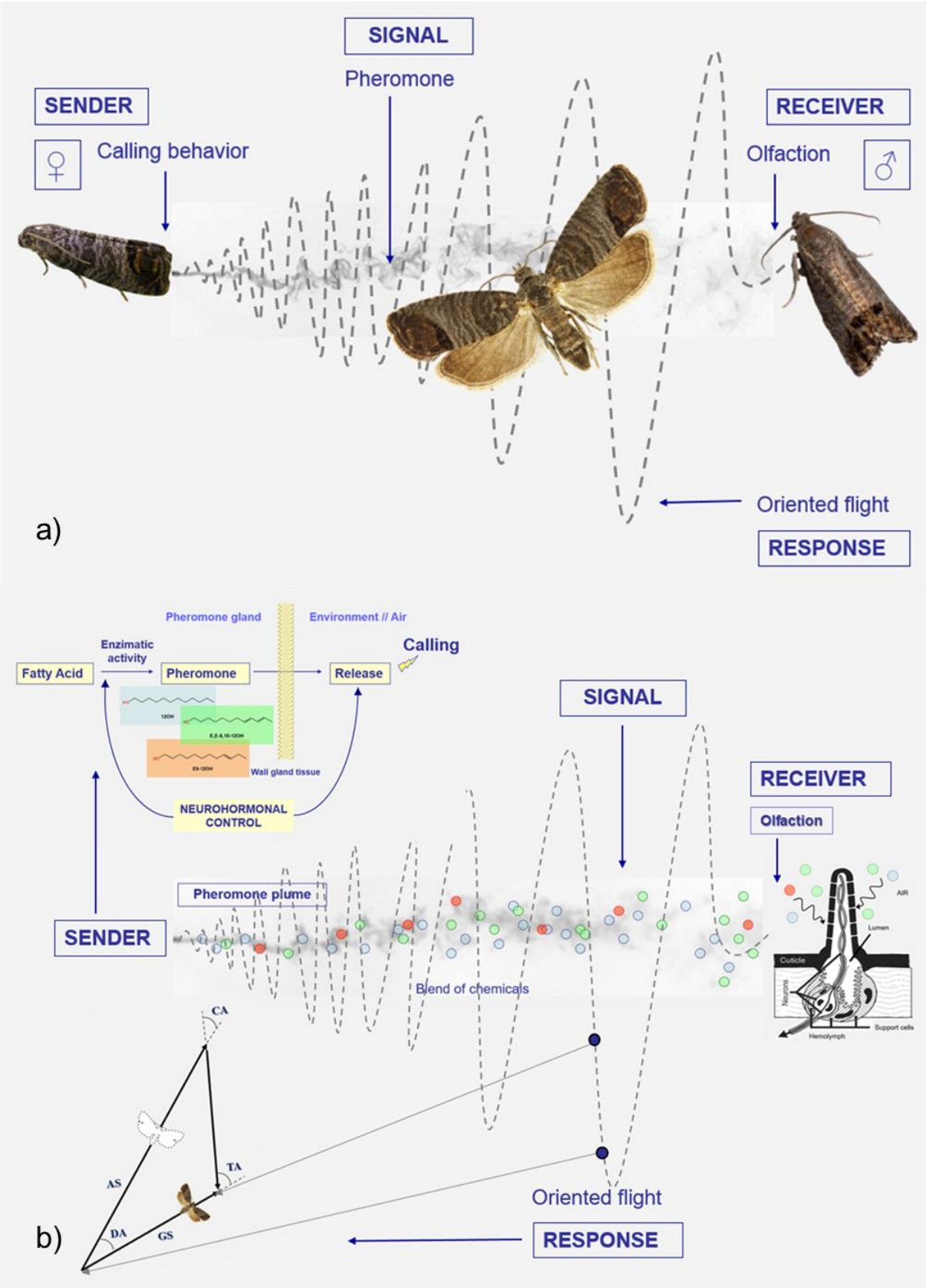
The total activity of an insecticide against pests includes both, its direct toxicity to one or more life stages and effects on physiology, biology and behaviour not necessary leading to mortality, the so-called sublethal effects of insecticides (Haynes 1988, Lee 2000, Desneux *et al.* 2007, Pisa *et al.* 2015, Guedes *et al.* 2016, 2017). Insects can come into contact with sublethal doses of insecticide when the initial application of the insecticide decreases to residue levels over the time, when structurally-derived residues exhibiting biological activity are generated, when the application is not aimed to other life stages or pest species, or from drift by blast sprayers in neighbor fields, among others. The consequences of these sublethal exposures are strong dose/concentration-dependent, and normally have deleterious effects. However, sometimes low insecticide doses can enhance reproduction or behaviour, a phenomenon that is named hormesis (Guedes and Cutler 2014). Hormesis is a special type of biphasic dose–response characterized by a low-dose stimulation and a high-dose inhibition. Hormesis must recorded over a certain time span (dose–time–response relationship), because an overcompensation response following an initial disruption in homeostasis may happen (Calabrese 2008). Theoretically, hormetic dose-responses are often present in all insect individuals and species, but the dose-response relationship is specific for the combination of species and individuals, so tolerant species need higher doses than susceptible ones for hormesis to occur (Calabrese and Baldwin 2002)

As the most commonly used insecticides interfere with neurotransmission, the effect of sublethal doses of insecticide on insect behavior is probably commonplace. Sex-pheromone mediated reproductive behavior is a likely target of sublethal effects because reproduction involves a complex series of behavioral and physiological events, which are coordinated by the insect's nervous and hormonal systems in a very precise manner (Haynes 1988, Tricoire-Leignel *et al.* 2012).

Sex-pheromone communication, mainly based on the insect olfaction system, includes the net displacement of one individual toward the odour source. Differences in this sexual activity between species is an effective isolation mechanism and an instrument in speciation, which mostly results in species-specific sex-pheromones and behaviour timing, which in case of moths could be very different between closely related species. Furthermore, moths show very specific daily activity patterns in their sexual activities (Groot 2014, and references therein). In moths, sexual behaviour usually starts by females releasing the sex pheromone, and then males respond to it with and oriented flight towards the female. Figure 3 shows a schematic representation of sex-pheromone communication system and the main parts involved.

Pheromone biosynthesis in female moths is mediated by a brain-released neurohormone (PBAN) that reaches the pheromone gland through the haemolymph and binds to specific receptors on the membrane of pheromone secretion cells (Jurenka and Rafaeli 2011). Usually, precursors of sex pheromone components are fatty acids like oleic and palmitoleic acids, which are synthesized by a combination of unique chain-shortening and desaturase steps (Roelofs and Wolf 1988). This biosynthesis occurs the novo every day and is synchronized with calling behavior (Groot 2014), but there are exceptions in which biosynthesis and release appear to be two independently controlled events (Raina 1993). During calling behavior, females visibly extrude their pheromone gland and pheromone components are released to the environment. Eventhough calling behavior is thought to follow a circadian pattern, several factors could influence it; like temperature, age, mating status, and pheromone autodetection, among others (Groot 2014). Sex-pheromone components released in nanogram amounts per hour, must be released in specific blend ratios in order to be discriminate by conspecific males from pheromone blends from other species. The pheromone plume originating from a single female could be perceived from tens to perhaps hundreds of meters by a male during their searching flight, and their flight response is triggered immediately upon perception of pheromone (Cardé 2016). Some factors have been found to influence the strength of the male-moths response after perception; like pheromone intermittent stimulation (Willis and Baker 1984, Baker *et al.* 1985), pheromone concentration

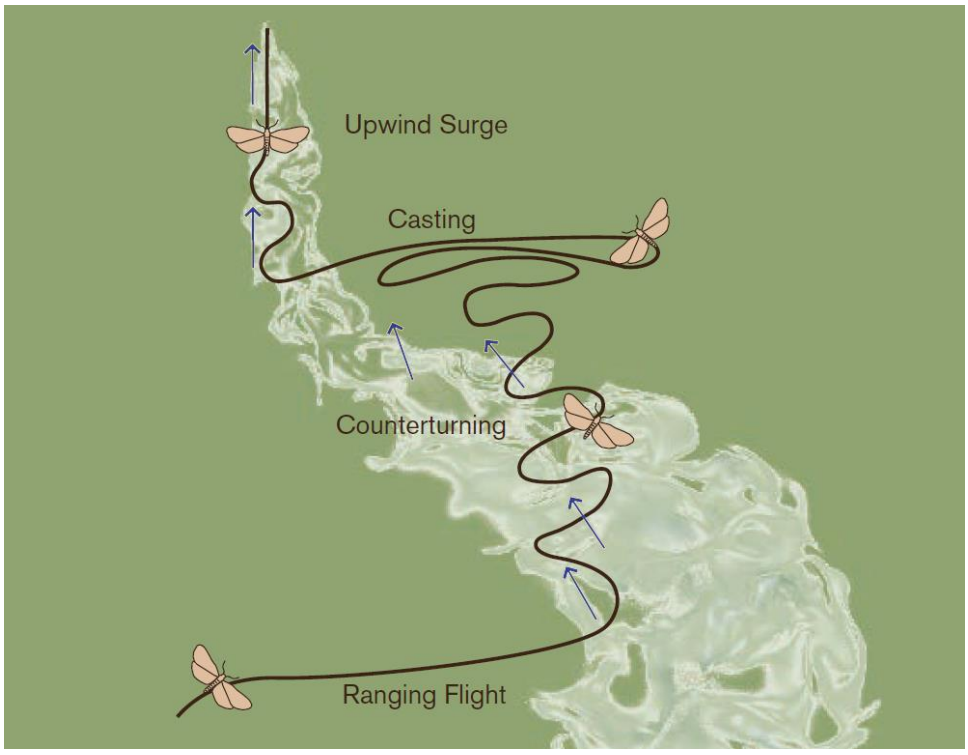
Figure 3.Schematic representation of sex-pheromone communication system in moths (a); and some physiological and behavioural parts involved in this system (b).



(Kuenen and Baker 1982, Charlton *et al.* 1993), pheromone plume structure (Mafra-Neto and Cardé 1994), plume composition (Bau *et al.* 1999), ratio of sex pheromone components in the plume (Willis and Baker 1988), wind velocity (Willis and Cardé 1990), illumination (Cardé and Knols 2000), flight height, and visual patterns (Kuenen 2013, Kuenen *et al.* 2014), among others.

The general odor-guided response model of a male moth finding and orienting along a pheromone plume consist on a first ranging flight to contact the plume, zigzag upwind flight while in plume contact, whereas if contact with plume is lost insect make casting flight and finally, an upwind surge when filaments of pheromone are contacted at high frequency rates (over 5 Hz) (Figure 4) (Cardé 2016). The term "zigzagging" during upwind flight is due to the characteristic meandering form of the flight, which results from the combination of two mechanisms: optomotor anemotaxis and an internal program of counterturning (Witzgall 1997). Optomotor anemotaxis allows the insect to assess its progress against wind direction using optical feedback by visual contacting with surrounding elements (Kennedy and Marsh 1974), whereas intermittent contact with airborne odor elicits and maintains a counterturning behaviour, thus chemotactic maneuvers are needed too (Kennedy *et al.* 1980, 1981).

Figure 4. Template of moth maneuvers as governed by sequential interactions with filaments of pheromone, encounters with "clean air" and wind flow (Cardé 2016).



Monitoring the temporal, spatial, and intensity parameters of pheromone plume and transmitting the messages of this dynamic environment to the insect's brain is achieved by the highly

sensitive olfactory receptor neurons (ORN) located in the antennal sensilla (Breer 1997). The sensillum wall is perforated by tiny pores that allow the access of volatile odor molecules, i.e. pheromones, plant volatiles, etc. In the antennal sensillum lymph there are pheromone-binding proteins (PBPs), a type of odorant-binding proteins (OBPs), which specifically bind with pheromone components and delivery them to the specific ORN (Prestwich and Du 1997). Chemosensory neurons respond to specific chemical stimuli with a change in the membrane potential, a receptor potential, which in turn elicits a distinct patter of action potentials, encoding the strength and duration of a pheromone stimulus. The afferent neuronal signals travel along the axons of the sensory neurons toward the olfactory antennal lobe (AL), where the pheromonal information converge and is processed in the macroglomerular complex (MGC), which after integration of the signal elicits the behaviour sex pheromone response (Breer 1997, Galizia and Rössler 2010).

These physiological and behavioural aspects from chemical communication have potential application in some control strategies. Based on semiochemicals, these strategies are high selective to target pests and present minimal risks to human health and environmental pollution. Thus, they are highly suitable for use in any IPM strategy. Some examples of efficient methods based on semiochemicals in controlling moth-pest species are (Howse 1998):

- Monitoring. It is not strictly a method for controlling pests, but otherwise it is a method which usually only traps males to monitor insect occurrence in orchards. The main functions of monitoring are: detection outbreaks, determination of emergence times of adult insects, mapping distribution, and evaluation of insect abundance changes. These functions provide useful information about timely insecticide treatments for pest control.

- Mass trapping. Consist on pest reduction by removing from the population individuals attracted to traps baited with, usually, pheromone lures.

- Mating disruption. Emitting large amounts of synthetic sex pheromone and so reducing the probability of mate finding is the operating principle of this technique. The exact way by which mating disruption operates is not well understood. Different mechanisms could be involved like masking of females pheromone plumes by persistent odour flood; males following false trails formed by synthetic pheromone dispensers; or saturation of male antennal receptors.

- Attract-and-kill. Like monitoring and mass trapping techniques, the use of toxic baits relies on attraction of one or both sexes to a lure, but in this case in combination with an insecticide-impregnated target. Two options are available in attract-and-kill strategies, (i) a trap with an

attractant semiochemical formulation with an independent tank that contains insecticide products, or (ii) an attractant and insecticide which are incorporated into a fully integrated matrix that can be applied as a stand-alone intervention. However, to reach good rates of pest control, mass annihilation requires the use of the most attractive lure, and becomes far more efficacious when using lures that attract females or both sexes.

Among these semiochemical-based techniques, mating disruption is less cost-effective than mass trapping and attract-and-kill, since much smaller amounts of pheromones are needed. In addition, the use of insecticides in attract-and-kill traps is less environmentally safer and have some public unacceptance (Witzgall *et al.* 2010).

Therefore, in an IPM context with a combined use of insecticides and environmentally safer methods like semiochemicals, it is not difficult to think that neurotoxic insecticides, which affect the normal functioning of the nervous system can interfere with semiochemicals, which are based on insect chemical communication that is strong-dependent of the correct functioning of nervous and hormonal systems. Indeed, in this ecosystem context of chemical and toxicological interactions, it is necessary the study of the whole insecticide effects, including sublethal effects on insect behavior and physiological functions.

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OUTLINE OF THE THESIS AND RESEARCH OBJECTIVES

Outline of the thesis

With this thesis, I want to gain basic knowledge about the ecotoxicology of different neurotoxic insecticides on three different moth pest species and the susceptibility differences between males and females. In addition, I want to analyse the possible metabolic mechanisms, like enzymatic activities, involved in the detoxification of these insecticides by investigating differences in their activity under the effect of enzyme inhibitors and comparing among species and sexes, and determining the important effect of time in the kinetics of inhibition. The second part of the thesis is dedicated to test sublethal effects of a neurotoxic insecticide on the sex-pheromone communication system of these species. For this goal, I test four sublethal doses of thiacloprid that cause between 0 to 20 % of insect mortality, and analyse the effects on the sender (females), the signal (sex pheromone), and the receiver (males). Specifically, sublethal effects were analysed on females calling behaviour, pheromone gland content and ratio composition, pheromone reception in the male's antennae and male flight behaviour. By comparing the effect of thiacloprid across phylogenetically related species, I hope to gain basic background information about the effect of sublethal insecticide doses on the pheromone communication system, being the first comparative study among *Tortricidae* species. The most noteworthy findings that this thesis unveils are: a) the higher tolerance of males to chlorpyrifos in all three species; b) differences on the inhibition kinetics of MFO by PBO across time; c) the first time of the complete observation on the calling period of *L. botrana*; and d) the first report of sublethal effects on Lepidopteran flight track analysis.

Research objectives by chapters

Chapter 1: "Comparative effect of three neurotoxic insecticides with different modes of action on adult males and females of three tortricid moth pests".

Determine which variables are important in toxicological responses and build dose-response curves for each combination of insecticide-species-sex that make up the framework to determine the sublethal doses for these combinations.

Chapter 2: "Enzymatic detoxification strategies for neurotoxic insecticides in adult tortricids".

Assess the metabolic mechanisms involved in detoxification of the neurotoxic insecticides tested in chapter one, among the main metabolic mechanisms (EST, GST and MFO), for each species and sex. Also, the evaluation of differences in enzymatic activity among groups (species-sex combinations), and other factors like adequate enzymatic inhibition and the influence of time in the inhibition-kinetic response.

Chapter 3: "Sublethal effects of neonicotinoid insecticide on calling behaviour and pheromone production of tortricid moths".

Test the sublethal effects of the neurotoxic insecticide thiacloprid on sex-pheromone communication system on signallers, by the evaluation of calling behaviour and amounts of pheromone gland content of the main components of the sex-pheromone blend and the ratios among them, under four sublethal doses with mortalities up to 20 % in females.

Chapter 4: "Sublethal doses of thiacloprid affect male flight responses to sex pheromone but not its detection in three tortricid moths".

Evaluate if four sublethal doses of thiacloprid, which are under 20 % of mortality, cause any effect on the sex-pheromone communication system of receivers by assessing male EAG responses to synthetic sex-pheromone stimulus, and their flight ability to the sex stimulus in wind-tunnel controlled conditions.

COMPARATIVE EFFECT OF THREE NEUROTOXIC INSECTICIDES WITH DIFFERENT MODES OF ACTION ON ADULT MALES AND FEMALES OF THREE TORTRICID MOTH PESTS

1

ABSTRACT

Insecticides are the dominant pest management method in fruit and vegetable crops worldwide due to their quick effect, low cost and relatively easy application, but they bear negative effects on human health and the environment. Insecticide mode of action (MoA), target species and sex are variables that could affect insecticide mortality. We recorded the mortality caused by three neurotoxic insecticides with different modes of action [chlorpyrifos (organophosphate, acetylcholinesterase inhibitor), λ-cyhalothrin (pyrethroid, sodium channel modulator) and thiacloprid (neonicotinoid, nicotinic acetylcholinesterase receptor agonist)] applied topically to adult males and females of three economically important tortricid species [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller)] that strongly depend on insecticide use for their control. Concentration and dose-mortality curves were recorded at 24 and 48 hours post application. Large mortality differences between insecticides (maximum 7800-fold for LD_{50}) were followed by much lower, yet important, differences between species (maximum 115-fold), and sexes (maximum 41.5-fold). Significant interactions between the three factors indicate that they are not independent from each other. Interestingly, with the organophosphate chlorpyrifos, males of the three species were less susceptible than females, which was unexpected since females are larger than males. Higher female sensitivity to organophosphates has been reported previously but only in *G. molesta*, not in other moth species. Our results highlight the importance of taking into account sex in dose-mortality studies with adult moths.

KEY WORDS: dose-response, neurotoxic insecticides, Tortricidae, adult insects, sex differences.

Chapter published Navarro-Roldán, M.A., J. Avilla, D. Bosch, J. Valls, and C. Gemeno. 2017. Comparative effect of three neurotoxic insecticides with different modes of action on adult males and females of three tortricid moth pests. J. Econ. Entomol. doi: 10.1093/jee/tox113.

Introduction

A fundamental aspect of insecticide pest control is determining the optimal quantity of toxicant needed to obtain maximum pest mortality while at the same time minimizing environmental and human impact (Guillette and Iguchi 2012, Guedes *et al*. 2015). Mortality curves are the most common method to assess the relationship between the quantity of toxicant and the level of mortality (Pasquier and Charmillot 2003, Cutler 2013). Both toxicant mode of action and insect species affect the slope and intercept of mortality curves but these are not the only variables that affect mortality curves. Some variables such as insect stage (egg, immature, or adult) or development are sometimes examined (Knight 2000, Sáenz-de-Cabezón Irigaray *et al*. 2005, Rodríguez *et al*. 2011), whereas other biological variables, such as sex (Kanga *et al*. 2001, Shearer and Usmani 2001, de Lame *et al*. 2001), or methodological issues, such as the mode of application or time of exposure (Preisler and Robertson 1989), are rarely considered. Because it is challenging to compare many variables in a single experiment, most comparative studies either test several insect species with one toxicant (Vandekerkhove and de Clercq 2004, Nayak and Daglish 2006, Ioriatti *et al*. 2009a), or several types of toxicants on a single species (Zotti *et al*. 2013, Grigg-McGuffin *et al*. 2015, Wu *et al*. 2015). Fewer studies, however, test the effect of several insecticides on different species (Beers *et al*. 2005, Fernandes *et al*. 2016, Rodriguez-Saona *et al*. 2016). In addition, the effect of sex is often neglected.

In the present study, we compare the effect of three neurotoxic insecticides with different modes of action (MoA) on adult males and females of three economically important moth species. We focus on the tortricid moths, *Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller), because they are key pests of relevant Mediterranean fruit crops, mainly apples, peaches, and grapes, but they also attack other hosts and have a relatively worldwide distribution (Ioriatti *et al*. 2011, Damos *et al*. 2015, Kirk *et al*. 2013). As toxicants, we chose three neurotoxic insecticides with different modes of action: chlorpyrifos, an organophosphate that acts on acetylcholinesterase (AChE), the enzyme that degrades the neurotransmitter acetyl-choline; λ-cyhalothrin, a pyrethroid that modulates sodium channels involved in action potential generation; and thiacloprid, a neonicotinoid that competitively modulates nicotinic acetylcholine receptors (nAChR) at the synapsis (Casida 2009, Insecticide Resistance Action Committee [IRAC] 2016). Neurotoxic insecticides act by contact and ingestion and could affect all insect life stages. Thiacloprid and chlorpyrifos affect larvae and adults of *C. pomonella* (Reyes and Sauphanor 2008); chlorantraniliprole affect eggs and larvae of *L. botrana* (Ioriatti *et al.* 2009b); several neonicotinoid and organophosphate insecticides affect all insect stages of *C. pomonella* and *G. molesta* (Magalhaes and Walgenbach 2011); and

our three test insecticides affect eggs and larvae of *C. pomonella* (Rodríguez *et al.* 2011).The three insecticides of our study are recommended by the Spanish Agriculture Ministry to control at least two of the three tortricid species each (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente [MAPAMA] 2017). Neurotoxic insecticides account for 54 % of total insecticide sales worldwide (Sparks and Nauen 2015). These insecticides have already been tested in these three moth species to assess resistance and sublethal effects, having larva as a common target insect stage. *Cydia pomonella* is resistant to chlorpyrifos (Reyes *et al*. 2011, Rodríguez *et al*. 2011), λ-cyhalothrin (Mota-Sánchez *et al*. 2008, Rodríguez *et al*. 2011); and thiacloprid (Rodríguez *et al*. 2011, Cichón *et al*. 2013), but there are not registered resistance cases for these active ingredients in *G. molesta* or *L. botrana*. Sublethal effects have been tested for chlorpyrifos in *C. pomonella* (Yang *et al*. 2013) and *L. botrana* (Pavan *et al*. 2014), for λ-cyhalothrin in *C. pomonella* (Yang *et al*. 2013) and *G. molesta* (Jones *et al*. 2011), and for thiacloprid in *C. pomonella* (Brunner *et al*. 2005) and *G. molesta* (Siegwart *et al*. 2011).

By comparing the effect of insecticides with different MoA across phylogenetically related species, and in both sexes, we hope to gain basic background information for further studies on the physiological mechanisms responsible for insecticide resistance and the effect of sublethal doses. At the same time, the response-mortality curves obtained will provide a diagnostic methodology to test possible resistance cases in field populations, using adults of the same species and the same insecticides tested in this study.

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 > 5 yr without introduction of wild individuals. Larvae were reared in artificial diet (Ivaldi-Sender 1974) in a rearing room maintained at 25 ± 1 ºC with a photoperiod of 16:8 (L:D) h. 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. Adult body mass was estimated by drying 40 frozen 1-d-old individuals of each sex and species for 2 d at 30°C, and then weighing them individually in an analytical balance (±0.1 mg precision).

Insecticides. 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), were the active ingredients used in the mortality bioassays. All the dilutions used in the bioassays were prepared from pure compound in at least two different occasions, 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 7 ºC. The same stock of acetone used to prepare the dilutions was also used as the negative control treatment.

Mortality bioassays. Newly emerged adults were separated from the pupal cages every day and received the insecticide treatments during the first half of the photophase at 0-24 h postemergence. Adults were placed individually or in pairs in 10-ml test tubes and received a brief (10 s) flow of industrial grade CO_2, which quickly 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 nonsterile clinical sample bottle (57 mm in diameter by 73 mm in height). Individuals receiving the same treatment were placed in groups of 3-10 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 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.

To select the final concentrations used in the dose-response curves we started testing 1:10 dilutions ranging from 10 μg to 10 pg per insect, with ~ 20 insects in each dose. High and low limits for each curve were roughly estimated this way and new doses (no < 5 for each treatment combination) were tested, also with ~ 20 insects per dose, until a reasonable probit fit was obtained for a given curve. Using the predicted values from the probit model, we chose six final test concentrations (plus acetone control) for each treatment combination and tested them with in

between 60 and 116 insects per concentration. Tests were performed on groups (i.e., repetitions) of at least three insects of the same treatment group (insecticide, dose, sex, and species), with different treatments tested each day depending on insect availability, until the desired sample size was achieved. A total of 6,802 insects were used to build the final curves.

Data analysis. All the statistical analyses were run in R software (R Core Team 2016). Weight differences among sex and species were analyzed with ANOVA followed by pairwise comparisons. For the analysis of mortality, we run generalized linear models (GLM) with a binomial family and a probit link. For statistical analyses, we used only the mortality at 24h as a function of insecticide dose (mortality data adjusted by the average dry body weight of each species and sex, i.e., lethal dose or LD). For discussion purposes we show in supplementary material the mortality at 48h as a function of insecticide dose, and 24-h mortality as a function of insecticide concentration (Figure S1 to S3).

Three levels of mortality analysis were carried out. First, a global model was built hierarchically to determine the effect of insecticide type, dose, species, and sex. We started with the simplest model containing no main effects, and followed it with a model including all main effects (insecticide type, dose, insect species, and sex), and then with successive models including all main effects and second-, third-, and fourth-order interactions. For model-selection, we used the likelihood ratio test (LRT) and the Akaike Information Criterion (AIC), preferring the model with the lower AIC value of pairs that were significantly different by LRT.

Second, we estimated intercepts, slopes, and LDs, and their errors for 1) insecticides independently of species and sex, 2) species within insecticide, independently of sex, and 3) sex within species and insecticide. For this, 13 GLM models were run: one to compare insecticides, three to compare species within each insecticide, and nine for each insecticide by species combination to estimate the effect of sex. Intercept and slope were extracted directly from the GLM models. To estimate LDs, we run the dose.p() function in the package "MASS" of R (Venables and Ripley 2002) on each of the 13 GLM models.

Finally, we performed pairwise comparisons (at same levels indicated above [a, b, c]), for slope, intercept, and LD_{10}, LD_{50}, and LD_{90}. For slope and intercept estimates, we run a generalized linear hypothesis test using the glht() function in the package "multcomp" of R (Hothorn *et al.* 2008). For comparison of the LDs, we calculated the Z-score of the GLM estimates and errors

and obtained a p-value by comparison with the Z-score of the standard Normal curve. R scripts and raw data are available at http://hdl.handle.net/10459.1/57672

Results

As expected, females were heavier than males in all three species, and *C. pomonella* was the heaviest of the three species, whereas males of *G. molesta* and *L. botrana* did not differ from each other, and neither did the females (Table S1).

Hierarchical model selection indicated that the most complex model, which contains all main variables plus second-, third-, and fourth-order interactions, provided the best fit to the data (Table 1). This 36-parameter model was significantly different from the next simpler model according to LRT, and also had a lower AIC value. Analysis of deviance for this model (Table 2) showed that the highest contribution of main effects was for insecticide dose ($P < 0.0001$) and insecticide type ($P = 0.019$), whereas neither species nor sex contributed significantly on their own ($P > 0.05$). Nine of the 11 second-order to fourth-order interactions were significant, indicating that the effect of individual variables was strongly dependent on the other variables.

Dose-response curves were constructed using the slope and intercept parameters estimated with the individual probit regression models of each curve (Figure 1, Table 3). Several qualitative features are already noticeable in this graph. A group of six green-color curves located on the left of the graph, which are separated by a gap from the rest of the curves on the right, consist of insects treated with λ-cyhalothrin. This illustrates that this insecticide is a more potent toxicant than the other two, as the model and pairwise comparisons (see below) confirmed. On the right half of the graph, the curves for insects treated with chlorpyrifos (blue) and thiacloprid (red) are intermixed over a relatively wide dose-range, with an apparent stronger effect of chlorpyrifos over thiacloprid. A distinct feature is the blue (chlorpyrifos) curve located at around the 100-ng dose on a background of red (thiacloprid) curves. This chlorpyrifos curve corresponds to *G. molesta* males and departs from the other chlorpyrifos curves, including *G. molesta* females, which cluster around a lower dose range. This illustrates a strong effect of sex on insecticide response.

Table 1. Model comparison for the analysis of percent mortality at 24h as a function of insecticide dose. Models with increasing numbers of parameter interactions (insecticide type, dose, species and sex) were compared pairwise using the likelihood ratio test (LRT) and Akaike Information Criterion (AIC).

Model type	Number of parameters	AIC[a]	LRT p-value
Null	1	4000.5	-
Main effects	7	3088.2	<0.00001
Main effects and 2nd-order interactions	20	1249.5	<0.00001
Main effects and 2nd and 3rd-order interactions	32	623.04	<0.00001
Main effects and 2nd, 3rd and 4th-order interactions	36	600.13	<0.00001

[a] Models with lower AIC indicate a better model fit

Table 2. Analysis of deviance table for the model with all main effects and second, third and fourth-order interactions. Main effects are: insecticide type (chlorpyrifos, λ-cyhalothrin and thiacloprid), moth species (*C. pomonella*, *G. molesta* and *L. botrana*), sex and insecticide dose (ng of insecticide per mg of insect dry weight).

Model terms	Df [a]	Deviance	Resid. Df [b]	Resid. Dev [c]	Pr (>Chi)
NULL			107	3588.7	
species	2	2.32	105	3586.3	0.3127
sex	1	1.40	104	3584.9	0.2360
insecticide	2	7.84	102	3577.1	0.0198
dose	1	912.81	101	2664.3	<0.0001
species:sex	2	19.62	99	2644.7	<0.0001
species:insecticide	4	959.85	95	1684.8	<0.0001
species:dose	2	483.91	93	1200.9	<0.0001
sex:insecticide	2	344.06	91	856.8	<0.0001
sex:dose	1	2.27	90	854.6	0.1321
insecticide:dose	2	54.96	88	799.6	<0.0001
species:sex:insecticide	4	568.98	84	230.6	<0.0001
species:sex:dose	2	0.27	82	230.4	0.8744
species:insecticide:dose	4	73.77	78	156.6	<0.0001
sex:insecticide:dose	2	7.43	76	149.2	0.0244
species:sex:insecticide:dose	4	30.92	72	118.2	<0.0001

[a] Degrees of freedom

[b] Residual degrees of freedom

[c] Residual deviance

Figure 1. Effect of insecticide type (chlorpyrifos, λ-cyhalothrin, and thiacloprid) and dose (ng of insecticide per gram of insect dry weight; in logarithmic scale) on the proportion of moribund and dead adult males and females of *Cydia pomonella*, *Grapholita molesta*, and *Lobesia botrana* 24 h after treatment. The symbols indicate the observed values (N = 60-116), while the curves are the estimated values from probit regression.

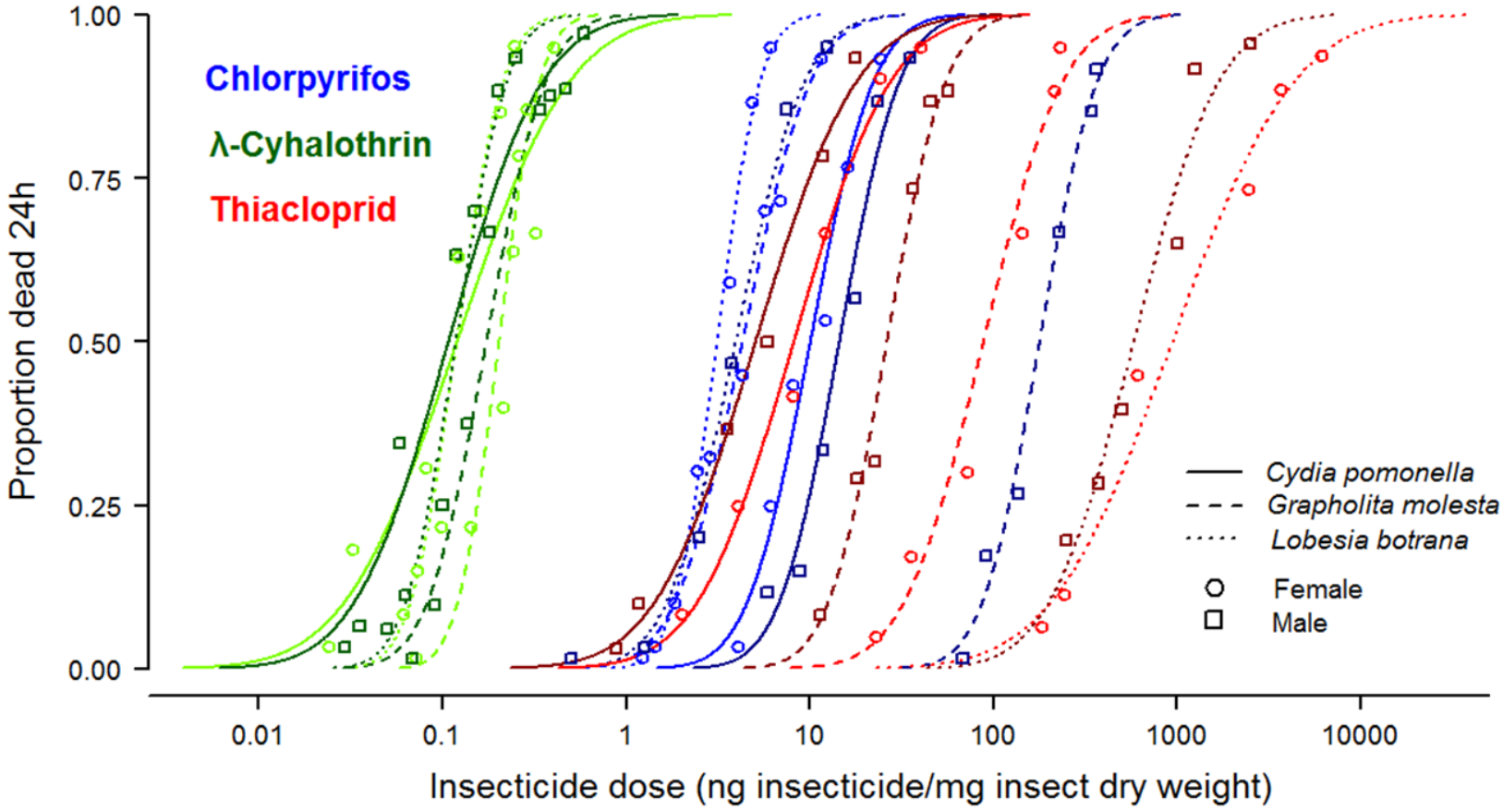

Table 3. Estimated intercepts and slopes of the probit regression models, and lethal doses LD_{10}, LD_{50} and LD_{90}, with their standard errors and 95 % confident intervals. Intercept and slope are "dimensionless" model parameter whereas LDs are in ng of insecticide per mg of adult insect dry weight. Estimations are provided by groups: for each insecticide (chlorpyrifos, λ-cyhalothrin and thiacloprid; independent of sex and species; top section), for each species (*C. pomonella*, *G. molesta* and *L. botrana*; within insecticide and independent of sex, middle section) and for each sex (within species and insecticide; bottom section). Heterogeneity factor (HF) indicates curve fit.

Insecticide	Species	Sex	n	Intercept		Slope		LD_{10}		LD_{50}		LD_{90}		HF[a]
				beta (SE)	(95 % CI)	beta (SE)	(95 % CI)	Dose	(95 % CI)	Dose	(95 % CI)	Dose	(95 % CI)	
Chlorpyrifos			2234	-0.63 (0.05)	(-0.72; -0.53)	0.58 (0.04)	(0.49; 0.66)	0.07	(0.04; 0.15)	12.30	(9.89; 15.28)	$2.09 \cdot 10^{+3}$	(937.64; $4.66 \cdot 10^{+3}$)	26.1
λ-Cyhalothrin			2364	2.40 (0.09)	(2.23; 2.59)	2.82 (0.11)	(2.63; 3.03)	0.05	(0.05; 0.05)	0.14	(0.13; 0.15)	0.40	(0.37; 0.44)	7.8
Thiacloprid			2204	-0.72 (0.06)	(-0.83; -0.60)	0.42 (0.03)	(0.37; 0.48)	0.05	(0.02; 0.13)	49.09	(36.54; 65.96)	$5.20 \cdot 10^{+4}$	($2.03 \cdot 10^{+4}$; $1.33 \cdot 10^{+5}$)	23.4
Chlorpyrifos	***C. pomonella***		720	-3.72 (0.26)	(-4.23; -3.23)	3.44 (0.23)	(3.00; 3.90)	5.12	(4.49; 5.85)	12.09	(11.27; 12.98)	28.55	(24.96; 32.66)	4.0
	G. molesta		744	-0.37 (0.09)	(-0.56; -0.19)	0.25 (0.06)	(0.14; 0.36)	$2.5 \cdot 10^{-4}$	($1.5 \cdot 10^{-6}$; $4.1 \cdot 10^{-2}$)	30.28	(13.20; 69.48)	$3.69 \cdot 10^{+6}$	($2.09 \cdot 10^{+4}$; $6.49 \cdot 10^{+8}$)	29.4
	L. botrana		770	-2.14 (0.14)	(-2.42; -1.87)	3.95 (0.25)	(3.49; 4.44)	1.65	(1.47; 1.84)	3.47	(3.25; 3.71)	7.33	(6.52; 8.24)	6.3
λ-Cyhalothrin	***C. pomonella***		736	2.13 (0.14)	(1.86; 2.41)	2.26 (0.13)	(2.01; 2.52)	0.03	(0.03; 0.04)	0.11	(0.10; 0.13)	0.42	(0.35; 0.51)	3.6
	G. molesta		862	3.09 (0.19)	(2.72; 3.47)	4.24 (0.26)	(3.73; 4.77)	0.09	(0.08; 0.10)	0.19	(0.18; 0.20)	0.37	(0.34; 0.41)	3.2
	L. botrana		766	4.41 (0.26)	(3.91; 4.93)	4.77 (0.27)	(4.25; 5.31)	0.06	(0.06; 0.07)	0.12	(0.11; 0.13)	0.22	(0.20; 0.24)	1.3
Thiacloprid	***C. pomonella***		725	-1.78 (0.13)	(-2.04; -1.53)	2.19 (0.14)	(1.92; 2.48)	1.68	(1.36; 2.07)	6.45	(5.75; 7.24)	24.77	(20.26; 30.28)	2.2
	G. molesta		733	-3.02 (0.24)	(-3.50; -2.55)	1.82 (0.14)	(1.55; 2.11)	9.01	(6.84; 11.86)	45.48	(40.12; 51.56)	229.63	(173.68; 303.60)	15.8
	L. botrana		746	-5.86 (0.38)	(-6.61; -5.13)	2.06 (0.13)	(1.81; 2.32)	167.34	(134.40; 208.37)	702.34	(623.71; 790.89)	$2.95 \cdot 10^{+3}$	($2.39 \cdot 10^{+3}$; $3.64 \cdot 10^{+3}$)	3.6
Chlorpyrifos	***C. pomonella***	**Female**	360	-3.70 (0.35)	(-4.42; -3.03)	3.69 (0.34)	(3.03; 4.39)	4.53	(3.82; 5.38)	10.08	(9.18; 11.08)	22.43	(18.85; 26.70)	1.3
		Male	360	-4.50 (0.42)	(-5.32; -3.72)	3.88 (0.35)	(3.21; 4.58)	6.77	(5.75; 7.97)	14.50	(13.24; 15.88)	31.05	(26.37; 36.56)	1.7
	G. molesta	**Female**	368	-2.17 (0.23)	(-2.65; -1.73)	3.43 (0.33)	(2.79; 4.11)	1.82	(1.49; 2.23)	4.31	(3.90; 4.77)	10.20	(8.51; 12.23)	0.7
		Male	376	-9.13 (0.74)	(-10.62; -7.71)	4.05 (0.33)	(3.43; 4.71)	86.29	(74.70; 99.69)	178.77	(163.55; 195.42)	370.36	(319.86; 428.84)	1.2
	L. botrana	**Female**	387	-2.67 (0.23)	(-3.14; -2.24)	5.39 (0.44)	(4.56; 6.28)	1.81	(1.62; 2.02)	3.13	(2.92; 3.35)	5.41	(4.83; 6.05)	0.3
		Male	383	-2.02 (0.20)	(-2.42; -1.65)	3.38 (0.30)	(2.83; 3.98)	1.65	(1.36; 2.01)	3.96	(3.53; 4.44)	9.48	(7.88; 11.40)	3.4
λ-Cyhalothrin	***C. pomonella***	**Female**	362	1.91 (0.19)	(1.54; 2.28)	2.07 (0.18)	(1.72; 2.44)	0.03	(0.02; 0.04)	0.12	(0.10; 0.14)	0.50	(0.37; 0.68)	4.8
		Male	374	2.39 (0.22)	(1.98; 2.84)	2.47 (0.20)	(2.10; 2.87)	0.03	(0.03; 0.04)	0.11	(0.09; 0.13)	0.36	(0.27; 0.46)	2.9
	G. molesta	**Female**	459	3.93 (0.41)	(3.18; 4.75)	5.67 (0.63)	(4.54; 6.94)	0.12	(0.10; 0.14)	0.20	(0.19; 0.22)	0.34	(0.31; 0.38)	2.8
		Male	403	3.00 (0.26)	(2.51; 3.50)	3.94 (0.32)	(3.33; 4.58)	0.08	(0.07; 0.10)	0.17	(0.16; 0.19)	0.37	(0.32; 0.43)	2.3
	L. botrana	**Female**	364	4.62 (0.41)	(3.85; 5.44)	5.01 (0.42)	(4.21; 5.87)	0.07	(0.06; 0.08)	0.12	(0.11; 0.13)	0.22	(0.19; 0.25)	1.9
		Male	402	4.26 (0.34)	(3.61; 4.94)	4.60 (0.35)	(3.93; 5.30)	0.06	(0.06; 0.07)	0.12	(0.11; 0.13)	0.23	(0.20; 0.26)	1.3
Thiacloprid	***C. pomonella***	**Female**	361	-2.20 (0.22)	(-2.64; -1.78)	2.41 (0.22)	(2.00; 2.85)	2.40	(1.83; 3.16)	8.18	(7.05; 9.50)	27.82	(21.71; 35.65)	0.6
		Male	364	-1.63 (0.16)	(-1.95; -1.32)	2.31 (0.20)	(1.93; 2.71)	1.41	(1.08; 1.84)	5.07	(4.33; 5.93)	18.19	(13.89; 23.84)	0.7
	G. molesta	**Female**	371	-5.71 (0.48)	(-6.68; -4.80)	2.94 (0.24)	(2.48; 3.42)	32.28	(26.16; 39.84)	88.17	(77.91; 99.77)	240.81	(198.04; 292.83)	1.8
		Male	362	-5.64 (0.53)	(-6.69; -4.63)	3.97 (0.36)	(3.28; 4.69)	12.52	(10.60; 14.79)	26.35	(24.14; 28.77)	55.45	(47.65; 64.53)	0.8
	L. botrana	**Female**	369	-5.69 (0.46)	(-6.63; -4.80)	1.91 (0.15)	(1.62; 2.22)	201.42	(147.00; 276.00)	941.34	(776.78; $1.14 \cdot 10^{+3}$)	$4.40 \cdot 10^{+3}$	($3.27 \cdot 10^{+3}$; $5.93 \cdot 10^{+3}$)	1.0
		Male	377	-7.58 (0.73)	(-9.03; -6.19)	2.74 (0.26)	(2.25; 3.26)	198.13	(154.86; 253.50)	581.53	(513.58; 658.47)	$1.71 \cdot 10^{+3}$	($1.37 \cdot 10^{+3}$; $2,13 \cdot 10^{+3}$)	1.9

[a] Heterogeneity factor = χ^2/dF

Intercepts, slopes, and LD estimates (Table 3) were compared among insecticides, species, and sexes producing a total of 103 statistical tests (Tables 4 and 5). To analyze the effect of insecticide (independently of species and sex), the intercept and slope of each resulting curve (including all data points, except acetone controls, for all insects treated with each insecticide) was compared with other insecticide resulting curves. A similar procedure was followed to analyze the effect of species (within insecticide and independent of sex) and sex (for each combination of insecticide and species).

Curves that have the same slope and intercept are considered to be equal, curves that have same slope but different intercept are considered parallel, and all other types of curves are neither equal nor parallel. In our experiment, equal curves occurred only in the comparison between sexes, in *C. pomonella* treated with chlorpyrifos and λ-cyhalothrin, and in *L. botrana* treated with λ-cyhalothrin (Table 4). Parallel curves were observed only in the comparison between species within insecticide, independently of sex. The curves of the three species treated with thiacloprid were parallel, and so were the curves of *G. molesta* and *L. botrana* treated with λ-cyhalothrin and the curves of *C. pomonella* and *L. botrana* treated with chlorpyrifos. All other curves were neither parallel nor equal. λ-cyhalothrin had lower intercept and higher slope than the other two insecticides, which did not differ from each other in these parameters (Table 4).

A second approach to analyze curves is by their LDs (Table 5). The maximum LD_{50} difference between two insecticides was 7800-fold, corresponding to *L. botrana* females treated with λ-cyhalothrin and thiacloprid. Between species, the maximum LD_{50} difference was 115-fold, corresponding to *L. botrana* and *C. pomonella* females treated with thiacloprid. Finally, the maximum difference between sexes was 41.5-fold, corresponding to *G. molesta* treated with chlorpyrifos (Table 3). Lethal doses LD_{50} and LD_{90} differed in all pairwise comparisons between insecticides, whereas LD_{10} did not (Table 5). Lethal dose comparisons among species were significant in 25 out of 27 pairwise tests. All the exceptions were in the insecticide λ-cyhalothrin, between *C. pomonella* and *L. botrana* for LD_{50} and between *C. pomonella* and *G. molesta* for LD_{90} (Table 5). Sex differences in LD occurred in eight out of nine comparisons in each chlorpyrifos and thiacloprid, but were rare in λ-cyhalothrin (Table 5). Because females of all three species were heavier than males (Table S1) it was expected that females would be less susceptible to the insecticides than males. This prediction was observed in two of the insecticides, λ-cyhalothrin and thiacloprid; however, in chlorpyrifos, the males of all three species had higher LD_{50} than females, with the notable difference of 41.5-times lower susceptibility for *G. molesta* males than females, as mentioned above (Table 3).

Table 4. Pairwise comparison of intercepts and slopes between a) insecticides (chlorpyrifos, λ-cyhalothrin and thiacloprid; independent of species and sex; top section), b) species (*C. pomonella*, *G. molesta* and *L. botrana*; within insecticide and independent of sex; middle section), and c) sex (within species and insecticide; bottom section). The numbers represent the difference between each pair of estimated values, and are followed in brackets by the p-values of these differences (*Tukey* test, p<0.05, after GLM).

Insecticide	**Species**	**Sex differences**		**Species differences**				**Insecticide differences**			
				C. pomonella		***G. molesta***		**Chlorpyrifos**		**λ-Cyhalothrin**	
		Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope
Chlorpyrifos	***C. pomonella***	0.80 (0.14)	-0.19 (0.71)	--	--	--	--	--	--	--	--
	G. molesta	6.95 (<0.0001)	-0.63 (0.18)	-3.35 (<0.0001)	3.18 (<0.0001)	--	--				
	L. botrana	-0.65 (0.03)	2.02 (<0.0001)	-1.58 (<0.0001)	-0.52 (0.13)	1.76 (<0.0001)	-3.70 (<0.0001)				
λ-Cyhalothrin	***C. pomonella***	-0.49 (0.10)	-0.41 (0.14)	--	--	--	--	-3.03 (<0.0001)	-2.25 (<0.0001)	--	--
	G. molesta	0.93 (0.05)	1.73 (0.01)	-0.96 (<0.0001)	-1.98 (<0.0001)	--	--				
	L. botrana	0.37 (0.49)	0.42 (0.45)	-2.28 (<0.0001)	-2.51 (<0.0001)	-1.32 (<0.0001)	-0.53 (0.16)				
Thiacloprid	***C. pomonella***	-0.57 (0.04)	0.10 (0.73)	--	--	--	--	0.09 (0.24)	0.15 (0.003)	3.12 (<0.0001)	2.40 (<0.0001)
	G. molesta	-0.08 (0.91)	-1.03 (0.02)	1.25 (<0.0001)	0.37 (0.07)	--	--				
	L. botrana	1.89 (0.03)	-0.83 (0.006)	4.08 (<0.0001)	0.14 (0.48)	2.84 (<0.0001)	-0.24 (0.22)				

Table 5. Pairwise comparison of lethal doses LD_{10}, LD_{50} and LD_{90} between a) insecticides (chlorpyrifos, λ-cyhalothrin and thiacloprid; independent of species and sex; top section), b) species (*C. pomonella*, *G. molesta* and *L. botrana*; within insecticide and independent of sex; middle section), and c) sex (within species and insecticide; bottom section). Numbers are the differences of the estimated values, and in brackets the p-values of these differences (Z-score, $p<0.05$, after GLM).

Insecticide	**Species**	**Sex differences**			**Species differences**						**Insecticide differences**					
					C. pomonella			***G. molesta***			**Chlorpyrifos**			**λ-Cyhalothrin**		
		LD_{10}	LD_{50}	LD_{90}	LD_{10}	LD_{50}	LD_{90}	LD_{10}	LD_{50}	LD_{90}	LD_{10}	LD_{50}	LD_{90}	LD_{10}	LD_{50}	LD_{90}
Chlorpyrifos	***C. pomonella***	-2.24 (0.00)	-4.42 (0.00)	-8.62 (0.01)	--	--	--	--	--	--	--	--	--	--	--	--
	G. molesta	-84.47 (0.00)	-174.46 (0.00)	-360.16 (0.00)	5.12 (0.00)	-18.19 (0.03)	$-3.69\cdot10^{+3}$ (0.00)	--	--	--						
	L. botrana	0.16 (0.42)	-0.83 (0.00)	-4.07 (0.00)	3.48 (0.00)	8.62 (0.00)	21.22 (0.00)	-1.65 (0.00)	26.81 (0.00)	$3.69\cdot10^{+3}$ (0.00)						
λ-Cyhalothrin	***C. pomonella***	-0.00 (0.50)	0.01 (0.37)	0.14 (0.10)	--	--	--	--	--	--	0.02 (0.31)	12.16 (0.00)	$2.09\cdot10^{+3}$ (0.00)	--	--	--
	G. molesta	0.04 (0.00)	0.03 (0.00)	0.03 (0.43)	-0.06 (0.00)	-0.07 (0.00)	0.05 (0.27)	--	--	--						
	L. botrana	0.00 (0.47)	0.00 (0.88)	-0.01 (0.62)	-0.03 (0.00)	-0.00 (0.52)	0.20 (0.00)	0.03 (0.00)	0.07 (0.00)	0.15 (0.00)						
Thiacloprid	***C. pomonella***	0.99 (0.01)	3.11 (0.00)	9.63 (0.02)	--	--	--	--	--	--	0.03 (0.48)	-36.79 (0.00)	$-4.99\cdot10^{+4}$ (0.00)	0.00 (0.90)	48.95 (0.00)	$-5.20\cdot10^{+4}$ (0.00)
	G. molesta	19.76 (0.00)	61.82 (0.00)	185.37 (0.00)	-7.33 (0.00)	-39.03 (0.00)	-204.86 (0.00)	--	--	--						
	L. botrana	3.28 (0.94)	359.81 (0.00)	$2.69\cdot10^{+3}$ (0.00)	-165.66 (0.00)	-695.89 (0.00)	$-2.92\cdot10^{+3}$ (0.00)	-158.33 (0.00)	-656.86 (0.00)	$-2.72\cdot10^{+3}$ (0.00)						

Discussion

Few dose-mortality studies have explored, as we have done in here, the combined effect of insecticides with different modes of action, on adults of several insect species, while simultaneously taking into account the effect of sex. Large mortality differences between insecticides (maximum 7,800-fold for LD_{50}) were followed by much lower, yet important, differences between species (115-fold) and sexes (41.5-fold), demonstrating that each of these three factors has a critical effect on adult mortality. Although these factors were not independent from each other, as shown by significant second-order to fourth-order model interactions, our results highlight the need to take into account sex as a very significant factor in the context of insecticide and species differences in dose-mortality studies of adult moths.

Insecticide dose-mortality curves of adult Lepidoptera are poorly represented in the scientific literature, probably because most insecticides are mainly designed to kill larval stages; however, neurotoxic insecticides could affect other life stages. Two studies using adult *G. molesta* and *C. pomonella,* and several studies on larva *C. pomonella* have shown, as we do in here, a stronger effect by pyrethroids than by other insecticide types (Linn and Roelofs 1984, Pasquier and Charmillot 2003, Mota-Sánchez *et al.* 2008, Rodríguez *et al*. 2012, Wu *et al*. 2015). Time effect after initial knock-down did not appear to be greatly affected by species or sex, but mostly by insecticide type. The 48-h recovery with thiacloprid (Figure S3), may involve the induction of detoxification enzymes (Terriere 1984), whereas the increased mortality of chlorpyrifos at 48h (Figure S3), may be related to the oxidative desulfurization of the P=S group to its corresponding P=O analog by cytochrome P450 monooxygenases, which would increase the toxicity of this insecticide over time (Yu 2008). The recovery observed in the laboratory may not be realized in the field because moribund insects are probably more susceptible to predation and environmental stress than nonintoxicated individuals.

Species differences in insecticide resistance could be explained either by the activity or quantity of insect-degrading enzymes that metabolize the insecticide before it arrives to the target protein, or by mutations at the insecticide's target site that lower its effect (Nauen and Denholm 2005). Resistance by degrading enzymes should be far more common than mutations at the target site because, at least in the case of neurotoxic insecticides, the target sites are proteins which play fundamental roles in nerve impulse generation and transmission, processes that are fairly conserved across lineages and that should be under strong stabilizing selection and resistant to mutations (Li *et al*. 2007). Changes in the activity or quantity of the main detoxifying enzyme

types (mixed-function oxidases [MFO], esterases [EST], and glutathione S-transferases [GST]) have been associated with resistance to a large number of insecticides in *C. pomonella* (Reyes *et al*. 2007, 2011; Morales *et al*. 2016). In addition, point mutations in the sodium channel and in the AChE have also been reported in this species as mechanisms of resistance (Reyes *et al*. 2007, Kanga *et al*. 2001). There are comparatively fewer reports of insecticide resistance in *G. molesta* (Glass 1960, Kanga *et al*. 2003, Jones *et al*. 2011) and *L. botrana* (Civolani *et al*. 2014). Lower cuticular penetration of carbofuran may explain resistance to this insecticide in *G. molesta* (Kanga *et al*. 1997).

For phytophagous insects, environmental toxins consist mainly of secondary plant metabolites acquired through ingestion (Li *et al*. 2007), and the function of detoxification enzymes is to make toxins more water soluble (Terriere 1984). It is plausible, then, that species using different food sources may have different detoxifying-enzyme activity levels (Yu 1982), and this may explain why they show different tolerance to insecticides. *Cydia pomonella*'s main agricultural host is apple fruit, *G. molesta*'s is peach shoots, and *L. botrana*'s is the flower and fruit of grape vines. The diversity of host species and host organs consumed by the larvae of these moth species may select for different detoxification mechanisms. For example, *C. pomonella*'s second major agricultural host, walnut fruit, produces high quantities of the naphthoquinone juglone, which is toxic to several insect species but not to the larvae of *C. pomonella* (Piskorski and Dorn 2011). Interestingly, the larva of *G. molesta*, which does not feed on walnuts, is also able to metabolize juglone (Piskorski *et al*. 2011), so ecological factors may not be the only determinants of the quantity and type of detoxification enzymes produced by each species.

One of the most striking findings of our study is the relatively large difference in susceptibility between males and females, and the higher tolerance of males to chlorpyrifos, in all three species. Higher male tolerance has been reported before in *G. molesta*, where the LC_{50} of females was between 3 and 10 times lower than the LC_{50} of males to three different organophosphate insecticides azinphos-methyl, malathion, and parathion-methyl; (Shearer and Usmani 2001). We confirm the higher male tolerance to organophosphates in *G. molesta*, and in addition, we show that higher male tolerance to organophosphates also occurs in the other two tortricids, *C. pomonella* and *L. botrana*. Higher female susceptibility seems to be restricted to organophosphates because, besides our results, *G. molesta* females are less susceptible to carbamates than males (Kanga *et al.* 2001, Shearer and Usmani 2001). The larger LC for females compared with males observed for the neonicotinoid thiacloprid could be explained by the larger body mass of females, but after correcting by body mass, the LD of females was still larger than that of males. This suggests that additional factors, such as differences in enzymatic activities

and quantities, might be playing a role in this case. The sex differences with chlorpyrifos (females more susceptible) cannot be accounted by body weight, and it is very likely that detoxification enzymes are involved in these differences. de Lame, *et al.* (2001) showed that the larger resistance of male *G. molesta* to three organophosphate insecticides (paraoxon-methyl, malaoxon, and diazinon-o-analogue) was the result of sex differences in both, degrading-enzyme activity levels and susceptibility of AChE to the insecticide. Kanga, *et al.* (2001) reported that Ace-1 insensitivity, the major mechanism of carbamate resistance in *G. molesta*, is both sex-linked and recessive. Point mutations are probably not involved in sex differences because both sexes share the same chromosomes, except for the W sex chromosome which is only present in females and codifies few gene products (Nguyen *et al.* 2013). A neo-sex chromosome in tortricids emerged from the fusion of the Z chromosome (the other sex chromosome, present in both sexes) and an autosome, and it bears genes encoding for detoxification enzymes (Nguyen *et al.* 2013). It has been suggested that the neo-sex chromosome may be responsible for both, a rapid evolution of this clade and a quick selection response to insecticides (Nguyen *et al.* 2013). Indeed, the expression of AChE genes is larger in males than in females of *C. pomonella* and *L. botrana* (Nguyen *et al.* 2013). It remains to be tested if the neo-sex chromosome is also responsible for the differential sex response of the three tortricid species to insecticides.

The results of our study have practical implications. First, our dose-mortality curves for susceptible strains provide a diagnostic baseline to test possible resistance cases in field populations using adult insects, as in other susceptibility-resistance studies that use larvae or adult insects (Pasquier and Charmillot 2003, Reyes *et al.* 2007, Jones *et al.* 2011, Wu *et al.* 2015). Second, resistance is expressed in both adults and larvae (Varela *et al.* 1993), but the use of adult instead of larvae in this kind of studies is advantageous because of the easier, faster, and cheaper procedure with adults than with larvae (Kanga *et al.* 1997). For example, adult individuals can be easily obtained in the field with monitoring traps (Bosch *et al.* 2016). Third, our dose-mortality results help estimate sublethal doses which could affect the behavior and physiology of these insects (Haynes 1988). Finally, differential sex response to insecticides should be considered in integrated pest management programs. Shearer and Usmani (2001) indicate that male pheromone trap catches may be unfit to monitor threshold population levels if males are less susceptible than females to insecticide.

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Supplementary material

Table S1. Differences in adult body dry weight by species and sex. Means followed by different letters are significantly different (*Tukey* test, $p<0.05$, after ANOVA).

Species	Sex	Mean	±	SEM (mg)	
C. pomonella	**Female**	12.41	±	0.63	a
	Male	8.47	±	0.34	b
G. molesta	**Female**	3.54	±	0.11	c
	Male	2.18	±	0.08	d
L. botrana	**Female**	4.09	±	0.10	c
	Male	1.98	±	0.04	d

Figure S1. Comparison of insecticide concentration (in ng; coloured curves) and dose (in ng of insecticide/mg of insect dry weight; grey colour curves, same as in Fig. 1 of the main text) for the 24h mortality of the three insecticides on the three moth species. Colours and symbols as shown in Fig. 1 of the main text, but in the present figure the x-axis is absolute insecticide concentration, instead of dose. As predicted (Figure S2), dose-response curves are displaced to the left from their homologous concentration-response curves, and the displacement is stronger for heavier insects (e.g., *C. pomonella* and the females)

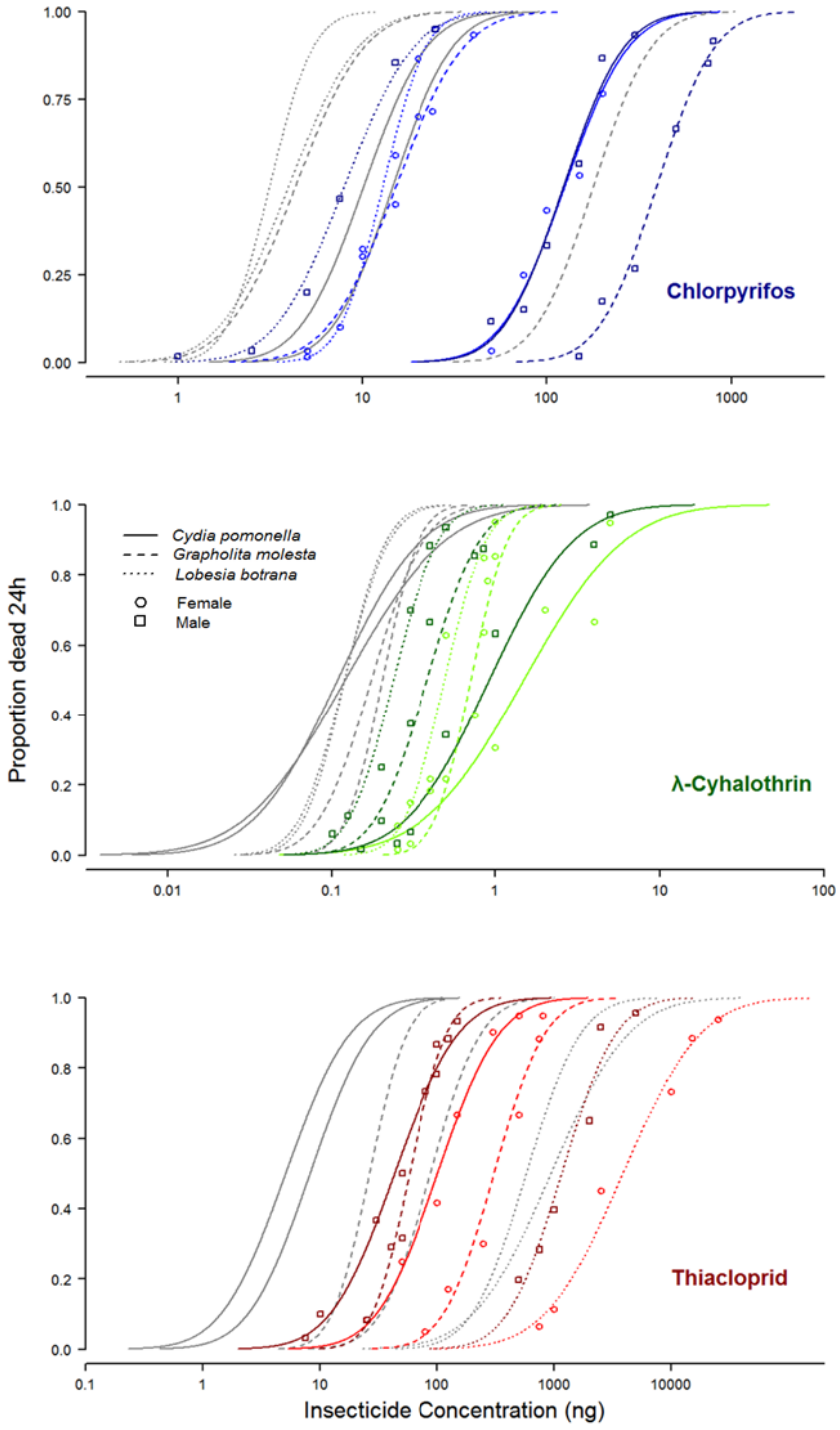


Figure S2. Theoretical relative position of mortality curves estimated with the absolute insecticide concentration (top chart) or the quantity of insecticide per mass unit (i.e., dose; bottom chart). The larger animal, *Cydia pomonella*, is represented with a continuous curve and the smaller animal, *Lobesia botrana*, is represented with a dashed curve. In case "A" the larger species is as susceptible as the smaller species in the concentration scale (top), which occurs if the larger species is more susceptible than the smaller species per unit of mass, as is shown in the dose scale on the bottom. In case "B", the large species is more susceptible than the smaller species in the concentration scale, and, correspondingly, in the dose scale this difference should be larger. In cases "C" and "D" the smaller species is more susceptible than the larger species in the concentration scale, which indicates that the smaller species is either as sensitive as, or less sensitive than the larger species per unit of mass, as shown in the dose scale.

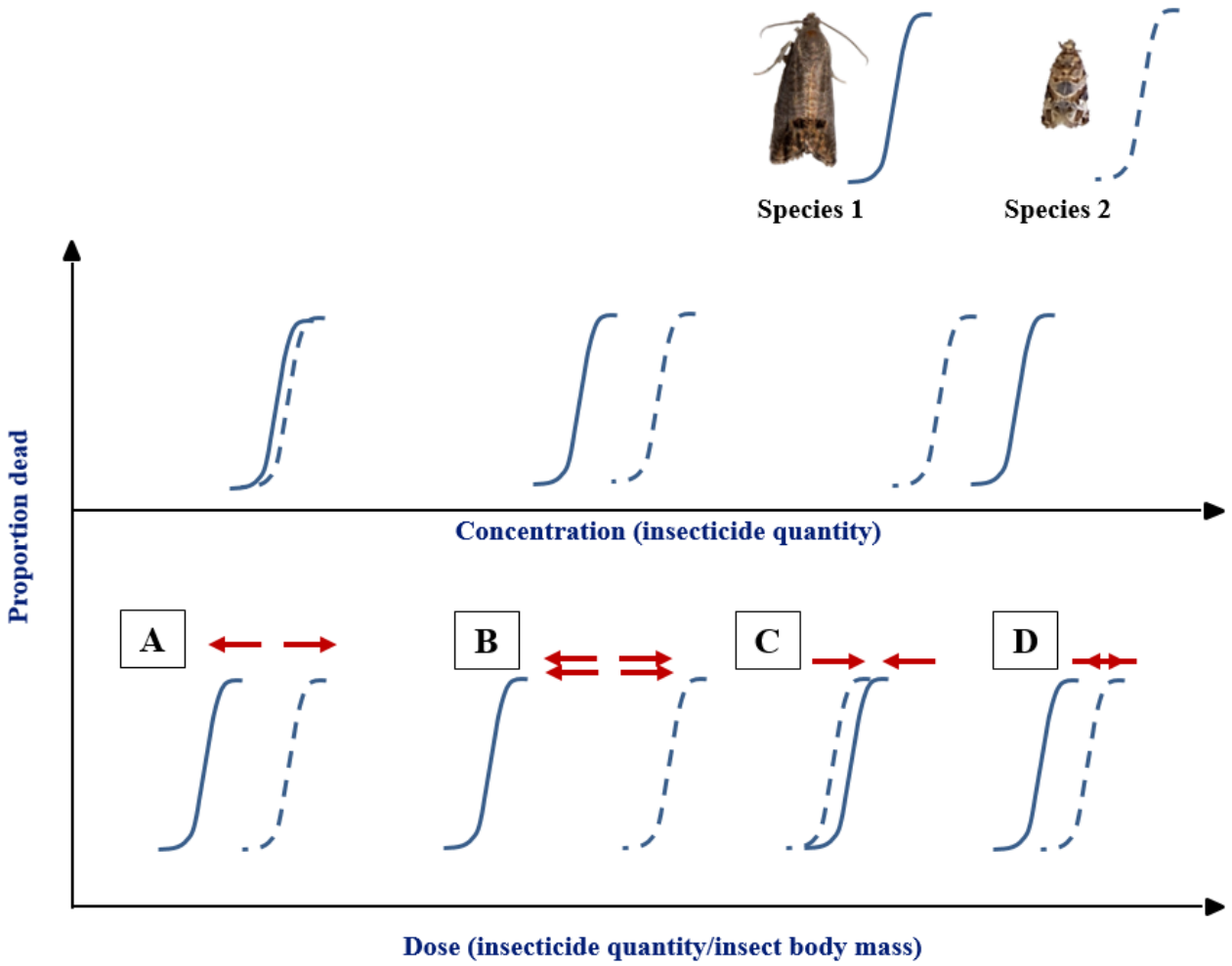
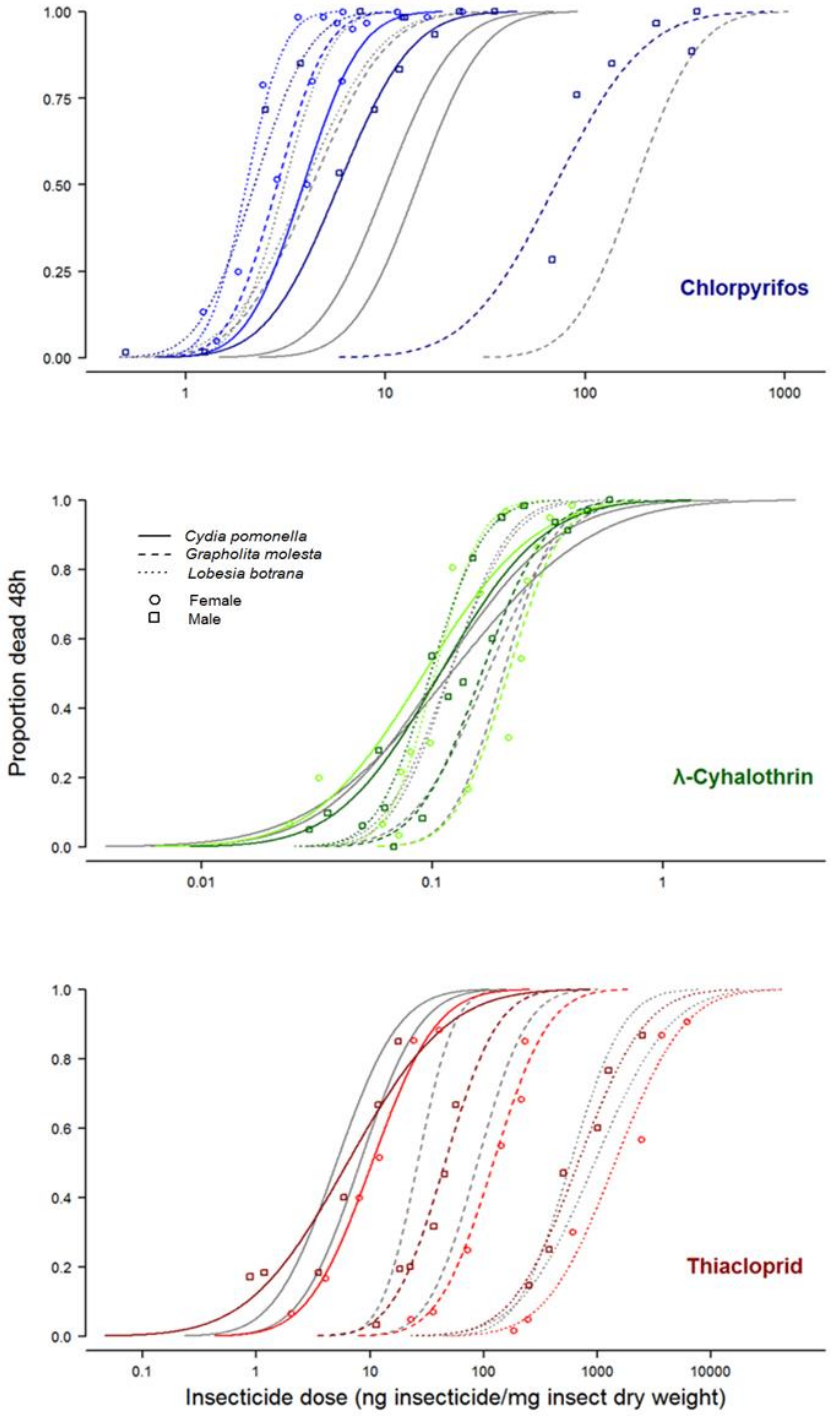


Figure S3. Effect of time after insecticide application on the proportion of mortality (dead + moribund). Grey curves represent mortality at 24 h (as shown in full detail in Fig. 1 of the main text). Colour curves represent mortality at 48 h. For thiacloprid, the curves experimented a displacement to the right in the dose axis, which indicates that part of the insects scored as moribund at 24 hours with this insecticide recovered at 48 hours. With chlorpyrifos the opposite was observed, in other words, mortality increased from 24 hours to 48 hours.



2

ENZYMATIC DETOXIFICATION STRATEGIES FOR NEUROTOXIC INSECTICIDES IN ADULT TORTRICIDS

ABSTRACT

The role of the three most important metabolic enzyme families [carboxylesterases (EST), glutathione-S-transferases (GST), and mixed-function oxidases (MFO)] in the detoxification of three neurotoxic insecticides [chlorpyrifos, λ-cyhalothrin, and thiacloprid] was studied on adult males and females of three tortricid moth pests [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller)]. The first approach was to determine if inhibition of these metabolic enzyme activities influence insecticide mortality, and the second approach was to quantify EST, GST and MFO activity in individuals treated with their respective enzyme inhibitors: S,S,S, tributyl phosphorotrithioate (DEF), diethyl maleate (DEM), and piperonyl butoxide (PBO). The mortality assay showed that phase I enzymatic activities (EST and MFO) were active in both sexes of the three species, whereas phase II enzymes (GST) were only active in *G. molesta*. In addition, EST played a role in the detoxification of all three insecticides and showed the highest differences between species, and MFO was involved in the detoxification of thiacloprid and the activation (i.e., bio-activation) of chlorpyrifos in both sexes of the three species. Enzymatic activity of control individuals showed differences among species and between sexes. In general, *L. botrana* females had the highest enzymatic activities. Enzymatic activities of individuals treated with enzyme inhibitors revealed significant inhibition of EST by DEF, whereas MFO and GST were not inhibited by PBO or DEM, respectively. In contrast, DEM enhanced GST activity in *G. molesta* males (i.e., induction). In addition, an unexpected inhibition kinetic was observed with PBO in *G. molesta* males where a slight inhibition occurred from 4-h post treatment onwards, whereas in *L. botrana* males a 9.5-fold activation (i.e., induction) appeared 16-h post-treatment. Our results indicate that the diverse

effect of enzyme inhibitors on insecticide synergism is related to the specific interactions among insecticides, detoxification enzymes and the enzyme inhibitors in each sex and species.

KEY WORDS: insecticide inhibitor, neurotoxic insecticide, detoxification, Tortricidae, adult, sex.

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Introduction

The capability to degrade toxic substances is essential for the survival of insect pests in the constantly changing chemical environment of the agroecosystem. All insects have detoxification abilities that vary among species and developmental stages (Yu and Hsu 1993). The three most important detoxification enzyme families in insects are cytochrome P450 monooxygenases (P450) [also called mixed-function oxidases (MFO)], carboxylesterases (EST), and glutathione-S-transferases (GST). These enzyme families are subject to gene amplification, overexpression, and coding sequence variations that could modify their detoxification abilities (Li *et al.* 2007). Metabolism of toxic compounds involves two phases, the first is the addition of a polar group to the substrate or its cleavage, and the second is the addition of sugar, aminoacid, sulphate or phosphate groups to the products resulting from phase I, in case this product is not hydrophilic enough to be excreted (B-Bernard and Philogène 1993). MFO and EST are involved in phase I, whereas GST is involved in phase II (B-Bernard and Philogène 1993).

Chemicals that inhibit detoxification enzymes could reduce the defensive system of the insect and thus are used in agriculture to lower insecticide lethal doses, extend the number of target species, or restore insecticide activity against resistant insect populations (B-Bernard and Philogène 1993, Ishaaya 1993). In addition, insecticide synergists (i.e., enzyme inhibitors) are used in research on the detoxification mechanisms, resistance, and mode of action of insecticides (Metcalf 1967). The most common inhibitors for EST, GST and MFO enzymes are S,S,S, tributyl phosphorotrithioate (DEF), diethyl maleate (DEM), and piperonyl butoxide (PBO), respectively (B-Bernard and Philogène 1993).

In a previous study (Navarro-Roldán *et al.* 2017), we reported mortality of adult males and females 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 significant differences among species and insecticides, but 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). This last result was unexpected given that females are larger than males and therefore should be less susceptible than them. Higher female susceptibility to organophosphates had been reported previously only in *G. molesta* (de Lame *et al.* 2001), but not (as far as we know) in other moth species.

Our main objective in the present study was to study the metabolic mechanisms involved in the defence of these insect species to the three insecticides. Two approaches were used to this end. The first one was to determine if enzymatic inhibition with DEF, DEM and PBO affect insecticide mortality, and the second one was to quantify EST, GST and MFO activity in individuals treated with the respective enzyme inhibitors.

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 (mortality bioassays) were maintained at 25 ± 1 ºC, and those used in Avignon (quantification of enzymatic activity) were shipped from Lleida as larvae or pupae and were maintained at 27.5 ± 0.5 ºC, both under a 16:8 hour light:dark photoregime. Pupae were separated by sex and checked daily for adult emergence, except for *C. pomonella*, which was sexed at the adult stage, also on a daily basis. Zero to 24-h post-emergence adults were treated 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 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 from Sigma-Aldrich, Spain). All the dilutions used in the bioassays were prepared from pure compound using acetone as solvent (CHROMASOLV®, for HPLC, ≥ 99.9 %, Sigma-Aldrich, Spain). Dilutions were stored in 2- or 4-ml acetone-rinsed glass vials at 7 ºC. The same stock of acetone used to prepare the dilutions was also used as the negative control treatment.

Effect of enzyme-inhibitors on insecticide mortality. Insecticides were applied at LC_{50} (Navarro-Roldán *et al.* 2017), and the inhibitors were applied at the highest concentration that produced the same mortality as solvent (Table 1), as determined in a preliminary test. The treatments that combined insecticide and inhibitor used mixtures of the two compounds at the

same concentration as when applied alone. The 96 treatment groups [solvent, enzyme-inhibitor, insecticide (LC_{50}), and insecticide (LC_{50}) + enzyme-inhibitor, species and sex], were tested with between 60 and 115 individuals per treatment. 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.

One or two adults were placed in 10-ml test tubes, anesthetized with a brief (10 sec) flow of industrial grade CO_2 and placed upside down under a stereo microscope. A 1-µl test solution (see below) 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 then transferred 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 with 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 in the rearing room.

Mortality was recorded 24 h post-treatment. Adults observed with the naked eye were 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.

Table 1. Concentration of enzyme inhibitor and insecticide used.

Species	Sex	Inhibitor (mg)[a]			Insecticide (ng)[b]		
		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

a Highest concentration of inhibitor that did not produce significantly larger mortalities than solvent (N = 30, *Fisher exact test*). A range of three to fifteen concentrations per inhibitor, species and sex, were used.

b Insecticide LC_{50} (Navarro-Roldán *et al.* 2017)

Activity of detoxification enzyme families. Enzymatic activity was quantified by measuring the quantity of artificial substrate that converted into 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 MFO. Abdomens were homogenized in the reaction

solution (see below) in 1.5-ml eppendorf tubes held in ice. MFO analysis was performed in the same day on fresh tissue and, whereas GST and EST samples were frozen (-80°C) and analysed at once to reduce sampling error (Reyes 2007). The amount of product was determined by absorbance (EST and GST) or fluorescence (MFO) 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 so that they fell within the range of the standard curves. *C. pomonella* female samples were diluted 10-fold, *L. botrana* male samples were undiluted and the rest were diluted 5-fold.

Carboxylesterases (EST). Abdomens were homogenized in 110 µl of Hepes buffer (50 mM, pH 7) and centrifuged at 10000 g for 15 min at 4 °C, and supernatants were stored at -80 °C until use. To measure total non-specific EST activity, 1 µl of supernatant or 1 µl of Hepes buffer (50 mM, pH 7.0) for blanks (3 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 and Weber 1972) in Hepes buffer (50 mM, pH 7.0). 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. Absorbance of the reaction product (α-Naphtol) was meassured at 590 nm. EST activity was expressed as nmol α-Naphtol /min/mg of total protein using a standard curve of α-Naphtol (0-18 nmoles/well). Between 55-59 insects per species and sex were used.

Glutathione S-Transferase (GST). 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 (3 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 and Stumpf 2002) plus 2.52 mM glutathione (GSH) in Hepes buffer (50 mM, pH 7). After 2 min of incubation at 25 °C absorbance of the reaction product (CDNB-glutathione (GSH) conjugate) was measured at 340 nm in the kinetic mode (every 30 sec for 3 min). Since the CDNB-glutathione 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.

Mixed-Function Oxidase (MFO). Abdomens were homogenised in of an incubation solution containing 7-ethoxycoumarin O-deethylation (ECOD) (0.4 mM) substrate (Bouvier *et al.* 2002) in Hepes buffer (50 mM, pH 7). 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 10000 g for 5 min at room temperature. The supernatant (approximately 200 µl) was placed in black microplates (96-wells, Corming Costar®, New York, U.S). Fluorescence of the enzymatic product [7-hydroxycoumarin (7-HC)] was quantified with 380 nm excitation and 465 nm emission filters. MFO activity was expressed as pg of 7-HC/min/mg of total protein by using a standard curve of 7-HC (0.5-4.5 nmoles/well). Between 57-60 insects per species and sex were used.

Effect 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 EST and GST (17-40 DEF and DEM-treated individuals respectively), and 1 h for MFO (16-25 PBO-treated individuals). Because we observed no effect of PBO on MFO activity 1h after application (see Results), and time has been shown to play a role in enzymatic inhibition (Young *et al.* 2005, 2006, Bingham *et al.* 2008), an additional test explored the effect of time after inhibitor application on MFO activity in *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, and there was an acetone control at 1 h (10-35 individuals per treatment).

Data analysis. All the statistical analyses were performed in R software (R Core Team 2016). We used generalized linear models (GLM) with Gaussian family functions for continuous variables (enzymatic activities), or binomial family functions for binomial variables (percentage of mortality). The glht() and/or the predictmeans() functions performed *Tukey´s* multiple pairwise comparisons. 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 between treatments it indicates a p-value < 0.05.

Results

Effect of enzyme-inhibitors on insecticide mortality. As expected, the enzyme-inhibitors alone did not increase mortality compared with solvent alone (Table 2), which shows that the inhibitor doses used were not toxic on themselves. DEF, the EST inhibitor, increased insecticide mortality in all cases, except in *L. botrana* females and *C. pomonella* males treated with thiacloprid (Table 2). DEM synergized the three insecticides but only in *G. molesta* (except in females treated with λ-cyhalothrin). PBO had the same effect on the three species and both sexes: it synergized thiacloprid, decreased the effect of chlorpyrifos, and did not affect λ-cyhalothrin (except in *G. molesta* females and *L. botrana* males).

Activity of detoxification enzyme families. EST, GST and MFO activities in abdominal extracts of susceptible male and female *C. pomonella*, *G. molesta* and *L. botrana* are shown in Figure 1. In general, *L. botrana* females had significantly higher levels of the three enzymes than any other insect group, except *L. botrana* males for EST and *G. molesta* males and females for GST. The only sex-differences observed were higher GST and MFO levels in *L. botrana* females than in males. EST activity was significant higher in *C. pomonella* than in *G. molesta*, whereas the opposite was in true for GST activity. MFO 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, MFO activity in *L. botrana* and *G. molesta* males was significantly higher than in *C. pomonella* males.

Effect of enzyme-inhibitors on the activity of detoxification enzymes. DEF inhibited EST activity in both sexes of the three species (up to 15.75-fold in *L. botrana* males), whereas DEM increased GST activity in *G. molesta* males (i.e., induction), and PBO did not have any effect on MFO activity (Table 3). A significant reduction of MFO activity was obtained 4 h after PBO treatment in *G. molesta* females, which was not significant different from control at 1 h, whereas in *G. molesta* males activity was significant reduced from 4-h onwards (Figure 2). Interestingly, MFO activity increased significantly with time in *L. botrana* (i.e., induction). In both sexes the effect started to be different at 12-h post-treatment. In males there was a 9.5-fold increase at 16-h after treatment (Figure 2).

Table 2. Mortality (%) of adult males and females of *C. pomonella*, *G. molesta* and *L. botrana* 24 h after treated with solvent acetone or insecticide (chlorpyrifos, λ-cyhalothrin or thiacloprid), with and without detoxification enzyme inhibitors (DEF, DEM and PBO). P-values indicate the difference between the treatment with enzyme inhibitor (columns 2, 4 and 6) and the treatments without enzyme inhibitor (column 1). (*Tukey*, after GLM).

Female species	**Insecticide**	**Inhibitor**						
		no inhibitor	DEF	p-value	DEM	p-value	PBO	p-value
	no insecticide	1.67	1.67	1.000	1.67	1.000	11.67	0.215
C. pomonella	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
	no insecticide	0.00	3.33	1.000	11.67	1.000	3.33	1.000
G. molesta	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
	no insecticide	1.74	6.67	0.371	1.67	1.000	0.00	1.000
L. botrana	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
Male species	**Insecticide**	**Inhibitor**						
		no inhibitor	DEF	p-value	DEM	p-value	PBO	p-value
	no insecticide	0.00	0.00	1.000	3.33	1.000	7.69	1.000
C. pomonella	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
	no insecticide	0.00	6.67	1.000	11.67	1.000	8.33	1.000
G. molesta	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
	no insecticide	0.00	14.29	1.000	0.00	1.000	5.71	1.000
L. botrana	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

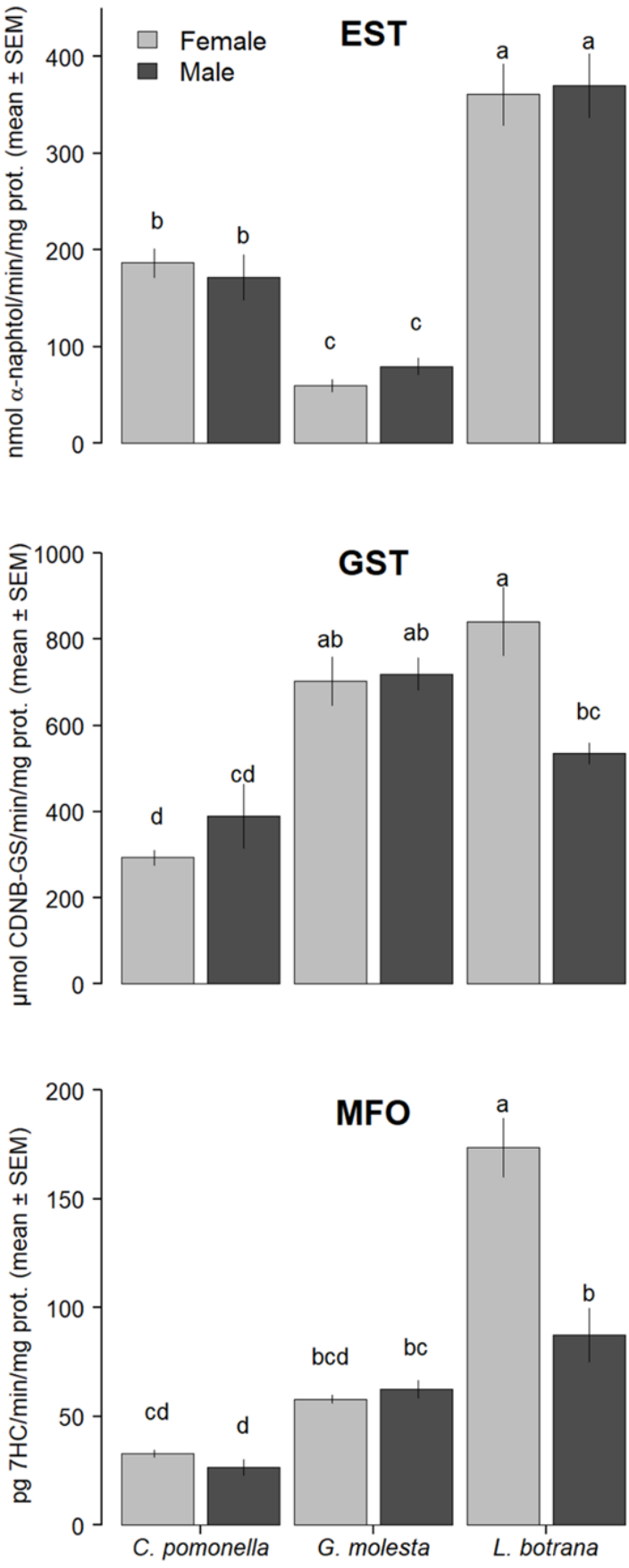


Figure 1. EST, GST and MFO enzymatic activities in the abdomens of adult *C. pomonella*, *G. molesta* and *L. botrana* from susceptible laboratory strains. Different letters indicate significant differences among bars for each enzymatic activity group (P<0.05, *Tukey* after GLM).

Table 3. EST, GST and MFO activities on adult abdomens of susceptible male and female *C. pomonella*, *G. molesta* and *L. botrana* adults, after application of DEF (24h), DEM (24h) and PBO (1h), respectively. P-values indicate differences between inhibitor-treated and control for each group (*Tukey*, after GLM).

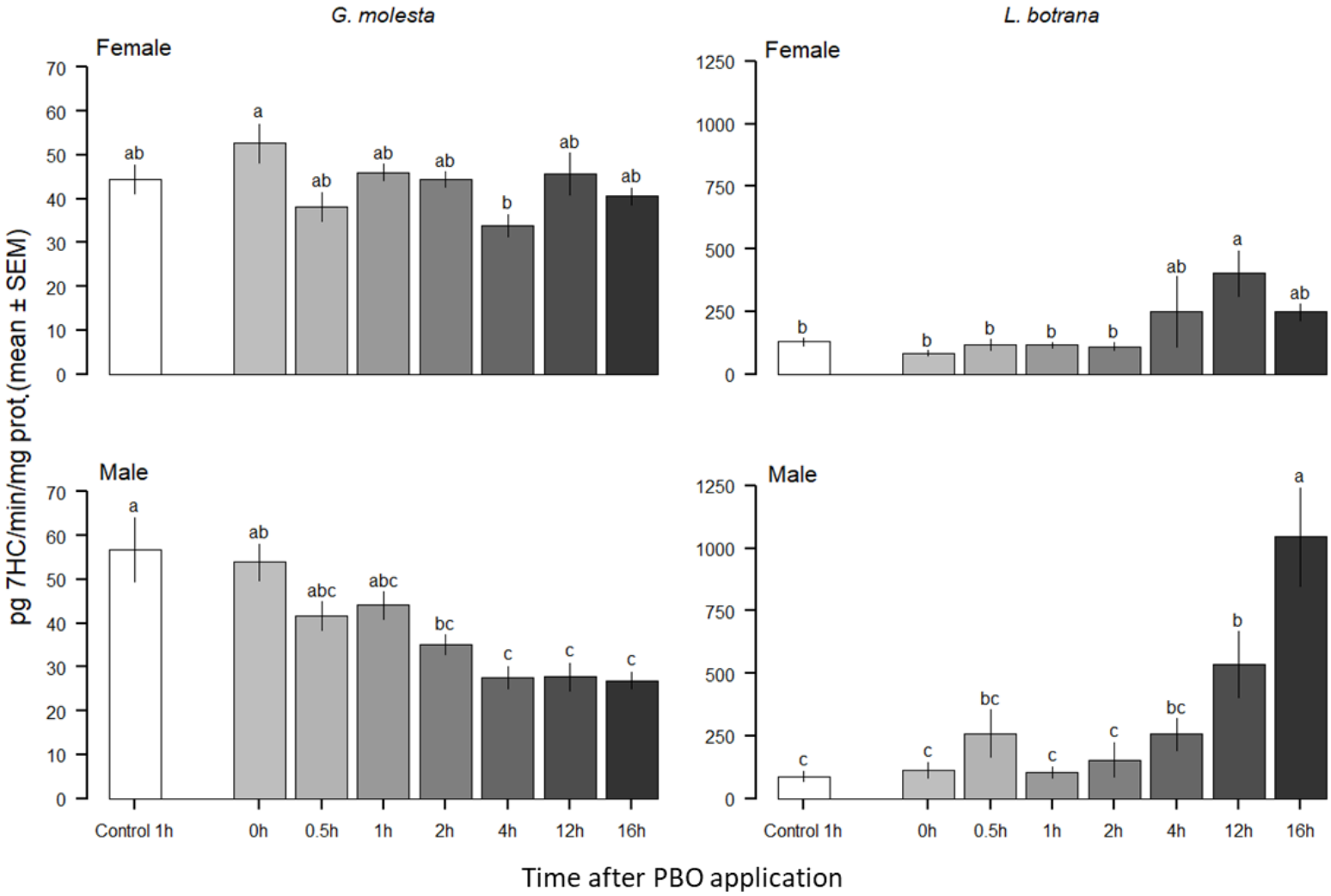
Species	Sex	Inhibitor [a]	EST [b]			GST [b]			MFO [b]		
			mean ± SEM (n)	p-value	ratio [c]	mean ± SEM (n)	p-value	ratio [c]	mean ± SEM (n)	p-value	ratio [c]
C. pomonella	female	(-)	566.38 ± 79.90 (39)	<0.001	7.76	2924.76 ± 1161.37 (40)	0.652	1.31	27.85 ± 2.37 (20)	0.880	1.02
	female	(+)	72.95 ± 13.41 (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)		

[a] (-) Control treatment (only with acetone), (+) Inhibitor treatment DEF, DEM or PBO for EST, GST and MFO, respectively.

[b] Enzymatic activities expressed in: EST (nmol α-naphtol/min/mg protein), GST (µmol of CDNB-GS/min/mg protein), and MFO (pg of 7-HC/min/mg protein).

[c] Enzymatic activity inhibition ratio control/inhibitor treatment.

Figure 2. Effect of time after application of PBO on MFO enzymatic activity in the abdomens of adult *G. molesta* and *L. botrana* from susceptible laboratory strains. Different letters indicate significant differences among groups (P<0.05, *Tukey* after GLM).



Discussion

The main aim of our study was to determine the metabolic mechanisms involved in insecticide detoxification in three tortricid moth pest. Mortality of individuals treated simultaneously with enzyme-inhibitors and insecticides showed that EST plays a general role in detoxification, acting on the three insecticides and on both sexes of the three moth species. 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, and so conferring resistance to a broad spectrum of insecticides (Devonshire and Moores 1982). The wide 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), and 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). The adaptability of EST in our test insects is limited by its relatively moderate level of resistance in comparison with MFO. However, it must be kept in mind that we used susceptible insect strains with a baseline resistance level, and so the enzyme activity levels may be different in populations under insecticide pressure.

The mortality tests also showed that phase I enzymatic activities (EST and MFO) were involved in detoxification in the three species and both sexes, whereas phase II enzymes (GST) were important only in *G. molesta*. We reviewed 92 cases of detoxification mechanisms in Lepidoptera and found that GST are involved in only 36 % of the cases, whereas EST and MFO are involved in 63 % and 64 % of the cases, respectively, so the GST enzymatic family appears to be less relevant in insecticide detoxification than the other two enzyme families. It is interesting, though, that in our enzymatic activity test with inhibitor-treated insects, the activity of GST in *G. molesta* was enhanced with the application of DEM, whereas the opposite was expected given that in mortality bioassays with insecticide plus enzyme-inhibitors the application of DEM increased the insecticide susceptibility in this species. The increase in detoxification activity in response to environmental stressors such as plant compounds, insecticides and herbicides, corresponds to an increase in enzyme production termed 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). For example, dose-dependent enzymatic activity induction (and inhibition) by insecticides has been shown in *Plutella xylostella* (L.) (Deng *et al.* 2016), and *C. pomonella* (Parra-Morales *et al.* 2017), both treated with the organophosphate chlorpyrifos. Induction of enzymatic

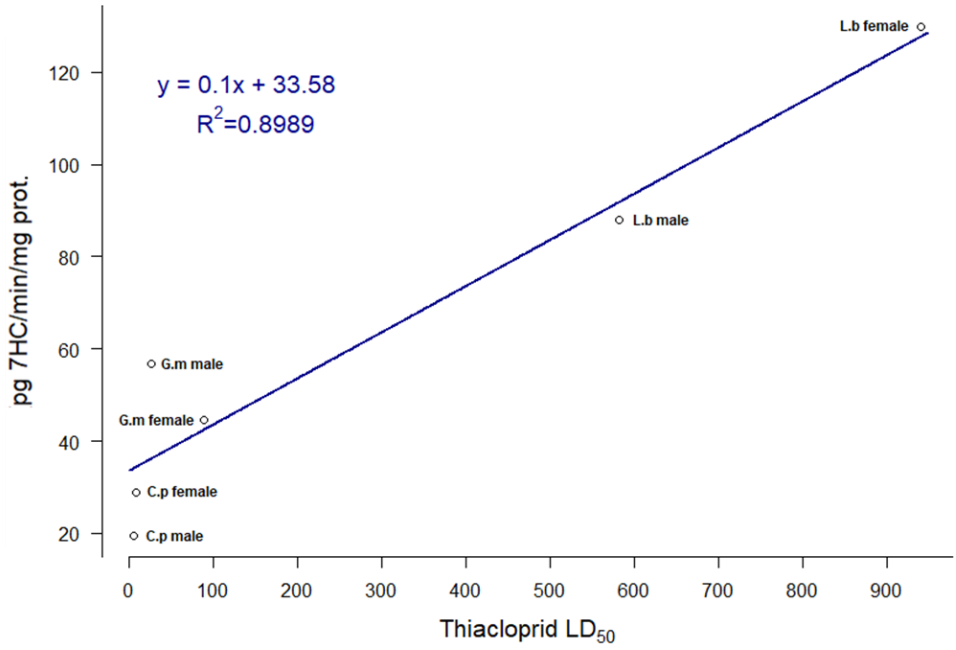
activities by an enzymatic inhibitor was also observed in *Drosophila melanogaster* (Meigen) (Willoughby *et al.* 2007).

MFO showed more diverse effects than the other two enzymatic families. It had a relatively higher impact than EST in detoxification of thiacloprid, whereas its involvement in the detoxification of λ-cyhalothrin was relative small. In the case of chlorpyrifos, PBO reduced mortality in the three species and both sexes, which was unexpected. This suggests that MFO increases the activity of chlorpyrifos, a phenomenon known as bio-activation, which has been described for organophosphates (Feyereisen 1999). The complex chemical reactions of MFO can lead to both insecticide bio-activation, as we have observed, or to detoxification (Levi *et al.* 1988), as in honeybees (Iwasa *et al.* 2004). Studies in several insect families reveal target-site mutation and MFO as predominant mechanisms on insecticide resistance (Bass *et al.* 2015). The innovative part of our results is the non-specificity of MFO for sex or species, but its specificity for thiacloprid.

In a previous study (Navarro-Roldán *et al.* 2017), we reported significant differences in mortality (LD_{50}) across insecticides (maximum 7,800-fold), species (maximum 115-fold), and sexes (maximum 41.5-fold), and so one objective of the present study was to determine if detoxification-enzyme activity levels could explain some of those differences. In order to determine if there is an association between enzymatic activity levels and mortality, we calculated for each enzyme family and insecticide, the correlation between the LD_{50}s of the three species and two sexes (Navarro-Roldán *et al.* 2017), and the enzymatic activity reported in this study. Of the nine regressions, only thiacloprid and MFO activity showed a significant relationship ($R^2 = 0.90$. Figure 3), which implies that MFO is the main mechanism of thiacloprid detoxification in these species. The highest MFO activity was for *L. botrana* females, which was also the most tolerant group to thiacloprid (Navarro-Roldán *et al.* 2017). A significant role of MFO in detoxification of thiacloprid has also been reported in *C. pomonella* adults (Reyes *et al.* 2007), and in combination with EST in the larvae (İşci and Ay 2017). The lack of correlation between enzymatic activity and mortality for the other insecticides and enzyme families may be due to a lower insecticide specificity and multiple target sites of the enzymatic groups, which would result in mutual interactions (Ahmad and Hollingworth 2004).

One unexpected finding from our previous study was a lower male than female susceptibility to the organophosphate insecticide chlorpyrifos (Navarro-Roldán *et al.* 2017). The expected pattern

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



was higher male susceptibility, because males are smaller than females, and this occurred with the other two insecticides. Lower male susceptibility to chlorpyrifos happened in the three moth species, and we wondered if detoxification enzymes could explain it. Shearer and Usmani (2001) reported higher female susceptibility to organophosphates in *G. molesta*. In a follow-up study, de Lame *et al.* (2001) reported that the larger tolerance of males may be linked to the larger acetylcholinesterase (AChE) and general EST activity levels in males than in females, which also may counteract the higher male AChE sensitivity to organophosphates. In contrast, we did not find significant differences in EST activity levels between sexes in our study, although a similar procedure was used in both studies. Our European laboratory strain was reared in artificial diet for more than 5 years without reintroduction of wild individuals, whereas the North American rearing (de Lame *et al.* 2001, Shearer and Usmani 2001) consist of less than three year-old colonies reared in green apples, so these two colonies may have slight differences in their detoxification enzyme systems. In addition to higher EST activity de Lame *et al.* (2001) proposed simultaneous involvement of other metabolic enzymatic families, and lower bio-activation by MFO of P=S compounds into the AChE-inhibitory P=O analogs in males to explain sex differences.

The efficacy of synergist-insecticide application is partially dependent upon pretreatment time, as in the case of PBO on pyrethroids in *Helicoverpa armigera* (Hübner) (Young *et al.* 2005, 2006) and *Bemisia tabaci* (Gennadius) (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). Because in the MFO-activity test, which showed no effect, we measured enzymatic activity only 1-h post-inhibitor treatment, whereas in the tests with the other two enzymes we left a 24-h incubation period, we explored if time after inhibitor application had an effect on the activity of PBO on MFO. In *G. molesta* there was limited inhibition 4 h post-exposure, but in *L. botrana* MFO activity increased up to 9.5 times after 16 hours of PBO application in males. Because the effect appeared after several hours, it suggests a biological rather than a chemical reaction, maybe due to a gene induction, as in the 32-fold induction of the Cyp6A2 gene and other MFO genes by PBO in *D. melanogaster* (Willoughby *et al.* 2007). These results could explain the lack of inhibitor effect in our first enzyme inhibition test. Interestingly, the changes in enzymatic activity across time revealed sex differences, like the inhibition after 4 h in *G. molesta* males and no effect in females, as well as different enzymatic activity between males and females of *L. botrana*, that may lead the way towards understanding of the sex differences in insecticide susceptibility reported previously (Navarro-Roldán *et al.* 2017).

Conclusion

Our comparative study shows specificity of detoxification enzymes, where GST was specific of *G. molesta* and MFO was specific for thiacloprid. The positive correlation between MFO activity and LD_{50} explains species-specific differences in susceptibility to thiacloprid reported previously (Navarro-Roldán *et al.* 2017). However, the sex differences reported cannot be explained with the enzymatic-activity results of the present study. Sex differences in enzymatic inhibition and induction observed in the kinetic experiment could help explain sex differences of insecticide susceptibility, but further kinetic investigations are needed that include the three species and the three enzyme inhibitors. We suggest the use of kinetic enzymatic inhibition assays in order to fully understand inhibitor-enzyme dynamics. Careful considerations must be given if enzymatic-inhibition want to be considered as insecticide synergist pre-treatments under field conditions because the presence of many "metabolic enzyme-inducers" (i.e., 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), could influence the insect's metabolic enzyme status in susceptible strain, like the ones we used in our assays. Our findings of enzymatic induction raise the

important question of the use of enzyme-inhibitors in agriculture, exploring the way to replace unspecific inhibitors by more specialised ones, i.e., CYP6 gene-family of MFO.

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SUBLETHAL EFFECTS OF NEONICOTINOID INSECTICIDE ON CALLING BEHAVIOR AND PHEROMONE PRODUCTION OF TORTRICID MOTHS

3

ABSTRACT

In moths, sexual behavior combines female sex pheromone production and calling behavior. The normal functioning of these periodic events requires an intact nervous system. Neurotoxic insecticide residues in the agroecosystem could impact the normal functioning of pheromone communication through alteration of the nervous system. In this study we asses if sublethal concentrations of the neonicotinoid insecticide thiacloprid, that competitively modulates nicotinic acetylcholine receptors at the dendrite, affect pheromone production and calling behavior in adults of three economically important tortricid moth pests [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller)]. Thiacloprid significantly reduced the amount of calling in *C. pomonella* females at $LC_{0.001}$ (a lethal concentration that kills only 1 in 10^5 individuals), and altered its calling period at LC_1, and in both cases the effect was dose-dependent. In the other two species the effect was similar but started at higher LCs, and the effect was relative small in *L. botrana*. Pheromone production was altered only in *C. pomonella*, with a reduction of the major compound, codlemone, and one minor component, starting at LC_{10}. Since sex pheromones and neonicotinoids are used together in the management of these three species, our results could have implications regarding the interaction between these two pest control methods.

KEY WORDS: sublethal, thiacloprid, calling behavior, pheromone, communication, Tortricidae.

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Introduction

In lepidopterans, reproduction shows a periodic pattern related to the duration of the daily light and dark cycles and involves a complex series of behavioral and physiological events including chemical communication mediated by sex pheromones (Groot 2014). Usually females release the sex pheromone and males fly towards females from tens or hundreds of meters (Cardé 2016). Closely related moth species with common phylogenetic origins are under competition for limited communication channels (Roelofs and Brown 1982). Reproductive isolation is instrumental in speciation (Smadja and Butlin 2009), and in the case of pheromone communication is modulated by species-specific differences in sex-pheromone composition and time of release (Byers 2006, Groot 2014)

Several factors influence calling behavior and pheromone production in moths (McNeil 1991, Raina 1993), including age (i.e., Webster and Cardé 1982, Gemeno and Haynes 2000, Kawazu and Tatsuki 2002, Mazor and Dunkelblum 2005, Ming *et al.* 2007), mating status (i.e., Foster and Roelofs 1994, Delisle *et al.* 2000, Mazo-Cancino *et al.* 2004), and pheromone autodetection (Holdcraft *et al.* 2016). Environmental stressors, such as sublethal doses of insecticides that intoxicate but do not kill the individual, could also affect pheromone production and release (Haynes 1988, Tricoire-Leignel *et al.* 2012), but this aspect has been tested in relatively few moth species.

Pesticides are often considered a quick, easy, and inexpensive solution to control insect pests. However, pesticides can cause negative effects on the environment and human health (Aktar *et al.* 2009). In Integrated Pest Management (IPM) the use of insecticides is often combined with environmentally safer methods (Damos *et al.* 2015), such as the use of sex pheromones for mating disruption (emitting large amounts of synthetic sex pheromone and so reducing the probability of mate finding), mass trapping (removing from the population individuals attracted to traps baited with pheromone lures), and monitoring the population for precise timing of control procedures (Witzgall *et al.* 2010). Because semiochemicals exploit insect chemical communication, and neurotoxic insecticides affect the normal functioning of the nervous system, it is plausible that the simultaneous use of semiochemicals and insecticides could affect each other in IPM strategies (Suckling *et al.* 2016). Indeed, several studies report alterations of the normal perception of and response to chemical signals in insects treated with sublethal doses of insecticides (Haynes 1988, Tricoire-Leignel *et al.* 2012).

In this context of potential semiochemical and toxicological interactions in the agroecosystem, we explored the effect of sublethal doses of a neonicotinoid insecticide on pheromone production

and release (i.e., calling behavior) in three tortricid moths. Our test species, *Cydia pomonella* (L.), *Grapholita molesta* (Busck) and *Lobesia botrana* (Denis & Schiffermüller), are main pests of apple, peach and grapevines, respectively, have a relatively worldwide distribution and are controlled with both semiochemicals and insecticides (Ioriatti *et al.* 2011, Kirk *et al.* 2013, Damos *et al.* 2015). Indeed these three species represent several of the most successful examples of pest control by means of mating disruption (Witzgall *et al.* 2010). For a toxicant, we used the neuroactive insecticide thiacloprid, a neonicotinoid that competitively modulates nicotinic acetylcholine receptors at the dendrite (Casida 2009). Thiacloprid is recommended for the control of *C. pomonella* and *G. molesta* in stone and pome fruits in Spain (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente [MAPAMA], 2017). Although aimed at eggs and larvae, thiacloprid residues from air blast spraying in fruit orchards could potentially intoxicate adults with residual sublethal doses, and therefore affect semiochemical control (Wise *et al.* 2006). Thiacloprid could potentially affect *L. botrana* in vineyards adjacent to fruit orchards treated with this insecticide (Harari *et al.* 2011). Baseline mortality with thiacloprid has been determined for the three species under laboratory conditions (Navarro-Roldán *et al.* 2017). *Cydia pomonella* and *G. molesta* belong to the tribe Grapholitinii and *L. botrana* to the tribe Olethreutini, both in the subfamily Oletheutrinae (Regier *et al.* 2012). By comparing the effect of thiacloprid across phylogenetically related species of diverse ecology we hoped to gain basic background information about sublethal effects of neurotoxic insecticides on sex pheromone signalers.

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) at 25 ± 1 ºC under a 16:8 h light:dark photoregime. Females of *G. molesta* and *L. botrana* were separated at the pupal stage and adult emergence was checked daily and always at the same hour. *C. pomonella* was sexed at the adult stage, also in a daily basis. Because adults were collected only once per day, they were 0-24 h old when separated from the pupae, 24-48 h old one day later, and so on.

Insecticide Application and Mortality Estimation. Thiacloprid (PESTANAL®, analytical standard, ≈ 100 % (a.i.), Sigma-Aldrich, Spain), was diluted using acetone (CHROMASOLV®, for HPLC, ≥ 99.9 %. Sigma-Aldrich, Spain) and stored at 7 ºC. The same stock of acetone used to prepare the dilutions was also used as the solvent control treatment. The four chosen sublethal concentrations of thiacloprid were $LC_{0.001}$, LC_1, LC_{10} and LC_{20}, according to Navarro-Roldan *et al.* (2017), with concentrations shown in Table S1.

Treatments were applied during the first half of the photophase at 0-24 h post-emergence (calling behavior test), or at 16-40 h post-emergence (pheromone gland test). One to three adults were placed in 10 ml clear polystyrene test tubes and received a brief (10 sec) flow of industrial grade CO_2 which quickly anesthetized them. Immediately after being anesthetized they were placed upside down under the field of view of a stereo microscope and a 1 µl test solution was applied to the ventral thoracic region of each individual using a high-precision, positive displacement, repeatable-dispensing micropipette (Multipette®-M4, Eppendorf, Germany). Treated females 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 1-mm-diameter holes to allow gas exchange, and a 1.5 ml Eppendorf tube® containing 10 % sugar solution and cotton lid was placed on the bottom to supply nutrients. Bottles with treated insects were placed in the rearing room until test time.

Mortality was determined 24 h post-treatment. Adults were observed with the naked eye and scored as: 1) alive if they flew or walked apparently unaffected; 2) as potentially moribund if they could barely walk or were laying on the floor of the bottle but still moved; 3) or as potentially dead if they laid immobile on the floor of the bottle. Mortality was estimated using the sum of the potentially moribund and dead individuals. The other individuals scored as alive were used in the calling and pheromone tests. No further anesthesia was needed.

Calling Behavior. Females were placed individually in 9 mm-long x 1.5 mm-diameter, 10 mL clear polystyrene test tubes that had both ends covered with 1.5 mm-diameter-mesh galvanized wire screen (Figure S1). Tubes were placed on a 42 cm-tall platform that could hold up to 13 tubes from bottom to top, leaving 2.5 mm between them (Figures S1 and S2). The platforms were painted white to facilitate observation of calling postures inside of the plastic tubes. Four platforms with test tubes were placed in a chamber with a continuous 0.4 ± 0.1 m s^{-1} air flow. The tubes were aligned with the air flow (flow through the tubes was not measured) to minimize

ambient pheromone levels, which could affect moth calling behavior (Holdcraft *et al.* 2016). Four 18-watt domestic fluorescent lamps (Standard daylight F18W/154-T8, Sylvania) that were placed between 20 and 52 cm above the highest and lowest female positions in the rack provided between 4700 and 1700 lux during photophase, respectively (TES-1330, Tes Electrical Electronic Corp.). During scothophase there was complete obscurity and calling was observed using a 660-nm LED (2.5V, 1.3 candles, 5 mm diameter, 30° view angle, LedTech, part number LURR5000H2D1) which was held manually near each female for observations.

G. molesta females call mainly before the beginning of the scotophase, *C. pomonella* females call mainly during the scotophase (Groot 2014), and *L. botrana* females call during the first hours of scotophase (Harari *et al.* 2011, 2015). However several factors (i.e., illumination, temperature etc.) could affect the calling period (McNeil 1991), so in order to determine the exact calling period of our laboratory colonies under our experimental conditions we performed preliminary observations on 69-75 untreated (i.e., no acetone or insecticide) individuals of each species over a 12-h period bracketing the expected calling times. The 12-h observation periods of *C. pomonella* and *L. botrana* started 2 h before the onset of the scotophase, covered all the scotophase and continued during the first 2 h of the photophase. The observation period of *G. molesta* started 8 h before the onset of the scotophase and extended 4 h into the scotophase. In order to observe the three species during the same 12 h time period, the photoregimes of *C. pomonella* and *L. botrana* were synchronized with each other and both were observed on the same day, the photoregime of *G. molesta* was delayed with respect to that of the other species. Observations of the two groups were made on alternate days, and were performed at 30 min intervals, except during the last 30 min of the photophase and the first 2 h of the scotophase when they were observed every 15 min to increase sample resolution for the relatively short (about 2 h) calling period of *L. botrana*. Females were placed in the observation setup at least 30 min before the first observation. The first observations during scotophase occurred between 5 to 10 minutes after lights off.

Once the calling period of our laboratory insects was determined (Figure S3), between 61 and 70 females treated with sublethal insecticide doses or acetone were observed during the same period as in the preliminary test to determine the effect of the insecticide dose on calling behavior. Calling behavior was categorized as either “weak calling” (the female walks or is slightly agitated, with an intention to adopt, or beginning to adopt, a calling posture consisting in rising its wings and extruding the abdomen tip), “medium calling” (incomplete calling stance: more or less stationary female with partially raised wings and abdomen tip partially extruded), or “strong calling” (full calling stance: mostly stationary female with fully raised wings, and protruded

abdomen tip readily visible). The specifics of the calling posture were slightly different and characteristic across species.

Pheromone Gland Content. Pheromone was extracted from females that were 40- to 64-hour-old and had been treated with sublethal insecticide doses, or acetone as control, 24 h earlier. Extractions were restricted to a 1 h period coinciding with peak calling time of each species: 30 to 90 min after the onset of scotophase in *C. pomonella*, 120 to 60 min before the scotophase in *G. molesta* and 0 to 60 min after the onset of scotophase in *L. botrana*. The tip of the abdomen containing the sex pheromone gland tissue was excised carefully by pulling it from the abdomen with fine forceps. Abdominal tips were deposited individually in solvent-rinsed and oven-dried conical-bottom glass vials (Total recovery vial, part number 186002805, Waters, USA) with Teflon-lined lids (part number 186000274, Waters, USA) containing 7 µl of a 1 ng/µl octadecane internal standard solution (> 99 % pure, Sigma-Aldrich, Spain) in *n*-hexane (> 97 % pure, VWR Chemicals, BDH-Prolabo, Spain). After 30 min at room temperature, the glands were removed from the vial and the extracts were stored at -20 °C until analysis (for a maximum of 10 days).

The remaining extract (approx. 0.5-3 µl) was injected in a Hewlett Packard 6890 gas chromatograph equipped with a flame ionization detector and a 30 m-long, 0.25-mm I.D., 0.25-µm film-thickness DB-Wax column (Agilent Technologies, Madrid, Spain). The constant helium flow through the column was 1 ml min^{-1}, and the injector and detector temperatures were 250 and 270 °C, respectively. The oven temperature program stayed at 70 ºC during 1 min and then increased to 170 °C at 30 °C min^{-1}, and from 170 °C to 230 °C at 10 °C min^{-1}, and remained at this temperature for 10 min. Retention time and quantification were estimated with the injection of synthetic standards and with the internal standard, respectively. The pheromone compounds of *C. pomonella* [Dodecan-1-ol (12:OH), (*E*)-9-Dodecen-1-ol (E9-12:OH), Tetradecan-1-ol (14:OH), and (*E*,*E*)-8,10-Dodecadien-1-ol (E,E-8,10-12:OH), Witzgall *et al.* 2008] eluted at 7.71 min, 8.00 min, 9.19 min and 9.36 min, respectively. The pheromone compounds of *G. molesta* [(*E*)-8-Dodecenyl acetate (E8-12:Ac), (*Z*)-8-Dodecenyl acetate (Z8-12:Ac), Dodecan-1-ol (12:OH), and (*Z*)-8-Dodecen-1-ol (Z8-12:OH), Knight *et al.* 2015] eluted at 7.46 min, 7.55 min, 7.71 min and 8.05 min, respectively. The pheromone compounds of *L. botrana* [(*E*)-9-Dodecenyl acetate (E9-12:Ac), (*Z*)-9-Dodecenyl acetate + 11-Dodecenyl acetate (Z9-12:Ac+11-12:Ac), (*E*,*Z*)-7,9-Dodecadienyl acetate (E,Z-7,9-12:Ac), and (*E*,*Z*)-7,9-Dodecadien-1-ol (E,Z-7,9-12:OH), Sans *et al.* 2017] eluted at 7.52 min, 7.62 min, 8.57 min and 9.12 min, respectively (Z9-12:Ac and 11-12:Ac eluted together). Between 19 and 21 females of each species were analyzed

for each treatment. For each individual the quantity of individual compounds and the ratio of the minor compounds to the major compound (E,E-8,10-12:OH in *C. pomonella*, Z8-12:Ac in *G. molesta* and E,Z-7,9-12:Ac in *L. botrana*) were calculated.

Statistical Analyses. All the statistical analyses were run in R software (R Core Team 2016). Mortality was analyzed with *Fisher's exact tests* and *Bonferroni* correction. To determine the effect of thiacloprid on the calling period, we calculated the first, mid and final times of calling for calling females. To determine the effect of thiacloprid on the amount of calling behavior we calculated the proportion of observations in which females called out of the total number of observations of the calling period estimated previously. For example, for an 8-h calling period and observations every 30 min there would be 960 observations for 60 insects. If calling appeared in 480 of these observations, then the amount of calling would be 50 %. Analyses were performed with generalized linear models (GLM), using Gaussian family functions for continuous variables (calling period and pheromone composition) and binomial family functions for binomial variables (amount of calling). The predictmeans() function performed *Tukey´s* multiple pairwise comparisons and provided parameter estimates and their standard errors and confidence intervals which are shown in tables and figures. Raw data and R scripts are available online (http://hdl.handle.net/10459.1/59531). Whenever the term "significant" is used in the text regarding differences between treatments it indicates a p-value < 0.05.

Results

Mortality in our tests (Table S1) was comparable to the dose-mortality curves used to determine the test concentrations (Navarro-Roldán *et al.* 2017). Acetone and $LC_{0.001}$ did not induce any mortality, and the maximum mortality with LC_1 was below 2.5 %. LC_{20} mortality ranged between 7 % and 21 %, and with LC_{10} it was between LC_1 and LC_{20} in all but one case (Table S1).

Calling Behavior. Under our test conditions, *C. pomonella*, *G. molesta* and *L. botrana* had distinct calling periods. *C. pomonella* called throughout the scotophase, *G. molesta* called from 4 h before the start of the scotophase to 0.5 h into the scotophase, and *L. botrana* called for 2.5 h starting at the beginning of the scotophase (Figure S3). Acetone did not appear to affect the amount or periodicity of calling with respect to untreated females (compare Figure 1 and Figure

S3). At least 80 % of the acetone-treated females called during peak calling time (all species), but there was a significant dose-dependent reduction of calling in treated females (Figure 1). In *C. pomonella* there was a strong reduction on the amount of calling which was already significant at the lowest concentration ($LC_{0.001}$), in the other two species the reduction started with LC_1 (Table 1), and although significant, the effect was very mild in *L. botrana*. Peak calling reductions with LC_{20} were 70.19 and 75.09 % for *C. pomonella* and *G. molesta*, respectively. In *L. botrana* calling was not reduced beyond LC_1, and reduction with respect to the control treatment was only 10 %. A small percentage (< 8 %) of the control females did not call a single time during the entire observation period, but this number increased with thiacloprid doses and peaked at LC_{20} with 53 % (*C. pomonella*), 61 % (*G. molesta*) and 20 % (*L. botrana*) non-calling females, respectively (Table S2). Individual differences in the number of calling observations per female and intensity of calling (weak, medium and strong) were observed (Figures S4, S5 and S6, data not analyzed). In general, "strong" calling coincided with peak calling time, whereas "weak" calling appeared to increase with insecticide dose.

Sublethal doses of thiacloprid modified calling periods (Table 2). LC_1, LC_{10} and LC_{20} advanced the end and midpoint calling times of *C. pomonela*'s (150 min, approx.), and delayed the start and midpoint calling times of *G. molesta* (74 min, approx.). No significant effect in calling period was observed in *L. botrana*.

Pheromone Gland Content. The two highest sublethal doses of thiacloprid, LC_{10} and LC_{20}, reduced significantly the quantity of the major pheromone component of *C. pomonella* (codlemone, E,E-8,10-12:OH) from about 5 ng to about 2 ng, and the minor component 12:OH from about 2 ng to about 1 ng, whereas the other two minor components of *C. pomonella* and the pheromone components of the other two species were unaffected (Figure 2). Reduction in the quantity of the major pheromone component of *C. pomonella* resulted in an increase in the relative proportion of two minor compounds, E9-12:OH and 14:OH, relative to codlemone (Table 3).This effect was significant only at the highest pheromone dose, LC_{20}. E9-12:Ac and 14:OH were 14 and 16 % relative to codlemone in acetone control females, and 56 and 40 % relative to codlemone in LC_{20} thiacloprid treated females. No further changes in the proportion of pheromone components were observed in *C. pomonella* or the other two species.

Figure 1. Effect of thiacloprid on the percentage of females calling of *C. pomonella*, *G. molesta* and *L. botrana* (N = 61-70). The grey area represents the scotophase.

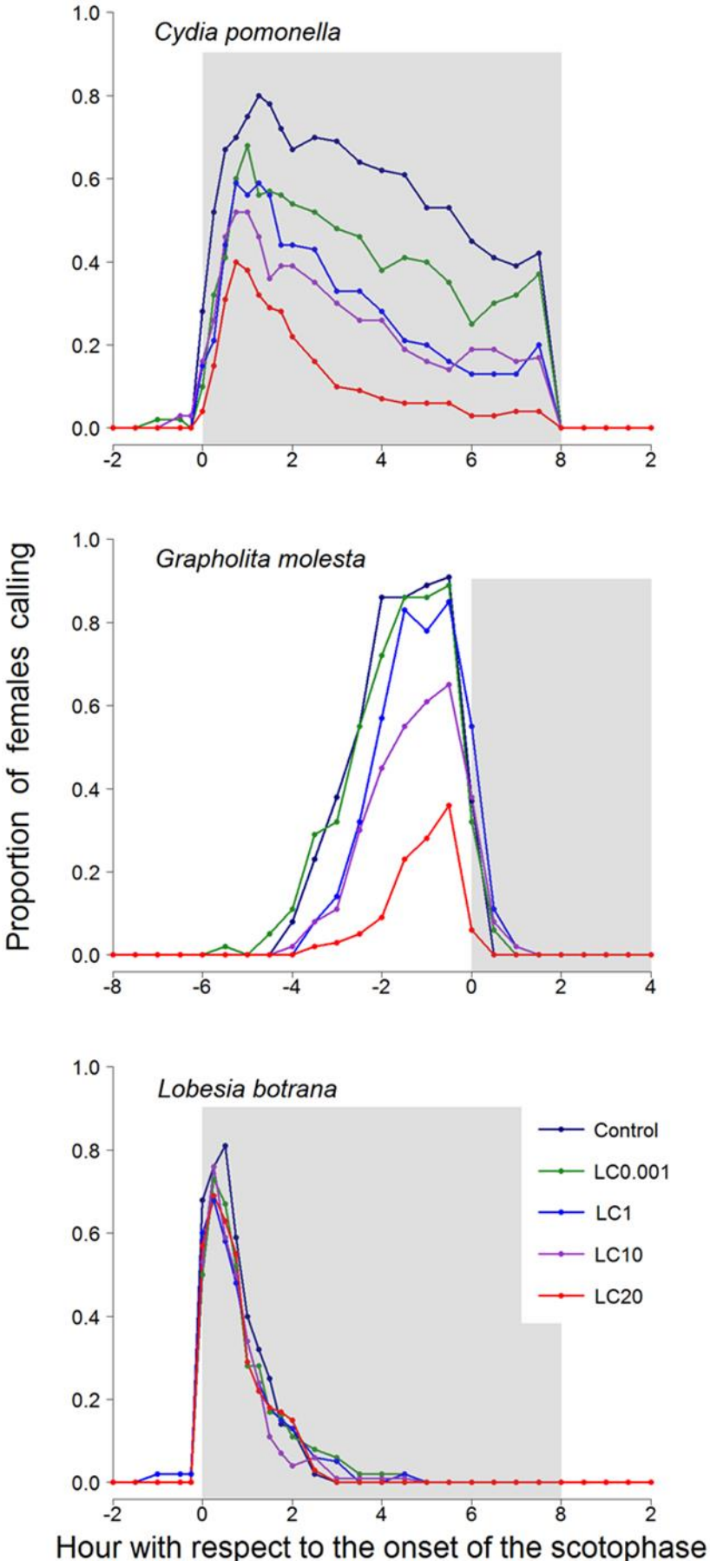

Table 1. Effect of thiacloprid on the percentage of calling observations during the calling period. Different letters within a species indicate significant differences among insecticide treatments (P<0.05, *Tukey* after GLM).

Species	Treatment	N ♀[a]	N Obs.[a]	N Tot.[a]	% calling (95 % CI)		
Cydia pomonella	Acetone	64	14	896	67.30	(64.16; 70.29)	a
	$LC_{0.001}$	63	14	882	49.43	(46.14; 52.73)	b
	LC_{1}	61	14	854	39.70	(36.46; 43.02)	c
	LC_{10}	69	14	966	34.16	(31.24; 37.21)	c
	LC_{20}	68	14	952	20.06	(17.64; 22.73)	d
Grapholita molesta	Acetone	65	5	325	81.54	(76.94; 85.39)	a
	$LC_{0.001}$	65	5	325	77.85	(73.00; 82.03)	a
	LC_{1}	65	5	325	67.08	(61.78; 71.97)	b
	LC_{10}	66	5	330	51.21	(45.82; 5657)	c
	LC_{20}	64	5	320	20.31	(16.25; 25.08)	d
Lobesia botrana	Acetone	63	6	378	59.62	(54.22; 64.11)	a
	$LC_{0.001}$	64	6	384	49.74	(44.75; 54.73)	ab
	LC_{1}	62	6	372	48.66	(43.60; 53.73)	b
	LC_{10}	70	6	420	49.29	(44.52; 54.06)	b
	LC_{20}	66	6	396	48.48	(43.59; 53.41)	b

[a] N ♀ = number of females; N Obs. = number of observations into the calling period of each species; N Tot. = total N consider in GLM analysis, which is the product between N♀ and N Obs.

Table 2. Effect of thiacloprid on the start, mid and end calling times relative to the onset of the scotophase (in minutes). Different letters within a column and species indicate significant differences among treatments ($P < 0.05$, *Tukey* after GLM). N = number of females.

Species	Treatment	N	Start (mean ± SEM)		Mid (mean ± SEM)		End (mean ± SEM)	
Cydia pomonella	Acetone	61	37.38 ± 7.08		183.44 ± 10.27	a	329.51 ± 19.38	a
	$LC_{0.001}$	54	43.61 ± 7.52		168.61 ± 10.92	ab	293.61 ± 20.6	ab
	LC_1	48	45.63 ± 7.98		134.38 ± 11.58	bc	223.13 ± 21.85	bc
	LC_{10}	45	25 ± 8.24		130.67 ± 11.96	bc	236.33 ± 22.57	bc
	LC_{20}	32	30.94 ± 9.78		105.23 ± 14.18	c	179.53 ± 26.76	c
Grapholita molesta	Acetone	63	-159.52 ± 6.51	a	-90.48 ± 3.72	a	-21.43 ± 3.61	
	$LC_{0.001}$	62	-162.1 ± 6.56	a	-92.42 ± 3.75	a	-22.74 ± 3.64	
	LC_1	61	-120 ± 6.62	b	-66.15 ± 3.78	b	-12.3 ± 3.67	
	LC_{10}	54	-115 ± 7.03	bc	-67.5 ± 4.01	b	-20 ± 3.9	
	LC_{20}	25	-85.2 ± 10.34	c	-56.4 ± 5.9	b	-27.6 ± 5.73	
Lobesia botrana	Acetone	58	6.98 ± 1.99		35.3 ± 3.24		63.62 ± 6.1	
	$LC_{0.001}$	55	8.45 ± 2.04		38.73 ± 3.32		69 ± 6.27	
	LC_1	51	5.59 ± 2.12		34.56 ± 3.45		63.53 ± 6.51	
	LC_{10}	60	9.5 ± 1.95		35.5 ± 3.18		61.5 ± 6	
	LC_{20}	53	5.94 ± 2.08		33.82 ± 3.39		61.7 ± 6.38	

Figure 2. Effect of thiacloprid on the quantity of individual pheromone components in the pheromone gland (N = 20-21). Different letters indicate significant differences among treatments for each compound and species (P<0.05, *Tukey* after GLM).

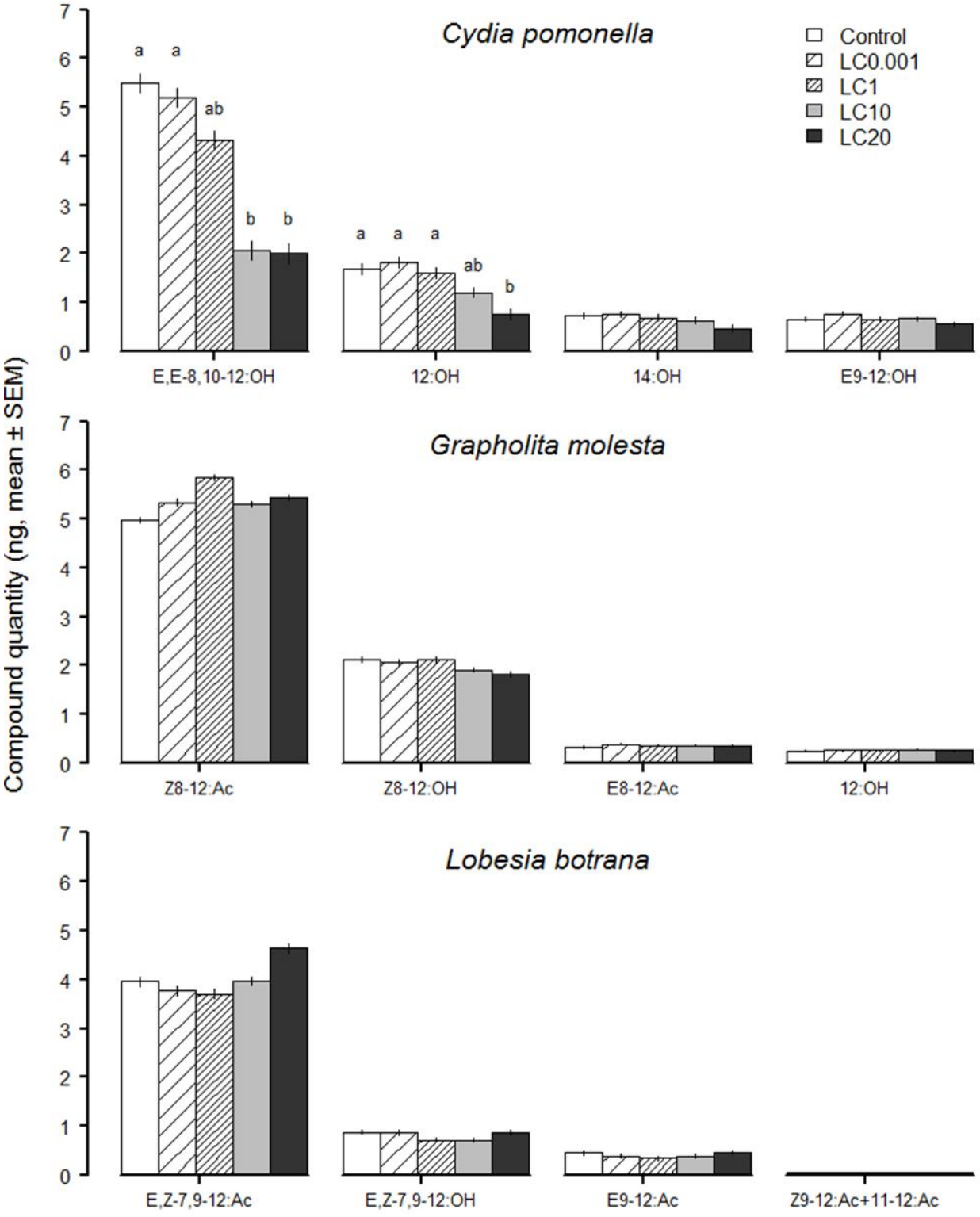

Table 3. Effect of thiacloprid on the ratio of minor pheromone components relative to the major pheromone compound. Minor compounds 1, 2 and 3 for *C. pomonella* (12:OH, E9-12:OH, 14:OH), *G. molesta* (12:OH, E8-12:Ac, Z8-12:OH) and *L. botrana* (E9-12Ac, Z9-12Ac + 11-12Ac, E,Z-7,9-12OH). Different letters within a column and species indicate significant differences among thiacloprid treatments (P<0.05, *Tukey* after GLM). N = number of females.

Species	Treatment	N	**Minor compound 1** % (mean ± SEM)	**Minor compound 2** % (mean ± SEM)		**Minor compound 3** % (mean ± SEM)	
Cydia pomonella	Acetone	21	31.41 ± 6.63	13.99 ± 7.00	b	15.98 ± 5.86	b
	$LC_{0.001}$	21	39.56 ± 6.63	21.08 ± 7.00	b	17.33 ± 5.86	ab
	LC_{1}	21	40.86 ± 6.63	23.34 ± 7.00	b	20.81 ± 5.86	ab
	LC_{10}	21	48.57 ± 6.63	39.81± 7.00	ab	28.15 ± 5.86	ab
	LC_{20}	20	45.35 ± 6.80	55.59 ± 7.18	a	40.00 ± 6.00	a
Grapholita molesta	Acetone	20	4.80 ± 0.33	6.06 ± 0.29		35.64 ± 2.04	
	$LC_{0.001}$	21	4.97 ± 0.32	6.89 ± 0.28		33.69 ± 1.99	
	LC_{1}	21	4.67 ± 0.32	5.94 ± 0.28		31.71 ± 1.99	
	LC_{10}	20	5.22 ± 0.33	6.30 ± 0.29		31.88 ± 2.04	
	LC_{20}	21	4.83 ± 0.32	6.06 ± 0.28		29.33 ± 1.99	
Lobesia botrana	Acetone	21	13.03 ± 1.86	1.42 ± 0.25		20.17 ± 1.90	
	$LC_{0.001}$	20	10.06 ± 1.90	1.36 ± 0.26		22.38 ± 1.95	
	LC_{1}	19	8.45 ± 1.95	1.09 ± 0.27		16.87 ± 2.00	
	LC_{10}	21	9.48 ± 1.86	1.72 ± 0.25		16.62 ± 1.90	
	LC_{20}	21	9.46 ± 1.86	1.01 ± 0.25		18.36 ± 1.90	

Discussion

Thiacloprid persists as surface residue on fruit and leaves (Wise *et al.* 2006), and has a half-life in the soil of 10 to16 days (Krohn 2001). Therefore, adult moths could be exposed to sublethal doses of thiacloprid even though the application is not aimed at them but to other life stages, or even at other pest species, or from drift by blast sprayers in neighbor fields. In the present study, sublethal doses of thiacloprid producing as low as 0.001 mortality significantly modified female pheromone signaling, but the effect was not the same on the three tortricid species.

In our study *C. pomonella* called throughout the scotophase as previously reported (Castrovillo and Cardé 1979, Weissling and Knight 1996). Reports on the calling period of *G. molesta* are only slightly different from ours (Baker and Cardé 1979, Stelinski *et al.* 2006, 2014), which could be explained by the effect that variations in light and temperature have on the calling period of moths (Baker and Cardé 1979, Castrovillo and Cardé 1979, Groot 2014). To our knowledge, our study provides the first complete observation on the calling period of *L. botrana*. Its onset of calling coincides with a previous report (Harari *et al.* 2015). Regarding pheromone gland composition, our estimations are generally similar to what has been previously reported (summarized in Table S3). Minor differences across studies could be attributed to population differences or to methodological aspects related to the extraction and analysis of compounds that are present in very low quantities in the pheromone glands. In general, the mortality caused by thiacloprid was similar to the expected levels of mortality estimated in a previous study (Navarro-Roldán *et al.* 2017).

The most dramatic phenotypic effect of sublethal thiacloprid doses in our test species was the significant reduction in the amount of calling in *C. pomonella* females treated with $LC_{0.001}$, a remarkably low concentration that kills only one in 10^5 females. The other two species were less sensitive, and the effect on *L. botrana*, although statistically significant was so mild that probably would not have a real effect in the field. The calling curves of the insecticide treatments for the most part fell within the boundaries of the acetone control curves, so the shift in calling period with thiacloprid was not as remarkable as the effect on the amount of calling. A detrimental effect of sublethal insecticide on calling behavior has been observed in other moth species with pyrethroid (Haynes and Baker 1985, Clark and Haynes 1992a, Yang and Du 2003, Shen *et al.* 2013, Quan *et al.* 2016) and organophosphate insecticides (Trimble *et al.* 2004). Insecticides do not always decrease calling behavior, as in the case of *Ostrinia furnacalis* (Güenee) and *Spodoptera litura* (Fabricius) treated with pyrethroids as larvae (Wei and Du 2004,

Wei *et al.* 2004). Yet, sublethal insecticide could increase the percentage of calling females, as in *Trichoplusia ni* (Hübner) treated with chlordimeform (Clark and Haynes 1992b). Regarding the timing of calling behavior, Haynes and Baker (1985) observed that for their highest permethrin dose (15 ng/moth, approx. an LC_{10}) the end of the calling period of *Pectinophora gossypiella* (Saunders) was reduced by 1 h. Surviving adults of *O. furnacalis* (Wei and Du 2004) and *Choristoneura fumiferana* (Clemens) (Dallaire *et al.* 2004) larvae treated with deltamethrin and tebufenocide, respectively, started to call 1 h later than control females.

The calling periods that we have observed in tortricids under laboratory conditions may be different under natural light conditions because our laboratory photoregime did not provide the smooth light:dark transition that occurs at dawn and sunrise in the field, and this factor alone is known to affect the periodicity of locomotor activity of other insects (Vanin *et al.* 2012). Captures of male *C. pomonella* in pheromone traps show a 4-h activity peak around dusk time under natural conditions (Knight *et al.* 1994), which suggests that the relatively long calling period of *C. pomonella* observed under artificial conditions could be narrower under more natural light conditions.

Unlike calling behavior, thiacloprid only affected pheromone production in one of the three species, *C. pomonella*, and it required higher doses than what was needed to affect calling behavior. The quantity of the major compound, codlemone, and one of the three minor compounds, 12:OH, were approximately halved compared to the acetone control at LC_{10} or LC_{20}, and the ratio with respect to codlemone of two minor compounds, E9-12:OH and 14:OH, increased 4 and 2.5-fold, respectively, at LC_{20}. Detrimental effects on pheromone production have been observed with deltamethrin in *O. furnacalis* (Yang and Du 2003) and with azinphos-methyl in *Choristoneura rosaceana* (Harris) (Delisle and Vincent 2002, Trimble *et al.* 2004). Changes in component ratios with sublethal doses of deltamethrin have been described in *S. litura* (Wei *et al.* 2004), and with a biopesticide mixture containing *Bacillus thuringiensis* (Berliner) and abamectin in *H. armigera* (Shen *et al.* 2013). Lack of effect of sublethal doses on pheromone production, as in *G. molesta* and *L. botrana*, has been described also in *T. ni* treated with cypermethrin and chlordimeform (Clark and Haynes 1992a,b).

It is interesting that thiacloprid affected calling behavior and pheromone production in *C. pomonella* but only calling behavior in *G. molesta* and *L. botrana*. In other species there is also a differential effect of insecticide on calling behavior and pheromone production (Clark and Haynes 1992a,b, Yang and Du 2003, Trimble *et al.* 2004, Wei and Du 2004, Shen *et al.* 2013). Pheromone biosynthesis in moths is mediated by a brain-released neurohormone (PBAN) that

reaches the pheromone gland through the haemolymph and binds to specific receptors on the membrane of pheromone secretion cells (Jurenka and Rafaeli 2011, Groot 2014). A likely mechanism by which the neurotoxic insecticide thiacloprid could alter pheromone production is by reducing PBAN secretion. In *O. furnacalis* an homogenate of the PBAN-producing tissues from females treated with the pyrethroid deltamethrin, which produced less pheromone than controls, resulted in a reduction of pheromone titer in the glands of the decapitated females in which it was injected, which suggests that deltamethrin reduced PBAN secretion in this species (Yang and Du 2003). It appears that juvenile hormone (JH) is involved in the regulation of calling behavior (Rafaeli 2009), and therefore insecticides may affect calling behavior and pheromone production differently. Since PBAN, JH and pheromone biosynthesis mechanisms are probably very similar in the three tortricid species (Roelofs and Rooney 2003, Jurenka and Rafaeli 2011), it remains to be determined why similar sublethal doses of thiacloprid resulted in differential effects in pheromone production and calling behavior among the three moth species.

Several questions need to be solved in order to determine the impact of our findings in IPM control. Males respond not to the pheromone in the gland but to the volatiles released by calling females, so we need to know if thiacloprid alters the composition of the pheromone blend emitted by females, as has been reported in *T. ni* with chlordimeform (Clark and Haynes 1992b). Obviously, the effect of thiacloprid on male response needs to be determined too, as insecticides are known to affect moth pheromone responses (Linn and Roelofs 1984, Wei and Du 2004, Wei *et al.* 2004, Zhou *et al.* 2005, Knight and Flexner 2007, Rabhi *et al.* 2016). Additionally, it needs to be determined if thiacloprid-treated females are as attractive to males as untreated ones, or less active at mating than untreated ones, as has been shown in other moth species (Delpuech *et al.* 1998, Wei *et al.* 2004, Knight and Flexner 2007, Reinke and Barrett 2007, Barrett *et al.* 2013, Quan *et al.* 2016). Mating in our test species is preceded by a courtship that may include contact chemical cues and short-range pheromones associated with male hair pencil displays (Jurenka and Rafaeli 2011), and these elements of mating behavior could also be affected by thiacloprid.

If thiacloprid is detrimental to these elements of mating behavior, its effect on reproduction may be even larger than what our results suggest, with a possible enhancement of semiochemical IPM control. For this reason, basic knowledge of insecticide effects on insect behavior, physiology, and reproductive success could be a critical issue if we want to optimize IPM strategies.

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Supplementary material.

Table S1. Mortality caused by sublethal doses of thiacloprid on females used in calling behaviour and pheromone gland experiments. Different letters in same column indicate significant differences among treatments in each species (P<0.05, *Fisher Exact test* after GLM).

Species	Treatment	Thiacloprid (ng)	Calling behaviour		Pheromone production	
			N	% dead 24 h	N	% dead 24 h
Cydia pomonella	Acetone	0	69	0.00 b	35	0
	$LC_{0.001}$	1.73	65	0.00 b	32	0
	LC_{1}	10.96	63	1.59 ab	38	0
	LC_{10}	29.64	74	5.41 ab	40	0
	LC_{20}	45.06	78	12.82 a	36	16.67
Grapholita molesta	Acetone	0	66	0.00 b	40	0
	$LC_{0.001}$	10.79	65	0.00 b	38	0
	LC_{1}	49.08	66	0.00 b	41	2.44
	LC_{10}	111.02	90	11.11 a	42	0
	LC_{20}	156.56	98	21.43 a	43	13.95
Lobesia botrana	Acetone	0	108	0.00 b	43	0
	$LC_{0.001}$	22.8	64	0.00 b	45	0
	LC_{1}	235.62	64	0.00 b	41	2.44
	LC_{10}	829.67	79	3.80 b	37	8.11
	LC_{20}	1409.6	92	14.13 a	43	6.98

Table S2. Percentage of females that did not call in any of the observations made during the 12h observation period. Different letters in same column indicate significant differences among treatments in each species (P<0.05, *Tukey* after GLM).

Species	Treatment	N	% no calling females (95 % CI)	
Cydia pomonella	Acetone	64	4.69 (1.24; 16.13)	c
	$LC_{0.001}$	63	14.29 (6.77; 27.66)	bc
	LC_{1}	61	21.31 (11.64; 35.77)	bc
	LC_{10}	69	34.78 (22.94; 48.86)	ab
	LC_{20}	68	52.94 (39.12; 66.33)	a
Grapholita molesta	Acetone	65	3.08 (0.75; 11.71)	b
	$LC_{0.001}$	65	4.62 (1.47; 13.56)	b
	LC_{1}	65	6.15 (2.29; 15.48)	b
	LC_{10}	66	18.18 (10.53; 29.55)	b
	LC_{20}	64	60.94 (48.37; 72.20)	a
Lobesia botrana	Acetone	63	7.94 (0.33; 1.79)	
	$LC_{0.001}$	64	14.06 (7.41; 25.08)	
	LC_{1}	62	17.74 (10.02; 29.48)	
	LC_{10}	70	14.29 (7.79; 24.75)	
	LC_{20}	66	19.70 (1.17; 31.23)	

Table S3. Review of studies reporting quantity (in ng) and blend ratios of female sex pheromone components of *C. pomonella*, *G. molesta* and *L. botrana*.

Cydia pomonella		**E,E-8,10-12:OH**	**12:OH**	**14:OH**	**E9-12:OH**	**Ratio pheromone blend**
Present study [a]		6.89 ± 1.08	1.87 ± 0.24	0.83 ± 0.15	0.68 ± 0.07	100 : 27.1 : 12.0 : 9.9
Witzgall *et al.* 2001		8.7 ± 2	-	-	-	100 : 18.4 : 3.8 : 5.1
El-Sayed *et al.* 1999a		-	-	-	-	100 : 20 : 5 : 10
Bartell *et al.* 1988		-	-	-		100: 90 : 30
Arn *et al.* 1985		2.1	1	0.04	0.2	100 : 47.6 : 1.9 : 9.5
Einhorn *et al.* 1984		-	-	-	-	100: 30-32: 10-17: 3-5
Grapholita molesta		**Z8-12:Ac**	**Z8-12:OH**	**E8-12:Ac**	**12:OH**	**Ratio pheromone blend**
Present study [a]		5.14 ± 0.35	2.19 ± 0.17	0.32 ± 0.02	0.25 ± 0.02	100 : 42.6 : 6.2 : 4.9
Knigth *et al.* 2015 [c]	French population	1.54 ± 0.18	0.33 ± 0.03	0.10 ± 0.01		100 : 21.2 : 6.2
	Italian population	2.07 ± 0.35	0.41 ± 0.04	0.14 ± 0.02		100 : 19.8 : 6.5
	Spanish population	1.95 ± 0.35	0.36 ± 0.03	0.13 ± 0.02		100 : 18.1 : 6.8
	USA population	1.47 ±0.49	0.21 ± 0.06	0.08 ± 0.03		100 : 15.3 : 5.6
Yang *et al.* 2002		8.28 ± 3.34	1.58 ± 0.40	0.56 ± 0.28	0.45 ± 0.52	100 : 19.1 : 6.8 : 5.4
El-Sayed and Trimble 2002		-	-	-		100 : 5.8 : 2.9
Han *et al.* 2001		-	-	-	-	100 : 1.9 : 7.2 : 2
Biwer *et al.* 1979		-	-	-		100 : 2 : 9
Cardé *et al.* 1979 [b]		-	-	-	-	100 : 30 : 7 : 6
Lobesia botrana		**E,Z-7,9-12:Ac**	**E,Z-7,9-12:OH**	**E9-12:Ac**	**Z9-12:Ac + 11-12:Ac**	**Ratio pheromone blend**
Present study [a]		4.35 ± 0.45	0.97 ± 0.14	0.48 ± 0.08	0.05 ± 0.00	100 : 22.3 : 11.0 : 1.1
Witzgall *et al.* 2005		0.87 ± 0.47	0.09 ± 0.04	0.03 ± 0.03	0.03 ± 0.02	100 : 10 : 3 : 3
El-Sayed *et al.* 1999b		-	-	-	-	100 : 5 : 1 : 11
Arn *et al.* 1988		-	-	-	-	100 : 25 : 0.5 : 8

[a] Acetone treated. In Figure 2 (main text) we show estimated values by GLM model.

[b] Volatile emission. The others are gland extracts.

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Figure S1. Set up for calling behaviour assay in the wind tunnel, a) front view, b) bottom-up view (detail of light source), c) view from downwind to show minimum overlap of tubes in the wind direction, d) test tubes and their dimensions.

Figure S2. Diagram of wind tunnel section. Lateral view (above) and view from above (bellow), showing the main dimensions.

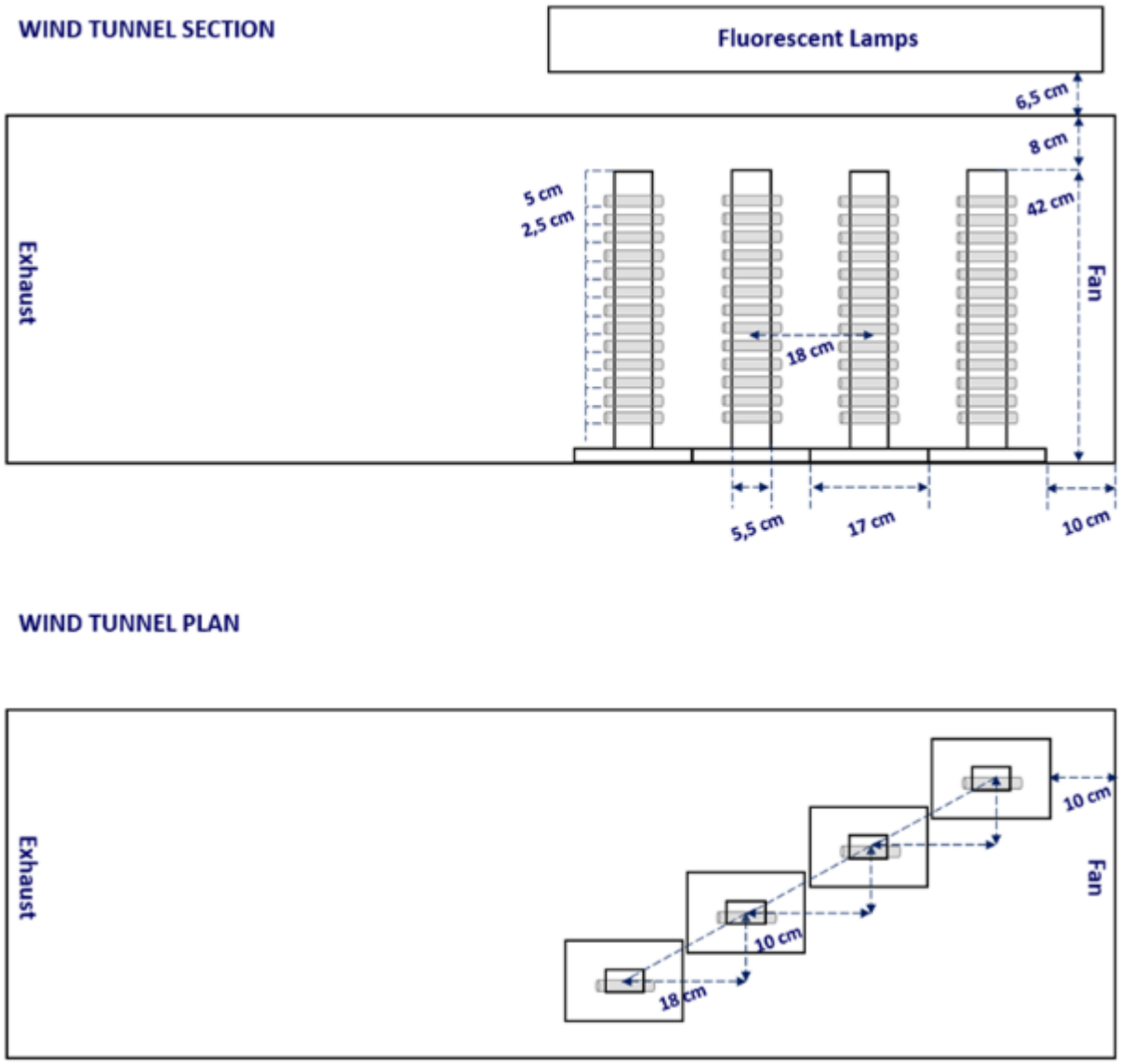


Figure S3 Calling period of untreated (no acetone or insecticide) females of *C. pomonella*, *G. molesta* and *L. botrana*, during a 12 h observation period (N = 69-75). The grey area represents the 8 h scotophase.

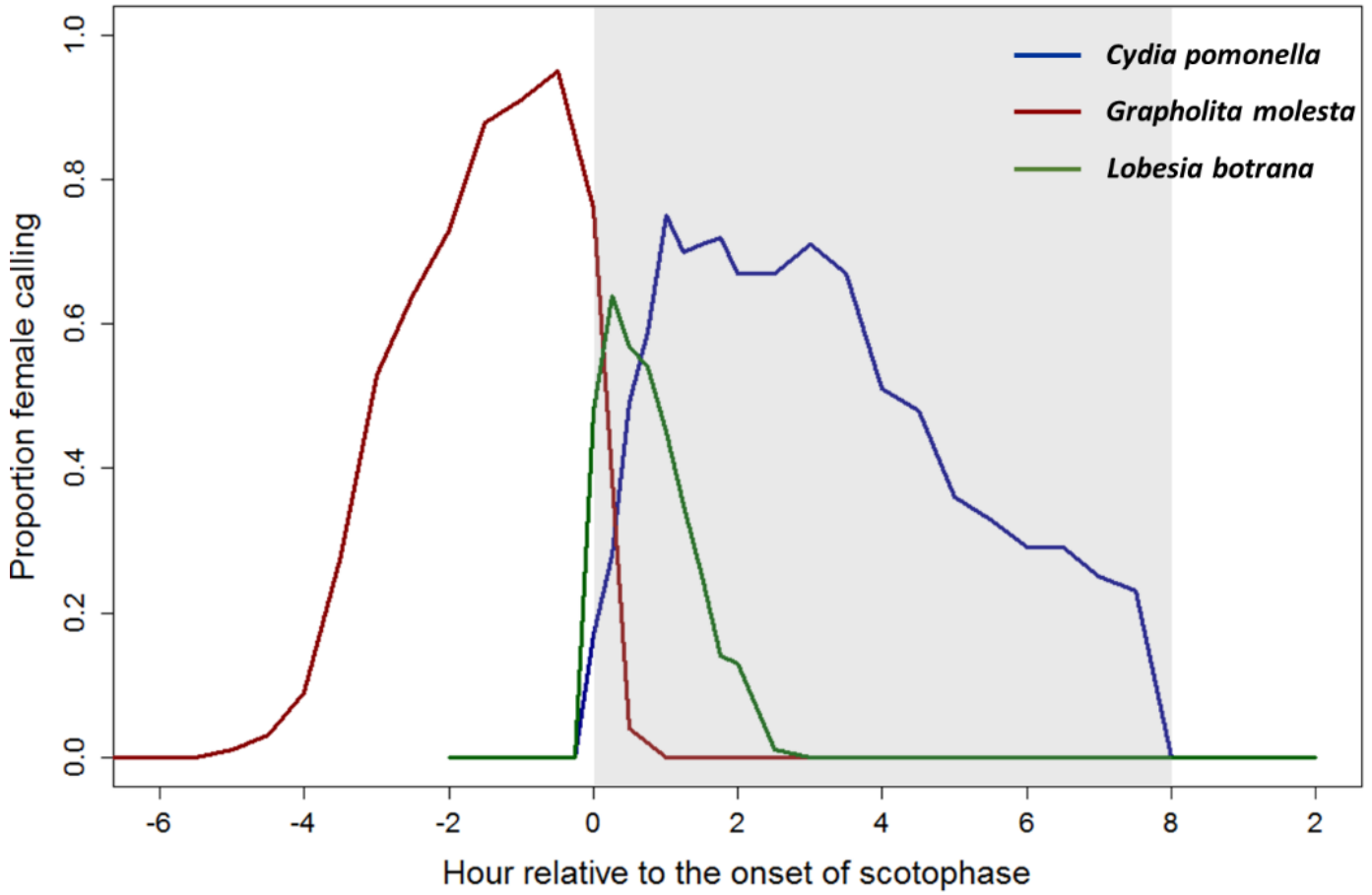


Figure S4. Individual female calling behaviour in *C. pomonella* treated with acetone or sublethal thiacloprid concentrations (LC_1 and LC_{20}). Each line corresponds with an observed female (N = 61-68). Colour scale represents calling intensity.

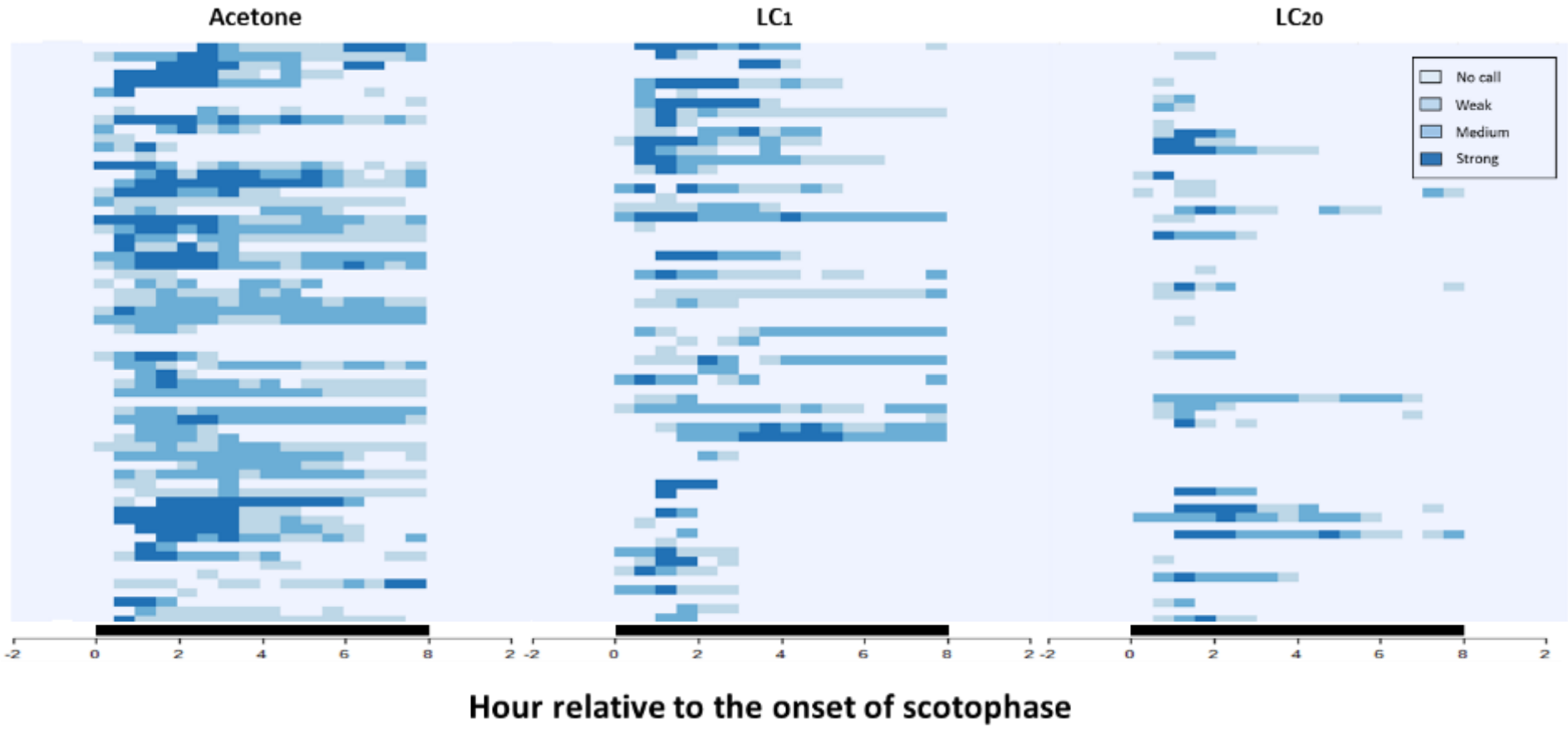


Figure S5. Individual female calling behaviour in *G. molesta* treated with acetone or sublethal thiacloprid concentrations (LC_1 and LC_{20}). Each line corresponds with an observed female (N = 64-65). Colour scale represents calling intensity.

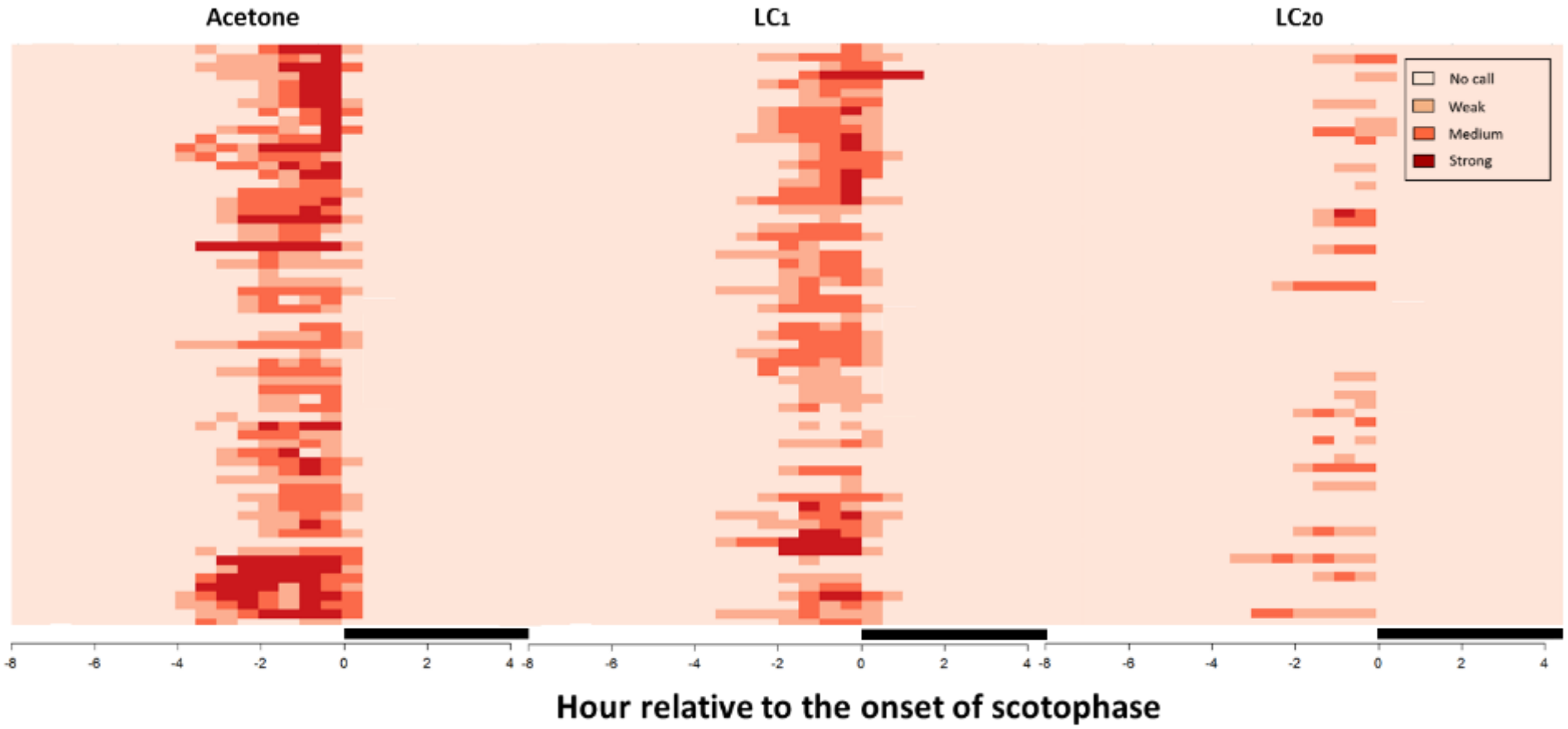
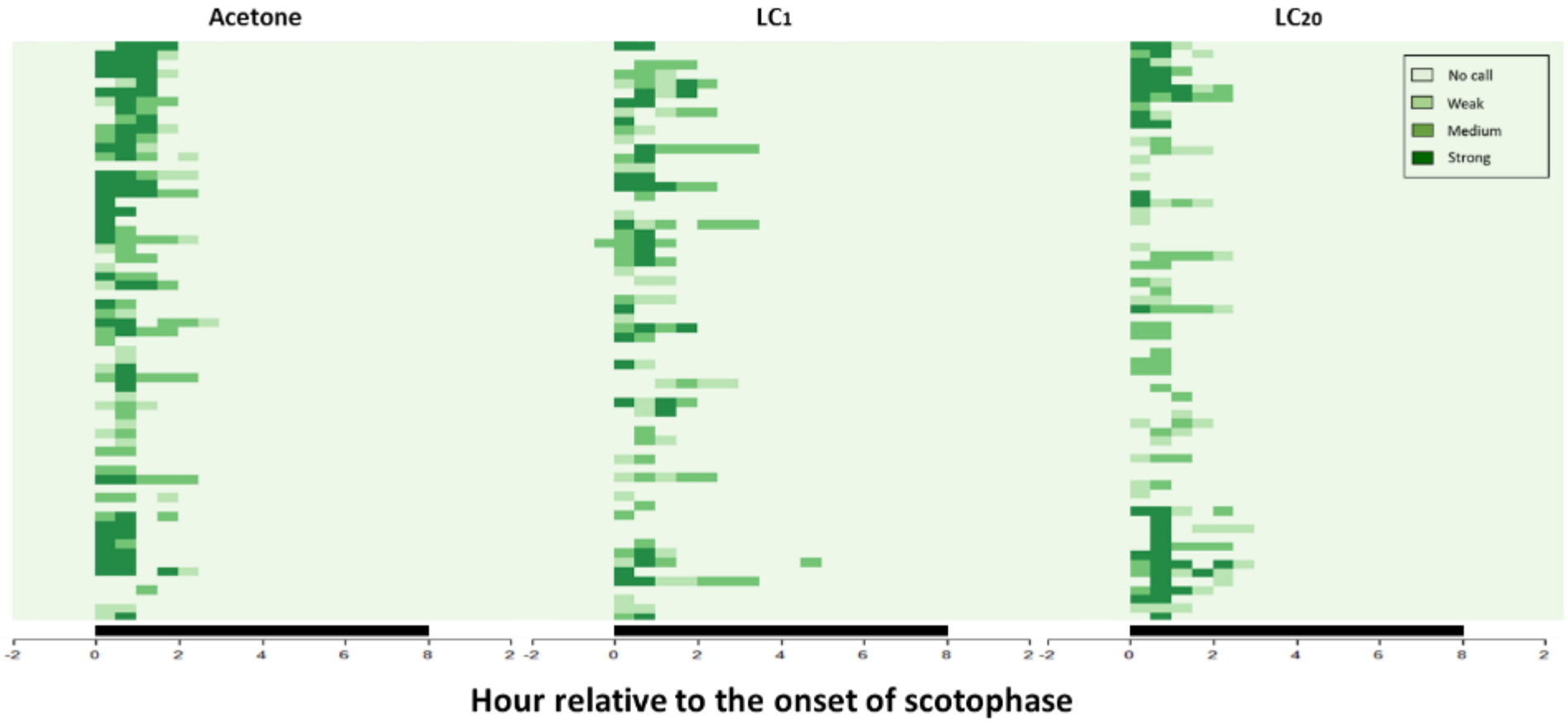


Figure S6. Individual female calling behaviour in *L. botrana* treated with acetone or sublethal thiacloprid concentrations (LC_1 and LC_{20}). Each line corresponds with an observed female (N = 62-65). Colour scale represents calling intensity.



SUBLETHAL DOSES OF THIACLOPRID AFFECT MALE FLIGHT RESPONSES TO SEX PHEROMONE BUT NOT ITS DETECTION IN THREE TORTRICID MOTHS

4

ABSTRACT

The effect of topically applied sublethal concentrations of thiacloprid on the flight behaviour and sensory detection of sex pheromone stimulus were investigated in males of three economically important tortricid moth pests [*Cydia pomonella* (L.), *Grapholita molesta* (Busck), and *Lobesia botrana* (Denis & Schiffermüller)]. In an Integrated Pest Management (IPM) context, these pests are mainly controlled with semiochemicals and insecticides, where residual accumulation of low doses of insecticides could influence olfactory behaviour. Thiacloprid, like other neonicotinoids, acts as agonists at the postsynaptic nicotinic-acetylcholine receptors (nAChRs), which are widely and predominantly distributed in the neuropil regions of the central nervous system (CNS). Results show that sublethal doses of thiacloprid had significant detrimental effects on both the percentage of males responding to the pheromone and several parameters of the flight tracks. These dose-dependent effects started already at the lowest lethal concentration $LC_{0.001}$, which kills only 1 in 10^5 individuals. The effects were not equal in the three species but the general trend was for a reduced percentage of response, slower flights and more drift in treated males than in control ones. Electroantennogram (EAG) responses to biologically appropriate insecticide doses showed no insecticide effect. This suggests that the behavioural effect of the insecticide is not mediated by changes in perception at the peripheral level. Since sex pheromones and neonicotinoids are used together in the management of these species, our results could have implications regarding the interaction between the two pest control methods.

KEY WORDS: sublethal, thiacloprid, male behaviour, pheromone communication, Tortricidae.

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Introduction

Since their appearance, chemical pesticides are the main tool used to control pest of fruit and vegetable crops worldwide, among other different technologies accepted in Integrated Pest Management (IPM), mainly due to their quick effect, low cost and relatively easy application (Waterfield and Zilberman 2012). Within insecticides, neonicotinoids are now the most widely used, owing to their flexibility of use (they can be applied in different ways), and their low toxicity to vertebrates and high toxicity to most of arthropods, which provides effective pest control (Jeschke *et al.* 2010). However, added to the upward risks of neonicotinoids like soil and water pollution, toxicity to non-target organism, or pest resistance (Goulson 2013), the direct toxicity of an insecticide against a pest species is not its only effect, but potential interactions with behaviour at sublethal doses (i.e., host finding, general locomotor and reproductive behaviour, etc.) must also be considered (Haynes 1988). Indeed, several studies report alterations of the normal perception of and response to chemical signals in insects treated with sublethal doses of insecticides (Tricoire-Leignel, *et al.* 2012 and references therein). The consequences of these sublethal exposures are strong and dose/concentration-dependent, and normally detrimental to the insect, but sometimes low insecticide doses could affect positively reproduction or behaviour, a phenomenon that is termed hormesis (Guedes and Cutler 2014).

Insecticide dose is not the only variable that influences sublethal effects, the insecticide mode of action (MoA) is important too. Many insect behaviours are associated to olfaction, which is totally dependent on nervous transmissions, and this is targeted by neurotoxic insecticides through different MoA. In the case of neonicotinoid insecticides, which are nicotinic acetylcholine receptor (nAChR) agonists, they strongly bind to nAChRs in the central nervous system of insects and this binding generates nervous stimulation at low doses, whereas at high doses it causes receptor blockage with the consequent paralysis and death of the insect (Matsuda *et al.* 2001, Casida and Durkin 2013).

Sex-pheromone communication involves the net displacement of one sex towards the odour source produced by the other sex. In Lepidopterans, it is usually females who release the sex pheromone and males who respond to it with and oriented flight towards the female. Differences in this sexual activity between species is an effective isolation mechanism and it is instrumental in speciation, and mainly results in species-specific sex-pheromone blends and behaviour timing, which could be very different between closely related species, as in the case of moths (Groot 2014 and references therein).

In the male moth, sexual behaviour depends on the detection of the female sex pheromone through olfactory receptor neurons (ORNs) located on their antennae. The projection from these neurons converge in the macroglomerular complex (MGC) of the antennal lobe (AL), which after integration of the signal elicit the behaviour of sex pheromone response (Galizia and Rössler 2010). The general odor-guided response model of a male moth finding and orienting along a pheromone plume consist first on a ranging flight to contact the plume and a zigzag upwind flight while in plume contact. If contact with the plume is lost the insect makes casting flight and finally, an upwind surge when filaments of pheromone are contacted at high frequency rates (over 5 Hz) (Cardé 2016). The usual method to analyse these flight tracks consist on the decomposition of flight maneuvers in the horizontal plane, described as the 2-D triangle of velocities by Marsh *et al.* (1978). Many factors have been found to influence the track of male moths, including intermittent pheromone stimulation (Willis and Baker 1984, Baker *et al.* 1985), pheromone concentration (Kuenen and Baker 1982, Charlton *et al.* 1993), pheromone plume structure (Mafra-Neto and Cardé 1994), plume composition (Bau *et al.* 1999), sex pheromone component ratios of the plume (Willis and Baker 1988), wind velocity (Willis and Cardé 1990), illumination (Cardé and Knols 2000), and flight height and visual patterns (Kuenen 2013, 2014). Several studies have reported the effect of insecticide on male flight response to the sex pheromone (Haynes 1988), but only one study has measured the effect of sublethal doses on flight track-related parameters, in which Carbaryl increased the distance between turns in upwind zigzag flights in *Grapholita molesta* (Busck) (Linn and Roelofs 1984). Other than this study, there are no further studies in Lepidoptera exploring the effect of sublethal doses of insecticide on flight track-related parameters.

Our study explores if male pheromone perception and response are affected by sublethal doses of insecticides, which could be useful in optimizing the combined use of semiochemicals and insecticides in IPM. We focus on tortricid moths pests, which are controlled both with semiochemicals and insecticides. These species are *Cydia pomonella* (L.), *G. molesta* and *Lobesia botrana* (Denis & Schiffermüller), which are main pests of apple, peach and grapevines, respectively; but they also attack other hosts and have a relatively worldwide distribution (Ioriatti *et al*. 2011, Kirk *et al.* 2013, Damos *et al.* 2015). As toxicant, we use the neuroactive insecticide thiacloprid, which is recommended for different life stages on insect control in stone and seed fruits attacked by *C. pomonella* and *G. molesta*, but not for *L. botrana* (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente [MAPAMA] 2017).

By comparing the effect of thiacloprid across phylogenetically related species, we hope to gain basic background information about sublethal effects on receivers, being the first comparative

report on these species, and follows up on a similar study made on females (Navarro-Roldán and Gemeno 2017). This is the first report of sublethal effects on Lepidopteran flight track analysis.

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, were maintained under laboratory conditions for more than 5 years without introduction of wild individuals. Larvae were reared in artificial diet (Ivaldi-Sender 1974) at 25 ± 1 ºC under a 16:8 hour light:dark photoperiod. 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. Thiacloprid (PESTANAL®, analytical standard, ≈ 100 % (a.i.). Sigma-Aldrich, Spain), was the active ingredients used in mortality test, flight behaviour and EAG response bioassays. All the dilutions used in the bioassays were prepared from pure compound, using acetone (CHROMASOLV®, for HPLC, ≥ 99.9 %. Sigma-Aldrich, Spain) as solvent and keeping them in. Dilutions were stored 2 or 4 ml acetone-rinsed glass vials at 7 ºC. The same stock of acetone used to prepare the dilutions was also used as the negative control treatment.

Four sublethal concentrations ($LC_{0.001}$, LC_1, LC_{10} and LC_{20}) plus control were tested on different bioassays. Sublethal concentrations were estimated for thiacloprid active ingredient (a.i.) according with results on (Navarro-Roldan, *et al.* 2017a). The values of sublethal concentrations (in nanogrames a.i. per microliter), for each species were: $LC_{0.001}$ (0.61, 4.88 and 32.29), LC_1 (4.24, 15.05 and 164.66), LC_{10} (12.04, 27.63 and 396.19) and LC_{20} (18.70, 35.68 and 573.40), respectively for *C. pomonella*, *G. molesta* and *L. botrana*.

Mortality test. Males received the treatments at 0-24 hours post-emergence during the last 1 to 5 h of photophase, depending on the species. One or two adults were placed in 10 ml test tubes that received a brief (10 seconds) flow of industrial grade CO_2, which quickly 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 with a high-precision, positive displacement, repeatable-dispensing micropipette (Multipette® M4Eppendorf, 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 cup was punctured to make 10 holes (1-mm-diameter each) that allow gas exchange, and a 1.5 ml eppendorf containing 10 % sugar solution and cotton lid was placed on the bottom of the cup to supply nutrients during the observation period. Bottles with treated insects were placed in the rearing room.

Mortality revisions were made at 24 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 floor but still moved, or as dead if they laid immobile on the floor of the bottle. Mortality was estimated with the sum of moribund and dead insects.

Pheromone stimulus in wind tunnel assays. Sex pheromone compounds were provided by Pherobank (The Netherlands) with an initial purity ≥ 99 %, and they were diluted in n-hexane (> 97 % pure, VWR Chemicals, BDH-Prolabo, Spain). The pheromone blend of *G. molesta* consists of a mixture of (Z)-8-Dodecenyl acetate (Z8-12:Ac), (E)-8-Dodecenyl acetate (E8-12:Ac), and (Z)-8-Dodecen-1-ol (Z8-12:OH) in a 100:6:10 ratio (Ammagarahalli and Gemeno 2015). For *C. pomonella* we used codlemone (E,E)-8,10-Dodecadien-1-ol (E,E-8,10-12:OH). For *L. botrana* we used gland extracts from 40- to 64-hour-old females. Extractions were restricted to a 1 h period coinciding with peak calling time (0 to 60 min after the onset of scotophase) (Navarro-Roldán and Gemeno 2017). The tip of the abdomen containing the sex pheromone gland tissue was excised and deposited in groups of 8 to 25 tips in solvent-rinsed and oven dried conical bottom glass vials (Total recovery vial, part number 186002805, Waters, USA) provided with Teflon-lined lids (part number 186000274, Waters, USA) containing 100 µl of *n*-hexane. After 30 min at room temperature, the glands were removed from the vial and the extracts of 100 pheromone glands where pooled (460 µl) and diluted with 540 µl of n-hexane for a final concentration of 1 pheromone gland extract per 10 µl. Stimuli were stored at -20°C.

Male flight behaviour. The flight tunnel consisted of a 170 × 45 × 45 cm (length × height × width) glass cage with a solid black floor and a sliding door on one of the long-sides. A 30-cm-diameter fan at the upwind end of the tunnel, and a 20-cm-diameter exhaust vent at the downwind end created a 0.32 m s^{-1} flow of unfiltered room air through the tunnel that was vented outside of the building. Temperature inside of the tunnel during tests was 23.83 ± 0.14 °C

(mean ± SEM). The flight tunnel was illuminated from above with two 25-watt incandescent white-light bulbs. A white fabric right underneath them homogenized their light. On the wind-tunnel wall opposite from the observers (left in the upwind direction) and at mid height along the tunnel´s length there were four CCTV infrared illuminators, each having 96 LED lights of 30° or 60° emitting at 850-nm (models IRE-30 and IRE-60, ECV Video Seguridad, Barcelona, Spain). They helped increase contrast of these small moths flying under the dim levels of visible light obtained by adjusting the intensity of the ceiling lights (20.2 ± 0.2 lux for *G. molesta* and 3.1 ± 0.1 lux for *C. pomonella* and *L. botrana*, at insect flight track height, mean ± SEM). Tests were carried out during the last 3 h of the photophase in *G. molesta*, from the second to the fourth h of scotophase in *C. pomonella* and during the first 2 h of the scotophase in *L. botrana*, corresponding to maximum female-calling periods (Navarro-Roldán and Gemeno 2017).

Males were placed individually in 10-cm-long × 3-cm-diameter glass tubes, with perforated aluminium lids covering both openings, and were transferred to the flight tunnel room, at least 30 min before the beginning of the test. Test odours were applied in 10 µl loads to 10 × 15 mm hexane-rinsed filter paper pieces (Whatman® No. 1, Sigma-Aldrich, Barcelona, Spain), with a final concentration of 100 ng of stimulus, except for *L. botrana*, which was a 1 female equivalent. The filter paper was held by a 30-mm alligator clip and was placed in a fume hood for 5 - 10 min to let dry before transferred to a 20 ml clean vial, where it remained until tested in the flight tunnel. The glass vial containing the test odour was opened and closed inside the flight tunnel to minimize contamination of the room. The base of the alligator clip was inserted vertically in the slot of a 25-mm binder clip, itself fixed to a 70-mm diameter aluminium metal plate located on top of a 12.5-cm-tall metal-wire platform (0.5-cm-mesh). The filter paper's flat surface faced the wind flow to attain a sufficiently turbulent odour plume. In a given day, several males of each treatment were exposed to a given filter paper, after which it was discarded and replaced by a fresh one, so that a filter paper was never exposed to the wind tunnel flow for more than one hour. After placing the odour stimulus in the upwind platform the male glass tube was placed in the flight tunnel on top of a metal-wire platform similar to the one used for the odour source and 1.30 m downwind from it. The aluminium lids were opened and we recorded for 2 min if the male took flight, started upwind oriented flight (zig-zagging up-wind flight) or landed on the filter paper containing the stimulus source. Males that did not take flight were tested outside of the tunnel to ensure that they could fly to eliminate false negatives. At the end of the day the interior of the flight tunnel was cleaned with ethanol and the exhaust fan was left on. All glass and metal utensils were thoroughly rinsed in acetone and oven-dried at 200 °C. Treatment order was randomized. N = 65 - 75 males per treatment.

Flight track analysis. Flights were recorded with a CCTV camera (Bosch DINION IP 5000 HD, Bosch Security Systems B.V., Eindhoven, The Netherlands) fitted with a 5.0-50 mm 1:14 objective and placed above the tunnel. The captured area had minimal angular distortion and spanned the width of the tunnel and 129 cm from the pheromone source to almost the point insect release at the insect´s flight level (Figure S1). Video was recorded at 25 frames per second (Bosh Video Client v1.6, Bosch Security Systems B.V., Eindhoven, The Netherlands) and the 2D (x,y) track coordinates were extracted with Blender v2.78 free online software (Blender Foundation 2007). The parameters of the triangle of velocities (Marsh *et al.* 1978) were calculated using flight track analysis software Track v1.1 (Bau and Guerrero 2013) (Table S1, Figure S2). Twenty videos were obtained for each thiacloprid dose except for LC_{10} in *G. molesta* and LC_{20} in *C. pomonella* and *G. molesta*, where the number of responding males was too low.

Electroantennography (EAG). Males were treated as in the wind tunnel experiment and tested 24 h later. They were held individually in modified alligator clips after 10 seconds of CO_2 anaesthesia and were placed in the EAG setup. Pulled glass capillaries with gold wire electrodes were filled with saline solution (1 % ClNa) and inserted in the moth´s mouth area (reference electrode) or placed over the left antenna's tip (recording electrode) from with some segments had been removed. The signal from the recording electrode was pre-amplified (10x gain, Universal Single Ended Probe, Syntech, Germany), filtered, and digitized (IDAC-4, Syntech, Germany), and recorded and analyzed in a PC (AutoSpike v.3.9, Syntech, Germany). The setup was mounted on an antivibration table (63–511, TMC Ametek, USA) shielded with a Faraday case to reduce low electrical noise.

Test odours were applied in 10 µl loads to 10 × 15 mm hexane-rinsed filter paper pieces (Whatman® No. 1, Sigma-Aldrich, Barcelona, Spain), let for 1 h in the fume hood, and introduced into disposable blue plastic 1000 µl pipette tips that were kept individually inside odour-clean 160-ml borosilicate glass tubes sealed with Teflon-lined caps until used.

A 0.6 l/min flow of charcoal-filtered and humidified air blew continuously over the insect preparation (CS-55, Syntech, Germany) through an 8-mm internal diameter steel tube placed 20 mm from the preparation. The tip of the pipette tip containing the odour stimulus was inserted 2-3 mm in the tube perpendicular to the direction of the continuous air flow through a hole in the tube´s wall located 11 cm from the preparation. A 0.3 l/m charcoal-filtered room airflow was

puffed through the odour cartridge for 0.5 s, continuous flow was decreased by 0.3 l/min during the puff. Stimuli were applied in increasing order of pheromone concentration (n-hexane, 1 ng, 10ng, 100ng, 1µg, 10 µg and 100 µg). Time intervals between puffs were 30 s between n-hexane and the low pheromone dose (1 ng), 60 s between the next pheromone dilutions (10 to 1µg), 120 s between 1 µg and 10 µg and 240 s between 10 and 100 µg, but longer if needed to let the spike activity return to pre-stimulation levels. Results of dose-response curves using acetone-treated insects (N = 20) served to select behaviourally relevant concentrations (i.e., lower than saturation levels) for the test with insecticide-treated insects (N = 20). Insecticide treatments were randomized, each antenna was treated with all the pheromone doses.

For the selection of the pheromone concentration we measured just EAG maximum depolarization (i.e., peak amplitude), but in the insecticide experiment we measured other EAG peak parameters: rise time, depolarization velocity (relationship between peak amplitude and rise time), recovering time, hyperpolarization time and maximum hyperpolarization, (Figure S3). Hyperpolarization was calculated for higher pheromone doses only (100 ng and 1 µg).

Data analysis. All the statistical analyses were run in R software (R Core Team 2016). Mortality was analysed using the Fisher's exact test with Bonferroni correction. The other tests were analysed with generalized linear models (GLM) using the appropriate distributions (binomial for percentages of response, and gaussian for the other dependent variables). For track analysis the wind tunnel was divided in three sections corresponding to zones where, after inspecting the videos, most males a) detected and started to fly towards the stimulus (section 1, 0 to 20 cm from downwind), b) progressed with typical zigzagging upwind flight (section 2, 20 to 100 cm from downwind) and c) approached the pheromone source (section 3, 100 to129 cm from downwind). Dividing the track in sections permitted control for the effect of distance, which is known to affect flight track parameters (Willis and Baker 1994). GLM models for the analysis of track parameters included the terms insecticide dose, wind tunnel section, and their interaction. Comparison of track parameters among species used only the control (acetone) individuals, and each section was analysed separately. For model-selection, we used the likelihood ratio test (LRT) and the Akaike information criterion (AIC), preferring the model with the lower AIC value of pairs that were significantly different by LRT. The predictmeans() function was used to perform *Tukey*'s multiple pairwise comparisons and provide the parameter estimates and associated standard errors shown in tables and figures for those parameters that were significant in the GLM models.

Raw data and R scripts are available online (*Repository UdL*). Whenever the term "significant" is used in the text regarding differences between treatments it indicates a p-value < 0.05.

Results

The level of mortality obtained with our test concentrations (Table S2) was comparable to the mortality estimated in a previous study (Navarro-Roldán *et al.* 2017a). Acetone and $LC_{0.001}$ did not induce any mortality, except in $LC_{0.001}$ for *G. molesta* in wind tunnel assay. The maximum mortality with LC_1 was below 2.7 %, except in the case of *G. molesta* insects used in EAG assays. LC_{20} mortality ranged between 6.5 % and 30.2 %, and with LC_{10} it was between LC_1 and LC_{20} in all cases.

Male flight behaviour. Sublethal doses of thiacloprid reduced take flight in *G. molesta* and *L. botrana*, and oriented flight and pheromone source contact in *C. pomonella* and *G. molesta* (Figure 1). The effect was notable in *G. molesta*, moderate in *C. pomonella* and limited in *L. botrana*. In *G. molesta* it was significant already at the second lowest dose, LC_1, affected all behavioural steps and caused reductions of up to 94 % of contact compared with acetone. In *L. botrana* and *C. pomonella* only the highest concentration of thiacloprid (LC_{20}) had a significant effect and only on take-flight in *L. botrana* and oriented flight and contact in *C. pomonella*, with a maximum reduction of 64 % for contact in *C. pomonella* (Figure 1). Thiacloprid delayed the starting times of all behavioural flight steps in *G. molesta*, as well as their time to complete the flight, and the time to take flight of *L. botrana* (Table 1).

The effect of wind tunnel section on flight track parameters was always significant (Tables S3 and 2B). Its interaction with flight track parameters was significant in 4 of the 30 GLMs, so mean comparison tests of the individual variables was performed in all 30 models (Table S3). Overall, males flew faster, more straight and with wider turns in the middle section (section 2) and they reduced their speed, flew more perpendicular to the wind line and performed more turns as they approached the pheromone (section 3), while section 1 parameters had intermediate values (Table 2B). Insecticide affected flight track parameters in all three species, but the effect was not homogeneous in all of them (Table 2A). Roughly, thiacloprid made males more suscep-

Figure 1. Effect of thiacloprid on the percentage predicted means of males that take flight, made oriented flight or contact with the pheromone source (N = 75-65). Different letters indicate significant differences among treatments for each compound and species (P<0.05, *Tukey* after GLM)

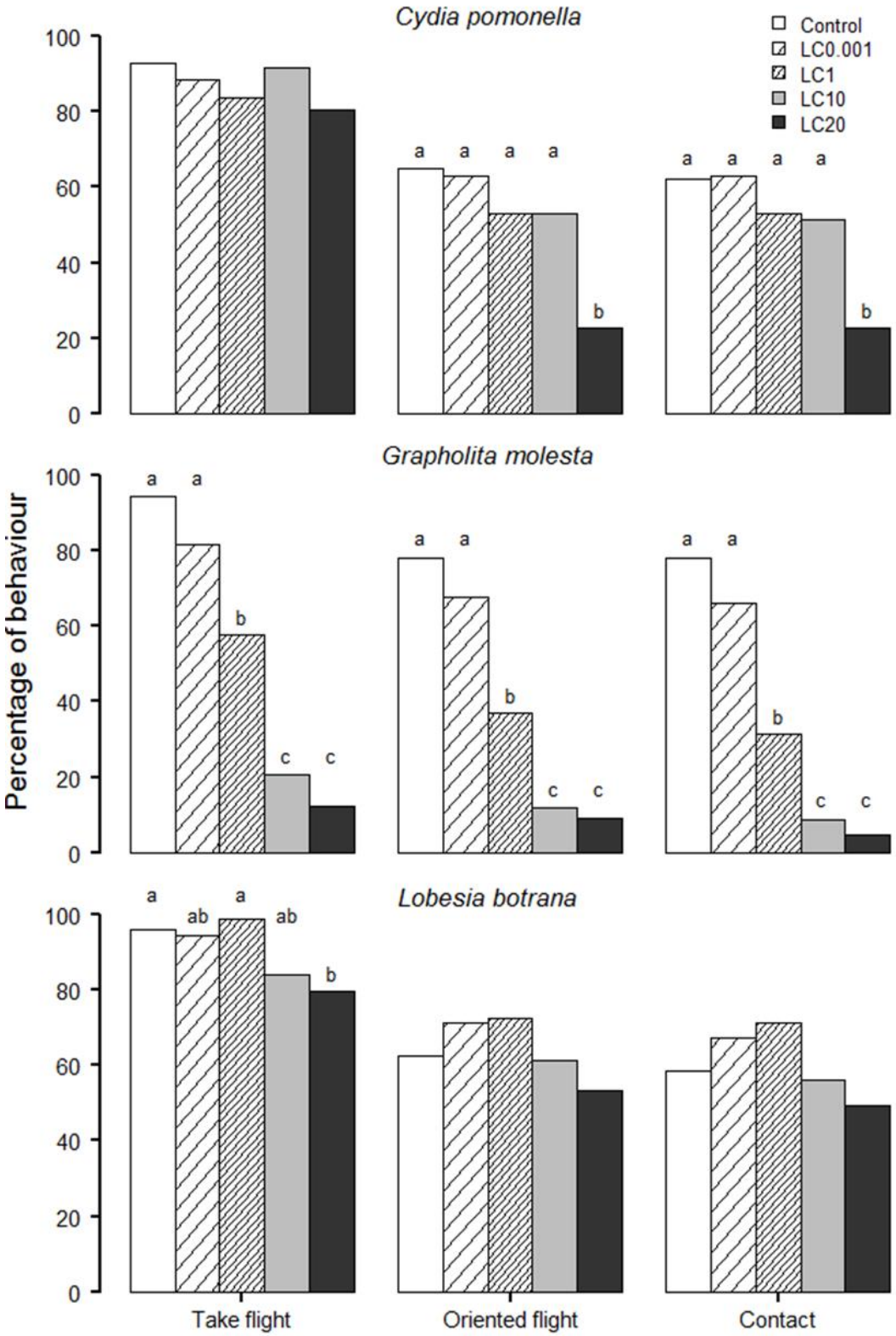


Table 1. Effect of thiacloprid on starting times (predicted means in seconds) of take flight (TF), oriented flight (OF), contact with the pheromone source (C) and mean time duration from TF to C on insects that complete the behaviour (D). Different letters within a column and species indicate significant differences among treatments ($P < 0.05$, *Tukey* after GLM). N = number of males with observed behaviour assessed.

Species	Treatment	N	TF (mean ± SEM)		N	OF (mean ± SEM)		N	C (mean ± SEM)		N	D (mean ± SEM)	
Cydia pomonella	Acetone	66	11.08 ± 2.29		46	23.54 ± 4.23		44	33.48 ± 4.41		44	25.93 ± 3.62	
	$LC_{0.001}$	62	12.27 ± 2.36		44	25.77 ± 4.32		44	37.11 ± 4.41		44	28.82 ± 3.62	
	LC_1	57	11.19 ± 2.47		36	17.86 ± 4.78		36	33.89 ± 4.88		36	26.47 ± 4.01	
	LC_{10}	64	15.64 ± 2.33		37	24.14 ± 4.72		36	39.56 ± 4.88		36	26.17 ± 4.01	
	LC_{20}	57	17.70 ± 2.47		16	24.31 ± 7.17		16	37.56 ± 7.32		16	23.13 ± 6.01	
Grapholita molesta	Acetone	69	7.57 ± 2.29	b	57	11.25 ± 2.78	b	57	17.91 ± 2.78	b	57	11.28 ± 2.21	b
	$LC_{0.001}$	58	13.09 ± 2.49	b	48	23.10 ± 2.03	a	47	31.64 ± 3.06	a	47	20.15 ± 2.43	a
	LC_1	42	29.07 ± 2.93	a	27	24.93 ± 4.04	a	23	33.35 ± 4.38	a	23	13.13 ± 3.48	ab
Lobesia botrana	Acetone	72	6.99 ± 2.03	b	47	17.02 ± 3.15		44	25.66 ± 3.06		44	21.39 ± 2.29	
	$LC_{0.001}$	69	9.23 ± 2.08	ab	52	17.42 ± 2.99		49	30.49 ± 2.90		49	21.33 ± 2.17	
	LC_1	72	10.15 ± 2.03	ab	53	15.55 ± 2.97		52	26.92 ± 2.81		52	21.00 ± 2.11	
	LC_{10}	63	11.33 ± 2.18	ab	46	20.50 ± 3.18		42	32.55 ± 3.13		42	25.33 ± 2.34	
	LC_{20}	58	15.97 ± 2.27	a	39	21.00 ± 3.46		36	29.39 ± 3.38		36	19.78 ± 2.53	

Table 2. Differences on the effect of thiacloprid on some parameters of the triangle of velocities (predicted means) independently of the flight section (part A), and differences among flight sections (predicted means) independently of the insecticide treatment (part B). ($P < 0.05$, *Tukey* after GLM). N = number of tracks analysed in each group.

A	Species	Treatment	N	TA (mean ± SEM)		CA (mean ± SEM)		DA (mean ± SEM)		GS (mean ± SEM)		AS (mean ± SEM)		T (mean ± SEM)		IT (mean ± SEM)		FL (mean ± SEM)		FD (mean ± SEM)		FV (mean ± SEM)	
		Acetone	20	60.00 ± 1.83		32.80 ± 1.07		27.20 ± 0.93	b	438.70 ± 13.17	a	642.79 ± 12.80	ab	10 ± 1.045	b	77.89± 3.92	ab	1297.80 ± 143.26	b	3.19± 0.33	bc	424.08 ± 12.81	a
		$LC_{0.001}$	20	58.33 ± 1.83		32.92 ± 1.07		25.41 ± 0.93	b	459.63 ± 13.17	a	667.46 ± 12.80	a	8 ± 1.054	b	81.15± 3.92	ab	1301.70± 143.26	b	3.04± 0.33	c	440.75 ± 12.81	a
	C.p	LC_1	20	62.49 ± 1.83		33.60 ± 1.07		28.89 ± 0.93	ab	413.22 ± 13.17	a	613.08 ± 12.80	bc	13 ± 1.04	a	77.14± 3.92	ab	1601.51± 143.26	ab	4.35± 0.33	ab	396.15 ± 12.81	a
		LC_{10}	20	61.68 ± 1.83		30.75 ± 1.07		30.93 ± 0.93	a	359.44 ± 13.17	b	568.32 ± 12.80	c	12 ± 1.045	a	66.72± 3.92	b	1627.05 ± 143.26	ab	4.69± 0.33	a	344.91 ± 12.81	b
		LC_{20}	15	62.37 ± 2.11		34.48 ± 1.23		27.90 ± 1.07	ab	427.47 ± 15.20	a	624.91 ± 14.77	ab	13 ± 1.049	a	83.43± 4.53	a	1963.21 ± 165.42	a	4.69± 0.38	a	412.37 ± 14.79	a
		Acetone	20	56.63 ± 2.02		34.07 ± 1.25		22.57 ± 0.96	b	575.68 ± 17.04	a	779.42 ± 17.50	a	6 ± 1.053	b	82.07± 4.98		1192.49± 114.66		2.28± 0.24		540.33 ± 15.92	a
	G.m	$LC_{0.001}$	20	56.56 ± 2.02		31.80 ± 1.25		24.76 ± 0.96	ab	498.57 ± 17.04	b	711.64 ± 17.50	b	6 ± 1.052	b	81.17± 4.98		1117.57± 114.66		2.59± 0.24		474.60 ± 15.92	b
		LC_1	20	57.43 ± 2.02		31.48 ± 1.25		25.96 ± 0.96	a	437.78 ± 17.04	c	648.60 ± 17.50	c	7 ± 1.045	a	71.87± 4.98		1172.06± 114.66		2.80± 0.24		418.84 ± 15.92	c
		Acetone	20	61.49 ± 1.75		30.69 ± 0.95	a	30.80 ± 1.38		399.46 ± 15.92	ab	609.07 ± 16.26	ab	12.85 ± 1.30		56.22± 3.64		1280.49± 131.12		3.87± 0.43		379.37 ± 14.87	ab
		LC0.001	20	59.09 ± 1.75		30.01 ± 0.95	ab	29.07 ± 1.38		428.35 ± 15.92	a	641.01 ± 16.26	a	11.87 ± 1.30		57.97± 3.64		1333.27± 131.12		3.74± 0.43		397.93 ± 14.87	a
	L.b	LC1	20	59.80 ± 1.75		29.29 ± 0.95	ab	30.51 ± 1.38		375.65 ± 15.92	ab	591.90 ± 16.26	ab	12.37 ± 1.30		55.14± 3.64		1200.45± 131.12		3.76± 0.43		353.32 ± 14.87	ab
		LC_{10}	20	60.27 ± 1.75		28.20 ± 0.95	ab	32.07 ± 1.38		349.76 ± 15.92	b	568.86 ± 16.26	b	15.63 ± 1.30		52.17± 3.64		1301.68± 131.12		4.62± 0.43		328.13 ± 14.87	b
		LC_{20}	20	55.82 ± 1.75		26.44 ± 0.95	b	29.38 ± 1.38		351.59 ± 15.92	b	580.27 ± 16.26	ab	12.83 ± 1.30		54.03± 3.64		1175.38± 131.12		3.93± 0.43		330.17 ± 14.87	b
B	**Species**	**Section**	**N**	**TA (mean ± SEM)**		**CA (mean ± SEM)**		**DA (mean ± SEM)**		**GS (mean ± SEM)**		**AS (mean ± SEM)**		**T (mean ± SEM)**		**IT (mean ± SEM)**		**FL (mean ± SEM)**		**FD (mean ± SEM)**		**FV (mean ± SEM)**	
		1	95	55.93 ± 1.23	b	29.81±0.80	b	26.11 ± 0.74	b	419.42± 10.49	b	638.02 ± 10.19	b	4 ± 1.05	c	70.89 ± 3.12	b	575.19 ± 114.10	b	1.50 ± 0.26	c	391.42 ± 10.20	b
	C.p	2	95	55.10 ± 1.23	b	32.12±0.80	b	22.97 ± 0.74	c	472.06 ± 10.49	a	686.77 ± 10.19	a	16 ± 1.03	b	85.13 ± 3.12	a	2100.50 ± 114.10	a	4.78 ± 0.26	b	469.84 ± 10.20	a
		3	95	71.67 ± 1.23	a	36.55±0.80	a	35.11 ± 0.74	a	367.58 ± 10.49	c	545.15 ± 10.19	c	19 ± 1.02	a	75.79 ± 3.12	ab	1999.07 ± 114.10	a	5.70 ± 0.26	a	349.69 ± 10.20	c
		1	100	48.00 ± 1.71	c	27.72±1.18	b	20.28 ± 0.96	b	506.73 ± 17.04	b	740.85 ± 17.50	b	2 ± 1.09	c	64.82 ± 4.22	b	373.25 ± 80.21	c	0.89 ± 0.18	b	459.08 ± 15.92	b
	G.m	2	100	54.78 ± 1.71	b	35.48±1.18	a	19.30 ± 0.96	b	616.83 ± 17.04	a	822.19 ± 17.50	a	10 ± 1.04	b	107.78 ± 4.22	a	1915.76 ± 80.21	a	3.28 ± 0.18	a	613.27 ± 15.92	a
		3	100	67.84 ± 1.71	a	34.14±1.18	a	33.70 ± 0.96	a	388.46 ± 17.04	c	576.61 ± 17.50	c	12 ± 1.04	a	62.52 ± 4.22	b	1193.11 ± 80.21	b	3.50 ± 0.18	a	361.42 ± 15.92	c
		1	100	53.74 ± 1.24	b	27.04±0.74	b	26.70 ± 0.89	b	413.43 ± 12.33	a	642.14 ± 12.60	a	4.21 ± 1.01	b	52.97 ± 2.55	b	452.25 ± 76.91	c	1.44 ± 0.28	b	382.97 ± 11.52	b
	L.b	2	100	57.21 ± 1.24	b	31.49±0.74	a	25.71 ± 0.89	b	450.00 ± 12.33	a	664.05 ± 12.60	a	18.49 ± 1.01	a	70.48 ± 2.55	a	2062.85 ± 76.91	a	5.18 ± 0.28	a	445.88 ± 11.52	a
		3	100	66.94 ± 1.24	a	28.25±0.74	b	38.69 ± 0.89	a	279.45 ± 12.33	b	488.48 ± 12.60	b	16.63 ± 1.01	a	41.86 ± 2.55	c	1259.66 ± 76.91	b	5.33 ± 0.28	a	244.49 ± 11.52	c

TA = Track angle (degrees); CA = Course angle (degrees); DA = Drift angle (degrees); GS = Ground speed (mm/s); AS = Air speed (mm/s); T = Turns (N); IT = Intern-turns (mm); FL = Flight length (mm); FD = Flight duration (s); FV = Flight velocity (mm/s).

tible to drift from the wind flow (higher DA), and so their speed was reduced (GS), but it did not affect the intended flight direction (CA), and therefore they maintained the same angle of flight with respect to the wind line (TA), and they flew faster to the pheromone source (FV) (Table 2). The observed means for the different parameters analysed for each insecticide treatment within tunnel section for each species are shown in Table S4. No flight track parameter, within a given section, was significatively different between species (Table 3). All three species showed the same differences among wind tunnel sections in flight speeds, turns and angles. However, there were notable differences in the shape of the flight track among species. In section 2 the flight of *G. molesta* was markedly different from the other two species in that males few faster, experiences less drift and performed wider turns, whereas in that same section, *L. botrana* are distinct from the other two species in their much larger number of turns (almost twice than the other two species), which are relatively shorter (the zigzagg is narrower) and they experienced more drift (Table 3).

Male EAG response. The lowest pheromone dose that produced significantly higher EAG peak amplitudes then hexane was 1 µg in the three species (Figure 2). We selected this pheromone dose and the three bellow (1, 10 and 100 ng) for the insecticide tests, as they are bellow saturation and probably in the range of what females emit. Thiacloprid did not affect EAG responses in peak amplitude (Figure 3), or any other EAG parameters (Table S5) at any of the pheromone doses tested.

Discussion

Male moth's response to sex pheromones is a complex sequence of events that concludes in male locating the female. When insects are at rest, signal detection by ORNs and the integration at AL, results in a behavioural sequence that starts with the activation of the insect, which take flight and, while in contact with the pheromone plume it makes an oriented flight consisting on a zigzag upwind that ends with contact with the pheromone source. During upwind flight, if the insect losses the pheromone plume, it makes casting flight until it re-contacts the stimulus (Cardé 2016). It seems obvious that the development of this behavioural sequence is a potential target for neurotoxicants because both the nervous and optomotor systems are implicated (Haynes and Baker 1985, Tricoire-Leignel *et al.* 2012). In an agricultural context with widespread use of insecticides, is not difficult to think that adult moths could be exposed to sublethal doses of

Table 3. Species differences in some parameters of the triangle of velocities (predicted means) within flight sections in wind tunnel, when insects are treated only with acetone. ($P < 0.05$, *Tukey* after GLM). N = number of tracks analysed in each group.

Section	Species	N	TA (mean ± SEM)		CA (mean ± SEM)		DA (mean ± SEM)		GS (mean ± SEM)		AS (mean ± SEM)		T (mean ± SEM)		IT (mean ± SEM)		FL (mean ± SEM)		FD (mean ± SEM)		FV (mean ± SEM)	
1	*C. pomonella*	20	58.40 ± 4.11		30.62 ± 2.36		27.78 ± 2.41	a	426.76 ± 35.06	b	636.39 ± 39.03	b	5 ± 1.11	a	68.72 ± 7.56		613.05 ± 87.00		1.65 ± 0.37		396.75 ± 29.94	b
	G. molesta	20	46.95 ± 4.11		29.13 ± 2.36		17.83 ± 2.41	b	605.59 ± 35.06	a	834.97 ± 39.03	a	2 ± 1.17	b	69.25 ± 7.56		401.84 ± 87.00		0.80 ± 0.37		536.40 ± 29.94	a
	L. botrana	20	57.34 ± 4.11		29.11 ± 2.36		28.23 ± 2.41	a	419.24 ± 35.06	b	636.89 ± 39.03	b	5 ± 1.10	a	50.03 ± 7.56		488.72 ± 87.00		1.70 ± 0.37		391.86 ± 29.94	b
2	*C. pomonella*	20	51.07 ± 2.77		30.77 ± 1.86	b	20.30 ± 1.45	b	502.12 ± 27.55	b	726.10 ± 28.46	b	12 ± 1.07	b	86.06 ± 7.00	b	1697.46 ± 201.06		3.57 ± 0.54	b	501.41 ± 27.91	b
	G. molesta	20	56.03 ± 2.77		38.34 ± 1.86	a	17.69 ± 1.45	b	706.25 ± 27.55	a	902.08 ± 28.46	a	10 ± 1.07	b	119.05 ± 7.00	a	2093.40 ± 201.06		3.08 ± 0.54	b	702.38 ± 27.91	a
	L. botrana	20	60.05 ± 2.77		33.93 ± 1.86	ab	26.12 ± 1.45	a	490.26 ± 27.55	b	693.71 ± 28.46	b	20 ± 1.05	a	75.65 ± 7.00	b	2263.66 ± 201.06		5.43 ± 0.54	a	485.61 ± 27.91	b
3	*C. pomonella*	20	70.54 ± 1.80		37.02 ± 1.42	a	33.52 ± 1.55	ab	387.21 ± 20.16	a	565.89 ± 18.14	a	15 ± 1.06	a	78.90 ± 6.01	a	1582.89 ± 142.33	a	4.34 ± 0.43	ab	374.08 ± 21.39	a
	G. molesta	20	66.92 ± 1.80		34.74 ± 1.42	a	32.18 ± 1.55	b	415.19 ± 20.16	a	601.20 ± 18.14	a	11 ± 1.07	b	57.91 ± 6.01	b	1082.23 ± 142.33	b	2.97 ± 0.43	b	382.23 ± 21.39	a
	L. botrana	20	67.09 ± 1.80		29.03 ± 1.42	b	38.06 ± 1.55	a	288.87 ± 20.16	b	496.60 ± 18.14	b	14 ± 1.06	a	42.98 ± 6.01	b	1089.08 ± 142.33	b	4.49 ± 0.43	a	260.64 ± 21.39	b

Figure 2. Pheromone dose curves for each male species using as stimuli the major pheromone component, using observed means. Letters in same color show significant differences for each species. (P < 0.05, *Tukey* after GLM). (N = 4-5, per dose and species).

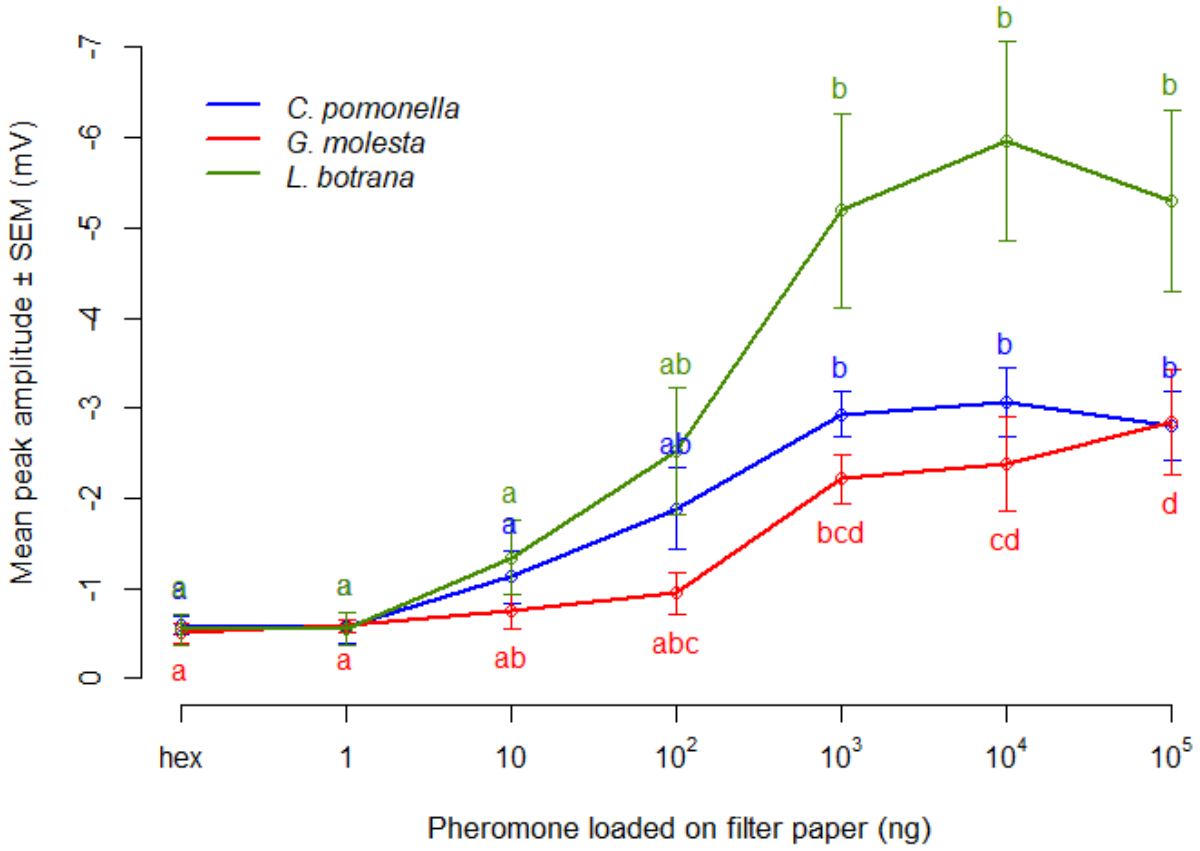
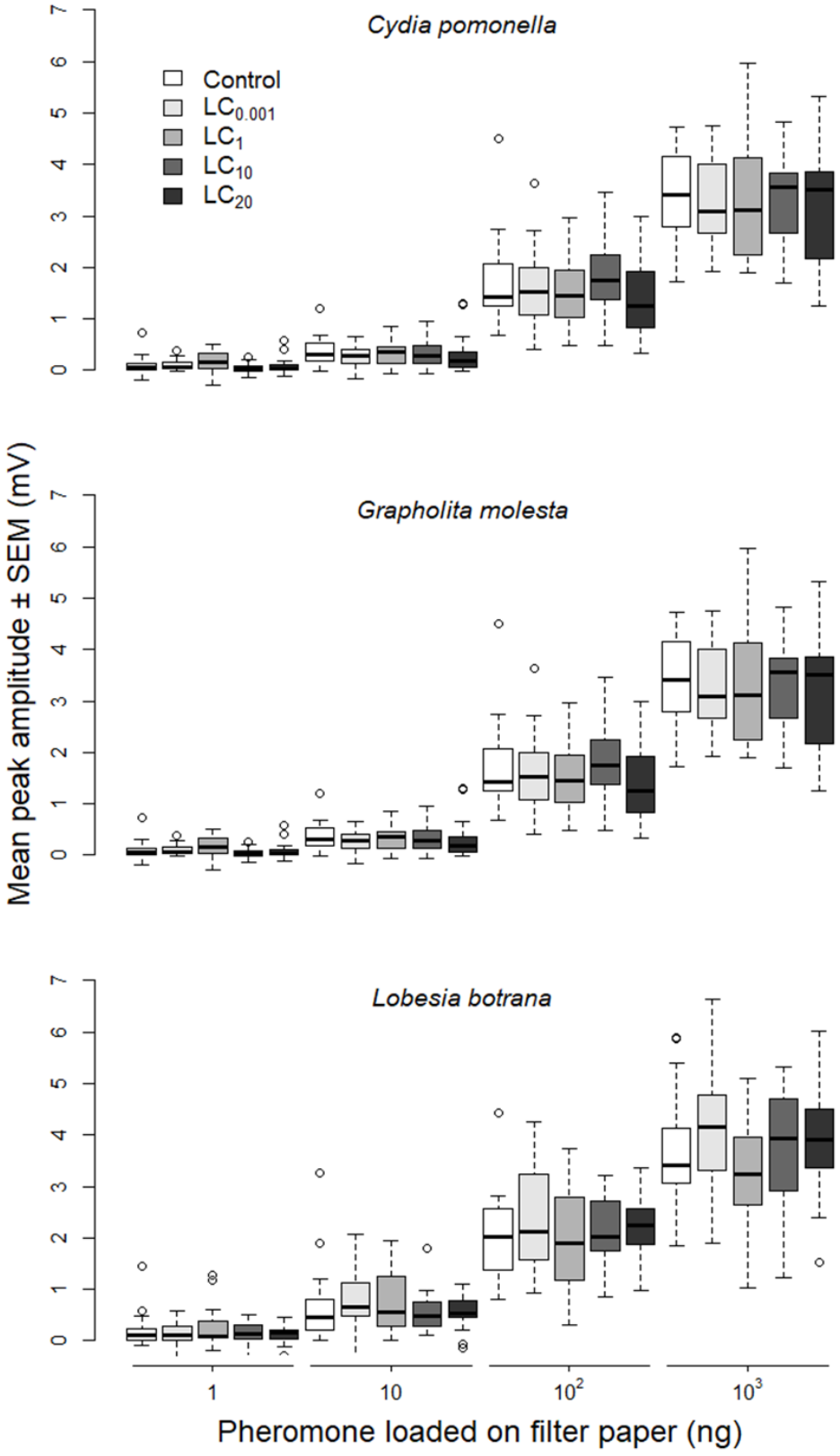


Figure 3. Effect of thiacloprid on mean peak amplitude (observed means in mV) for different doses of major pheromone component. Insecticide treatment was no significant. (N = 20, per dose and species).



insecticides even though the application is not aimed at them. In the present study, sublethal doses of neonicotinoid thiacloprid, doses that did not generate mortality, significantly modified male's flight behaviour in response to an odour stimulus, but not the antennal perception of the stimulus.

Reports on wind tunnel assays with the same species show that the response observed in terms of take flight, oriented flight and contact with the pheromone source, in males that were not treated with insecticide, is similar to what we found for males treated only with acetone (Figure 1) (Witzgall et *al.* 2001, Coraccini *et al.* 2004, Judd *et al.* 2005, Evenden and McLaughlin 2005, Varela *et al.* 2011, Ammagarahalli *et al.* 2017). However, in *L. botrana* there are references which show higher percentages of behaviour than ours (El-Sayed *et al.* 1999), and other studies that report relatively lower percentages of response than we did (von Arx *et al.* 2011, Sans *et al.* 2017). Slight differences across studies could be attributed to population differences or to methodological aspects related to the pheromone-blend or the stimulus concentration used, or small differences in wind tunnel conditions. Regarding to track analysis, *G. molesta* is one of the most studied species, starting with the anemotactic zigzagging flight analysis using the triangle of velocities described (Marsh *et al.* 1978). Track parameter results for *G. molesta* were similar in our study and in previous ones with regard to angles (Willis and Baker 1994), number of turns, inter-turn distances and ground speeds (Kuenen 2013, Kuenen *et al.* 2014). There are fewer studies with *C. pomonella* and *L. botrana*, but in general they report similar results for angles and velocities to ours (El-Sayed 2004, Witzgall *et al.* 2001, Coracini *et al.* 2004). Results of track and course angles and ground speeds for *L. botrana* were slight different from those reported elsewhere (Witzgall and Arn 1990, El-Sayed *et al.* 1999, 2000), but similar ratios between AS and wind speed (WS) have been reported (Witzgall 1997).

Although it was not the main aim of our study, our data allow us to compare the flight track parameters of the three species since they were flown in almost identical (except for light intensity) wind tunnel conditions. All three species showed the same differences in flight speeds, turns and angles among wind tunnel sections. If we focus in the middle section, which is the one where the males do most of the approach to the pheromone, it is clear that the flight of *G. molesta* is different from the other two species in that males fly faster, experience less drift and perform wider turns, while *L. botrana* are distinct in their much larger number of turns (almost twice than the other two species), narrow turn distances and relatively slow flight, which was noticeable already with the naked eye. Differences in flight pattern of these closely related species could be explained by their different daily periods of sexual activity (diurnal or nocturnal, Navarro-Roldán and Gemeno 2017), or their occurrence in different agroecosystems

(fruit trees and vineyards), where wind or other environmental characteristics may have shaped their flight pattern over evolutionary time.

Approach to pheromone source reduced velocity and inter-turn length, and increased the angle of flight with respect to wind direction and the number of turns in all three species. The same was observed in 33 cm sections of a more than 200 cm-long wing tunnel with *G. molesta* males (Willis and Baker 1994). The authors concluded that the structure of the pheromone plume was the factor that modulates this behaviour.

EAG assays in our three species reveals that the peak amplitudes that we observed are similar to those found for *C. pomonella* at pheromone concentrations of 10 and 100 ng (Judd *et al.* 2005), and 100 ng in *G. molesta* (Stelinski *et al.* 2006), but no similar references were found for *L. botrana*.

Insecticides at sublethal doses can either stimulate or depress general locomotory behaviour (i.e., walking or flying), and often insects poisoned become uncoordinated or even convulsive, and thus sublethal doses of insecticides can affect their mating behaviour (Haynes 1988). In our wind tunnel results, male's flight behaviour was affected in the three species tested, but the effect was not the same in all of them. Insecticide effects on, at least, one part of male flight behaviour have been reported in *Choristoneura fumiferana* (Clemens) (Dallaire *et al.* 2004), *Helicoverpa armigera* (Hübner) (Shen *et al.* 2013), *Ostrinia furnacalis* (Guenée) (Wei and Du 2004), *Spodoptera litura* (Fabricious) (Wei *et al.* 2004), when all the species were treated in larvae stages; and in *C. pomonella* (Hoelscher and Barret 2003), *G. molesta* (Linn and Roelofs 1984), *O. furnacalis* (Zhou *et al.* 2005) and *Pectinophora gossypiella* (Saunders) (Haynes and Baker 1985, Haynes *et al.* 1986), when insects were treated in the adult stage. The study of Linn and Roelofs (1984) is particularly germane to our study because they tested several insecticides on *G. molesta* and found, like we did, significant negative effects on male responses (percentage of males responding and time to contact the source) at low insecticide doses, although they did not study many flight track-related parameters.

Most flight track analysis of moths in the wind tunnel use a single camera that films the flight track from above and so a two-dimensional (2D) track is produced. Some studies, with moths and other species, have used two cameras to render a three-dimensional (3D) track, and although this is technically challenging and not often used (Cardé 2016), it has been employed under field conditions even before digital technology was widely available the for relatively high-speed video needed for this kind of analysis (Baker and Haynes 1996). When the moth flies it not only displaces or zigzags in the horizontal plane, it also moves in the vertical plane, and therefore 2D

tracks could be missing important information regarding the moth´s flight maneuvers. Luckily, 3D studies have been performed with two of our three species, and they have shown that most of the displacement of *G. molesta* was in the horizontal plane (Baker and Haynes 1996), and the same when stimulus is from a female in *L. botrana* (Witzgall and Arn 1990), therefore 2D track analysis were representative of flight tracks. In addition, the study of Baker and Haynes (1996) shows that the flight track of *G. molesta* is very similar under wind tunnel and field conditions.

The reduction in velocity caused by insecticide implies negative orthokinetic response. During flight, the goal of an insect is to make a net displacement from one point to another, but air causes drift. In order to correct this net-path displacement over the ground, a flying insect could alter its course angle (CA) and AS (Marsh *et al.* 1978). Reduced AS during flights enhances the effect of wind-induced drift (Witzgall 1997), a fact that we observe in *C. pomonella* and *G. molesta*. Reducing AS was reported to pheromone concentration increases in *G. molesta* (Kuenen and Baker 1982) and *Lymantria dispar* (L.) (Charlton *et al.* 1993, Kuenen and Cardé 1994), which suggest that treated males that shown reduced airspeed may integrate stimulus as if it were in a higher than normal pheromone concentration. Alternatively, males maybe more reliant on visual cues, which are known to influence AS and GS in *G. molesta* (Kuenen *et al.* 2014).

Witzgall (1997) suggest that better stimulus generates straighter upwind flights in *G. molesta* and *L. botrana*, in our case LC_{20} generated narrower CA (i.e., more straight upwind), in *L. botrana* males which in this context could indicate that the quality of the stimulus integration in intoxicated males was better than in males treated with acetone. In *L. botrana* DA remained invariable, perhaps because insects can counteract drift by modifying their CA and AS, as in *G. molesta* (Willis and Baker 1988), *L. dispar* (Willis *et al.* 1991, Kuenen and Cardé 1994), *Spodoptera littoralis* (Boisduval) (Bau *et al.* 1999), *Sesamia nonagrioides* (Lefèbvre) (Bau *et al.* 1999, Riba *et al.* 2001). and *Manduca sexta* (Linnaeus) (Rutkowski *et al.* 2009). However, thiacloprid widened DA in *G. molesta* and *C. pomonella*, which could denote lower capability to oppose resistance to the wind force. Willis and Baker (1988) showed that males increase drift in order to be more sensitive to wind velocity and direction and control their zigzag angle to the wind, and their flight via optomotor feedback. In addition, increased DA may provide more lateral displacement and therefore more visual information (Charlton *et al.* 1993). Finally, changes in turn rates of *G. molesta* and *C. pomonella* can be indicative of changes in their flight speed, similar to what has been reported for *G. molesta* (Kuenen and Baker 1982).

Unlike flight behaviour, thiacloprid did not affect EAG responses in any of the three species (Figure 3, Table S5). Normally, EAG responses to sex pheromone stimuli are not affected by sublethal doses of insecticide (Lucas and Renou 1992, Wang *et al.* 2011, Barret *et al.* 2013). Lower EAG responses were found in malathion-teated *O. furnacalis* males, independently of pheromone ratio and concentration (Zhou *et al.* 2005). Changes in the response of individual olfactory receptor neuron activity were found by DDT or blockage by deltamethrin in males of *Mamestra brassicae* (Linnaeus) and *Mamestra suasa* (Schiffermüler) (Lucas and Renou 1992). A reduction in EAG sensitivity to 12:OH in *C. pomonella* males poisoned with methoxyfenozide has been reported (Barret *et al.* 2013).

Thiacloprid sublethal effects observed could be owing to harmful process on both peripheral (PNS) or central nervous system (CNS), and on muscular system (MS). Nevertheless, insects after take flight were capable of maintain the flight, even so insects that did not show flight behaviour were tested to flight out of the wind tunnel. In addition, once oriented flight was initiated there was no critical decrease in contact with pheromone source; the highest decrease was experimented in *G. molesta* males (Figure 1). For that reasons, probably, sublethal effects of thiacloprid were not acting at the MS. Similarly that no significant differences between insecticide doses on EAG reveals no sublethal effects on PNS stimulus perception, thus the ORNs are still able to detect semiochemical signals even males were poisoned with sublethal doses of thiacloprid. Neonicotinoid insecticides, like thiacloprid, act as agonists at the postsynaptic nAChR by conformational changes of these receptors of acetylcholine (Ach), which is the endogenous agonist and excitatory neurotransmitter of the cholinergic nervous system. In insects, the nAChR is widely and predominantly distributed in the neuropil regions of the CNS (Matsuda *et al.* 2001, Tomizawa and Casida 2003), thus, thiacloprid must be active at the CNS. Since we have shown that thiacloprid did not affect pheromone sensing (EAGs) but it did affect flight, it is possible that it affected the centres involved in sex pheromone stimulus integration and the guidance mechanism. Similar conclusions were assumed in Rabhi *et al.* (2016), whom in addition, hypothesized that neonicotinoid clothianidin effects at CNS to a pheromone responses might be interpreted by the involvement of differences in nAChR subtypes and their ligand insecticide-affinities. Thiacloprid could also affect the specific motor pathways involved in flight.

Sublethal doses of thiacloprid negatively affects male's flight behaviour (this study) and female's calling behaviour in the three species (Navarro-Roldán and Gemeno 2017). The negative effect of thiacloprid on these elements of mating behaviour might interfere on mating success, as has been shown with other insecticides in other pest species (Nansen and Phillips

2004, Wei *et al.* 2004, Knight and Flexner 2007, Reinke and Barret 2007, Quan *et al.* 2016). The effects of sublethal doses could be temporary, as in male *P. gossypiella*, which recovered from sublethal pyrethroid (permethrin) infoxication after 4 days (Haynes and Baker 1985), or males of *C. rosaceana* which recovered from azinphosmethyl after 6 or 24 h (Trimble *et al.* 2004). However, thiacloprid has a relative good residual activity on fruit and leaves (Wise *et al.* 2006), and therefore, there is a significant probability of toxicant re-contact. Basic knowledge in sublethal effects of insecticide on insect behaviour, physiology, and reproductive success is important for proper development of IPM control strategies.

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Supplementary material.

Table S1. Selected parameters of triangle of velocities.

Parameter	**Abrev.**	**(Units)**	**Interpretation**
Track angle	TA	(degrees)	Angle formed between two vectors: wind direction and track length (TL) (observed insect track between two frames)
Course angle	CA	(degrees)	Controlled by the insect is the angle between wind direction and the course length (CL), which is the length that separate the position of the insect and the goal position per vector (between two frames)
Drift angle	DA	(degrees)	Angle formed by the shift of the displacement intention and the real displacement of the insect. In other words, angle formed between CL and TL.
Air speed	AS	(mm/s)	Speed with the insect cover the CL
Ground speed	GS	(mm/s)	Speed with the insect cover the TL
Turns	T	(N)	When the insect change the track direction
Intern-turn reversal	IT	(mm)	Average of the displacement that the insect made in Y-axis. Is a measurement of the amplitude of the insect displacement from the centre of the wind tunnel.
Total flight length	FL	(mm)	Complete path made by the insect
Total flight duration	FD	(s)	Time needed by the insect to complete the FL
Total flight velocity	FV	(mm/s)	Velocity during FL

Table S2. Mortality caused by sublethal doses of thiacloprid on males used in wind tunnel and EAG experiments. Different letters in same column indicate significant differences among treatments in each species (P<0.05, *Fisher Exact test* after GLM).

Species	Treatment	Thiacloprid (ng)	Wind tunnel			EAG		
			N	% dead 24 h		N	% dead 24 h	
Cydia pomonella	Acetone	0	109	0.00	b	54	0.00	b
	$LC_{0.001}$	0.61	110	0.00	b	54	0.00	b
	LC_{1}	4.24	112	1.79	ab	54	0.00	b
	LC_{10}	12.04	117	5.13	ab	56	0.00	b
	LC_{20}	18.70	125	10.40	a	59	23.73	a
Grapholita molesta	Acetone	0	73	0.00	b	61	0.00	c
	$LC_{0.001}$	4.88	73	1.37	b	61	0.00	c
	LC_{1}	15.05	73	0.00	b	61	6.55	bc
	LC_{10}	27.63	75	9.33	ab	61	18.03	ab
	LC_{20}	35.68	79	17.72	a	63	30.16	a
Lobesia botrana	Acetone	0	75	0.00	b	62	0.00	
	$LC_{0.001}$	32.29	74	0.00	b	62	0.00	
	LC_{1}	164.66	75	2.67	ab	63	0.00	
	LC_{10}	396.19	80	6.25	ab	63	4.76	
	LC_{20}	573.40	81	9.87	a	63	6.35	

Table S3. Model selection for predicted means and pairwise comparisons in parameters of 2-D triangle of velocities (Marsh *et al.* 1978), and other track parameters.

Species	Parameter[a]	Selected model	GLM formula[b]	Model analysis of deviance[c]						
				Variable	Df	Deviance Resid.	Df Resid.	Dev	F	Pr(>F)
C. pomonella	TA	Simplest	(ta ~ ttm)	ttm	4	730.55	280	56,099	0.9116	0.4576
		Simplest	(ta ~ sec)	sec	2	16,564	282	40,266	58.002	< 0.001
	CA	Simplest	(ca ~ ttm)	ttm	4	417.34	280	19149	1.5256	0.1948
		Simplest	(ca ~ sec)	sec	2	2,227.1	282	17,340	18.11	< 0.001
	DA	*No interaction*	(da ~ ttm + sec)	ttm	4	1,002.3	278	14,342	4.857	< 0.001
				sec	2	7,547.1	282	15,344	73.144	< 0.001
	GS	*No interaction*	(gs ~ ttm + sec)	ttm	4	340,392	278	2,891,234	8.1824	< 0.001
				sec	2	518528	282	3,231,627	24.9289	< 0.001
	AS	*No interaction*	(as ~ ttm + sec)	ttm	4	327,565	278	2,730,868	8.3365	< 0.001
				sec	2	983,504	282	3,058,432	50.0599	< 0.001
	T[d]	**Interaction**	(t ~ ttm * sec)	ttm	4	113.10	278	1157.5	-	< 0.001
				sec	2	1,062.27	282	1,270.6	-	< 0.001
				ttm:sec	8	48.27	270	1,109.3	-	< 0.001
	IT	*No interaction*	(it ~ ttm + sec)	ttm	4	9,282.3	278	255,658	2.5234	0.0413
				sec	2	9,938.8	282	264,940	5.4037	0.0050
	FL	*No interaction*	(fl ~ ttm + sec)	ttm	4	15,665,904	278	342,312,930	3.1807	0.0141
				sec	2	138,203,683	282	357,978,834	56.1192	< 0.001
	FD	**Interaction**	(fd ~ ttm * sec)	ttm	4	151.92	278	1,845.4	5.8888	< 0.001
				sec	2	897.18	282	1,997.3	69.5520	< 0.001
				ttm:sec	8	103.93	270	1,741.4	2.0143	0.0450
	FV	*No interaction*	(fv ~ ttm + sec)	ttm	4	321,397	278	2,736,070	8.1639	< 0.001
				sec	2	706,952	282	3,057,467	35.9151	< 0.001
G. molesta	TA	Simplest	(ta ~ ttm)	ttm	2	28.089	177	43,137	0.0576	0.944
		Simplest	(ta ~ sec)	sec	2	12,203	177	30,962	34.88	< 0.001
	CA	Simplest	(ca ~ ttm)	ttm	2	239.94	177	16,523	1.2851	0.2792
		Simplest	(ca ~ sec)	sec	2	2,064.7	177	14,698	12.432	< 0.001
	DA	*No interaction*	(da ~ ttm + sec)	ttm	2	354.9	175	9,631.6	3.2239	0.0422
				sec	2	7,772.4	177	9,986.4	70.6096	< 0.001
	GS	**Interaction**	(gs ~ ttm * sec)	ttm	2	573,137	175	3,175,349	16.4518	< 0.001
				sec	2	1,565,252	177	3,748,486	44.9303	< 0.001
				ttm:sec	4	196,757	171	2,978,592	2.8239	0.0265
	AS	*No interaction*	(as ~ ttm + sec)	ttm	2	513,625	175	3,216,932	13.97	< 0.001
				sec	2	1,877,949	177	3,730,557	51.08	< 0.001
	T[d]	*No interaction*	(t ~ ttm + sec)	ttm	2	14.45	175	468.94	-	< 0.001
				sec	2	501.86	177	483.39	-	< 0.001
	IT	Simplest	(it ~ ttm)	ttm	2	3,827	177	263,665	1.2845	0.2793

		Simplest	(it ~ sec)	sec	2	77,980	177	189,512	36.416	< 0.001
	FL	Simplest	(fl ~ ttm)	ttm	2	180,003	177	139,627,375	0.1141	0.8922
		Simplest	(fl ~ sec)	sec	2	71,474,880	177	68,332,498	92.57	< 0.001
	FD	Simplest	(fd ~ ttm)	ttm	2	8.0433	177	593.57	1.1992	0.3039
		Simplest	(fd ~ sec)	sec	2	250.12	177	351.49	62.976	< 0.001
	FV	**Interaction**	(fv ~ ttm * sec)	ttm	2	443,791	175	2,789,887	14.5994	< 0.001
				sec	2	1,934,822	177	3,233,679	63.6497	< 0.001
				ttm:sec	4	190,860	171	2,599,027	3.1394	0.0160
	TA	Simplest	(ta ~ ttm)	ttm	4	1,089.3	295	54,215	1.4818	0.2077
		Simplest	(ta ~ sec)	sec	2	9,368.9	297	45,935	30.288	< 0.001
	CA	*No interaction*	(ca ~ ttm + sec)	ttm	4	667.69	293	15,860	3.0838	0.0165
				sec	2	1,061.66	297	16,527	9.8069	< 0.001
	DA	Simplest	(da ~ ttm)	ttm	4	346.77	295	33,798	0.7567	0.5542
		Simplest	(da ~ sec)	sec	2	10,448	297	23,696	65.476	< 0.001
	GS	*No interaction*	(ga ~ ttm + sec)	ttm	4	267,147	293	4,453,030	4.3944	0.0018
				sec	2	1,612,506	297	4,720,177	53.0498	< 0.001
L. botrana	AS	*No interaction*	(as ~ ttm + sec)	ttm	4	190,398	293	4,648,975	2.9999	0.0189
				sec	2	1,830,601	297	4,839,373	57.6865	< 0.001
	T[d]	**Interaction**	(t ~ ttm * sec)	ttm	4	37.98	293	1,646.5	-	< 0.001
				sec	2	1,106.17	297	1,684.5	-	< 0.001
				ttm:sec	8	37.82	285	1,608.7	-	< 0.001
	IT	Simplest	(it ~ ttm)	ttm	4	1,153	295	234,360	0.3628	0.8350
		Simplest	(it ~ sec)	sec	2	41,641	297	193,872	31.895	< 0.001
	FL	Simplest	(fl ~ ttm)	ttm	4	1,093,000	295	304,295,696	0.2649	0.9004
		Simplest	(fl ~ sec)	sec	2	129,702,158	297	175,686,539	109.63	< 0.001
	FD	Simplest	(fd ~ ttm)	ttm	4	31.793	295	3,219.9	0.7282	0.5733
		Simplest	(fd ~ sec)	sec	2	969.83	297	2,281.9	63.114	< 0.001
	FV	*No interaction*	(fv ~ ttm + sec)	ttm	4	224,366	293	3,886,967	4.2282	< 0.001
				sec	2	2,123,126	297	4,111,333	80.0208	< 0.001

[a] TA = Track angle (degrees); CA = Course angle (degrees); DA = Drift angle (degrees); GS = Ground speed (mm/s); AS = Air speed (mm/s); T = Turns (N); IT = Intern-turns (mm); FL = Flight length (mm); FD = Flight duration (s); FV = Flight velocity (mm/s)

[b] GLM formula: 1st term = parameter; ~ = function to; 2nd term: a) simplest model = only the variable [ttm = insecticide dose, or sec = track section]; b) No interaction model = both variables (ttm and sec) without interaction; and, c) Interaction model = both variables and their interaction

[c] Df = degrees of freedom; Deviance Resid. = deviance of residuals; Df Resid. = degrees of freedom of residuals; Dev = deviance; F = F coefficient; Pr(>F) = probability value of F coefficient

[d] In parameter T the p-value was made it using the Chi squared test, because this parameter use a Poisson distribution

Table S4. Observed means of different parameters of the triangle of velocities.

Species	Section	Treatment	TA	CA	DA	GS	AS	T	IT	FL	FD	FV
		Acetone	58.40 ± 4.17	30.62 ± 2.21	27.78 ± 2.31	426.76 ± 28.34	636.39 ± 33.27	4.95 ± 0.85	68.72 ± 6.97	613.05 ± 100.48	1.654 ± 0.27	396.75 ± 24.53
		$LC_{0.01}$	51.33 ± 3.63	28.76 ± 2.13	22.57 ± 2.01	468.05 ± 34.23	697.10 ± 36.25	2.80 ± 0.52	76.15 ± 8.20	383.61 ± 40.65	0.968 ± 0.11	430.05 ± 28.56
	1	LC_1	58.34 ± 3.80	32.21 ± 2.16	26.13 ± 2.23	428.69 ± 23.64	637.69 ± 24.83	5.60 ± 1.30	78.36 ± 7.41	718.92 ± 164.51	1.88 ± 0.41	401.65 ± 20.56
		LC_{10}	53.77 ± 3.50	25.60 ± 1.83	28.17 ± 1.91	343.23 ± 14.01	576.97 ± 14.59	4.00 ± 0.85	52.47 ± 4.94	456.93 ± 104.21	1.41 ± 0.28	323.25 ± 13.03
		LC_{20}	58.46 ± 3.64	32.57 ± 2.09	25.89 ± 2.07	431.44 ± 20.88	642.73 ± 21.13	4.80 ± 1.47	79.31 ± 5.43	611.17 ± 134.32	1.57 ± 0.39	407.15 ± 20.04
		Acetone	51.07 ± 2.60	30.77 ± 1.77	20.30 ± 1.29	502.12 ± 24.64	726.10 ± 23.14	12.50 ± 1.39	86.06 ± 6.70	1697.46 ± 135.55	3.57 ± 0.37	501.41 ± 24.85
		$LC_{0.01}$	53.88 ± 1.93	32.94 ± 1.47	20.94 ± 1.07	519.46 ± 24.23	735.54 ± 22.74	12.90 ± 1.03	89.86 ± 6.76	1781.12 ± 136.34	3.58 ± 0.30	516.93 ± 24.56
C. pomonella	2	LC_1	58.72 ± 2.34	33.95 ± 1.43	24.76 ± 1.81	466.56 ± 25.20	671.76 ± 24.37	19.45 ± 3.10	87.12 ± 5.47	2329.41 ± 295.95	5.65 ± 0.99	464.23 ± 25.54
		LC_{10}	57.63 ± 2.72	31.23 ± 1.68	26.41 ± 1.57	403.64 ± 18.47	616.83 ± 19.64	21.10 ± 2.54	76.19 ± 5.15	2407.59 ± 307.39	6.242 ± 0.77	399.67 ± 18.78
		LC_{20}	53.91 ± 3.13	31.57 ± 2.15	22.34 ± 1.50	464.78 ± 25.60	682.02 ± 23.99	16.40 ± 2.89	84.77 ± 7.61	2214.08 ± 448.09	4.85 ± 0.86	463.08 ± 25.72
		Acetone	70.54 ± 1.05	37.02 ± 1.28	33.52 ± 1.19	387.21 ± 20.31	565.89 ± 16.69	15.35 ± 1.59	78.90 ± 7.83	1582.89 ± 192.92	4.338 ± 0.45	374.08 ± 21.51
		$LC_{0.01}$	69.79 ± 1.61	37.07 ± 1.60	32.72 ± 1.08	391.38 ± 20.91	569.74 ± 16.04	16.20 ± 1.95	77.45 ± 7.05	1740.36 ± 243.86	4.57 ± 0.54	375.25 ± 21.49
	3	LC_1	70.42 ± 1.44	34.63 ± 1.31	35.79 ± 1.34	344.41 ± 16.65	529.81 ± 13.81	19.05 ± 2.62	65.95 ± 5.04	1756.19 ± 266.92	5.52 ± 0.77	322.57 ± 18.28
		LC_{10}	73.63 ± 0.83	35.43 ± 1.31	38.20 ± 1.15	331.44 ± 18.02	511.15 ± 13.99	21.85 ± 1.66	71.50 ± 6.78	2016.64 ± 227.86	6.42 ± 0.54	311.80 ± 19.15
		LC_{20}	74.75 ± 1.31	39.29 ± 2.25	35.46 ± 1.46	386.19 ± 30.95	549.96 ± 22.99	26.20 ± 3.22	86.20 ± 12.22	3064.38 ± 667.62	7.67 ± 1.01	366.89 ± 33.89
		Acetone	46.95 ± 4.77	29.13 ± 3.05	17.83 ± 2.19	605.59 ± 44.05	834.97 ± 48.37	2.00 ± 0.57	69.25 ± 10.06	401.84 ± 83.00	0.80 ± 0.15	536.40 ± 36.01
	1	$LC_{0.01}$	48.69 ± 3.11	28.43 ± 2.08	20.27 ± 1.99	503.42 ± 39.48	738.38 ± 36.88	1.9 ± 0.55	69.76 ± 7.03	336.28 ± 39.38	0.83 ± 0.14	462.89 ± 35.62
		LC_1	48.36 ± 3.35	25.61 ± 1.89	22.75 ± 1.77	411.17 ± 24.19	649.21 ± 26.41	2.80 ± 0.56	55.44 ± 6.24	381.63 ± 65.09	1.04 ± 0.17	377.95 ± 18.40
G. molesta		Acetone	56.03 ± 3.26	38.34 ± 2.23	17.69 ± 1.15	706.25 ± 23.46	902.08 ± 28.35	9.95 ± 1.26	119.05 ± 7.43	2093.40 ± 234.46	3.08 ± 0.37	702.38 ± 23.78
	2	$LC_{0.01}$	53.45 ± 2.98	34.71 ± 2.02	18.74 ± 1.34	628.14 ± 31.26	837.10 ± 31.99	9.55 ± 1.39	112.35 ± 8.36	1845.36 ± 194.90	3.202 ± 0.45	624.32 ± 31.89
		LC_1	54.86 ± 2.04	33.40 ± 1.75	21.46 ± 0.85	516.11 ± 24.19	727.40 ± 19.91	11.90 ± 0.76	91.93 ± 6.38	1808.53 ± 129.81	3.55 ± 0.20	513.13 ± 23.92
		Acetone	66.92 ± 2.59	34.74 ± 1.71	32.18 ± 1.90	415.19 ± 22.78	601.20 ± 22.89	10.65 ± 1.10	57.91 ± 5.68	1082.23 ± 123.50	2.97 ± 0.31	382.23 ± 24.13
	3	$LC_{0.01}$	67.54 ± 2.04	32.26 ± 1.56	35.28 ± 1.86	364.15 ± 23.56	559.46 ± 22.24	12.25 ± 1.25	61.41 ± 5.99	1171.07 ± 128.02	3.72 ± 0.38	336.58 ± 24.36
		LC_1	69.08 ± 1.72	35.42 ± 1.61	33.66 ± 1.48	386.05 ± 23.83	569.18 ± 19.97	13.60 ± 1.72	68.25 ± 6.67	1326.02 ± 146.45	3.80 ± 0.45	365.46 ± 24.61
		Acetone	57.34 ± 3.23	29.11 ± 1.60	28.23 ± 2.70	419.24 ± 30.72	636.89 ± 33.52	5.10 ± 1.68	50.03 ± 4.64	488.72 ± 75.62	1.7 ± 0.55	391.86 ± 28.14
		$LC_{0.01}$	50.72 ± 5.30	27.40 ± 2.86	23.32 ± 2.82	507.41 ± 48.65	734.03 ± 55.87	3.50 ± 1.30	57.29 ± 10.06	493.74 ± 83.22	1.29 ± 0.31	458.28 ± 34.26
	1	LC_1	55.47 ± 3.62	26.86 ± 1.44	28.61 ± 2.65	384.63 ± 22.32	614.05 ± 26.83	3.60 ± 0.70	49.78 ± 3.84	416.77 ± 44.66	1.35 ± 0.22	360.75 ± 20.04

		LC_{10}	54.16 ± 3.29	26.93 ± 1.75	27.23 ± 2.41	376.65 ± 24.52	607.76 ± 25.09	4.80 ± 1.40	51.73 ± 5.30	447.61 ± 74.11	1.51 ± 0.35	353.18 ± 23.85
		LC_{20}	50.99 ± 3.77	24.89 ± 1.96	26.10 ± 2.48	379.22 ± 32.39	617.96 ± 34.80	4.05 ± 0.75	56.03 ± 6.20	414.39 ± 48.84	1.37 ± 0.21	350.80 ± 28.21
		Acetone	60.05 ± 2.38	33.93 ± 1.49	26.12 ± 1.83	490.26 ± 33.46	693.71 ± 33.03	19.90 ± 3.14	75.65 ± 6.85	2263.66 ± 218.93	5.43 ± 0.78	485.61 ± 33.97
		$LC_{0.01}$	61.07 ± 2.32	34.23 ± 1.64	26.84 ± 1.82	480.76 ± 31.40	680.79 ± 30.21	18.15 ± 2.22	74.23 ± 6.12	2309.54 ± 182.97	5.36 ± 0.57	476.39 ± 32.25
L. botrana	2	LC_1	55.72 ± 2.22	30.94 ± 1.31	24.78 ± 1.67	449.36 ± 26.02	668.25 ± 26.73	16.50 ± 1.94	70.87 ± 5.29	1899.02± 148.50	4.65 ± 0.50	445.98 ± 26.40
		LC_{10}	57.15 ± 2.07	30.16 ± 1.36	26.99 ± 1.80	414.53 ± 30.63	632.47 ± 29.85	22.00 ± 3.96	67.80 ± 6.20	2157.36 ± 233.05	6.05 ± 0.90	409.66 ± 31.24
		LC_{20}	52.05 ± 1.92	28.20 ± 1.55	23.84 ± 1.31	415.11 ± 26.04	645.03 ± 24.28	15.90 ± 1.44	63.87 ± 5.02	1684.66 ± 112.18	4.43 ± 0.38	411.78 ± 26.74
		Acetone	67.09 ± 1.37	29.03 ± 1.22	38.06 ± 1.48	288.87 ± 16.98	496.60 ± 13.60	13.55 ± 1.61	42.98 ± 3.84	1089.08 ± 91.11	4.49 ± 0.49	260.64 ± 18.10
		$LC_{0.01}$	65.47 ± 2.00	28.41 ± 1.37	37.06 ± 1.81	296.89 ± 20.79	508.22 ± 17.26	13.95 ± 1.93	42.39 ± 5.21	1196.54 ± 287.34	4.58 ± 0.64	259.12 ± 21.51
	3	LC_1	68.21 ± 1.52	30.06 ± 1.44	38.15 ± 1.27	292.96 ± 15.78	493.39 ± 11.48	17.00 ± 2.18	44.76 ± 6.04	1285.55 ± 150.04	5.29 ± 0.78	253.23 ± 16.38
		LC_{10}	69.50 ± 1.26	27.49 ± 1.24	42.01 ± 1.57	258.09 ± 15.05	466.35 ± 12.55	20.10 ± 3.13	36.98 ± 3.38	1300.05 ± 169.30	6.31 ± 0.85	221.53 ± 15.84
		LC_{20}	64.43 ± 2.27	26.24 ± 1.89	38.19 ± 1.44	260.45 ± 19.59	477.82 ± 13.71	18.55 ± 3.33	42.20 ± 5.58	1427.09 ± 330.21	5.98 ± 1.01	227.94 ± 20.80

TA = Track angle (degrees); CA = Course angle (degrees); DA = Drift angle (degrees); GS = Ground speed (mm/s); AS = Air speed (mm/s); T = Turns (N); IT = Intern-turns (mm); FL = Flight length (mm); FD = Flight duration (s); FV = Flight velocity (mm/s)

Table S5. Observed means of different depolarization and hyperpolarization parameters of EAG analysis.

Species	Pheromone dose [a]	Insecticide dose	Rise time (ms) (mean ± SEM)	Depolarization velocity (mV/s) (mean ± SEM)	Recovering time (ms) (mean ± SEM)	Maximum hyperpolarization (mV) (mean ± SEM)	Hyperpolarization time (ms) (mean ± SEM)
C. pomonella	1	Acetone	613.085 ± 17.389	0.138 ± 0.056	726.175 ± 53.347	NA	NA
		LC0.001	624.225 ± 12.877	0.166 ± 0.043	677.205 ± 33.316	NA	NA
		LC1	614.255 ± 15.122	0.274 ± 0.076	651.285 ± 35.412	NA	NA
		LC10	599.835 ± 9.264	0.075 ± 0.036	696.17 ± 38.675	NA	NA
		LC20	624.21 ± 15.564	0.130 ± 0.057	644.63 ± 43.476	NA	NA
	10	Acetone	625.705 ± 11.943	0.571 ± 0.105	773.1 ± 44.796	NA	NA
		LC0.001	607.235 ± 8.31	0.457 ± 0.084	715.57 ± 37.083	NA	NA
		LC1	627.575 ± 13.68	0.495 ± 0.084	687.99 ± 31.124	NA	NA
		LC10	609.705 ± 11.558	0.545 ± 0.097	701.775 ± 35.297	NA	NA
		LC20	613.395 ± 10.458	0.530 ± 0.143	754.44 ± 51.767	NA	NA
	10^2	Acetone	572.53 ± 10.691	3.122 ± 0.373	995.7 ± 34.28	1,648.267 ± 67.474	0.532 ± 0.079
		LC0.001	581.26 ± 9.946	2.827 ± 0.321	971.435 ± 39.685	1,532.35 ± 63.186	0.563 ± 0.101
		LC1	560.915 ± 11.017	2.665 ± 0.255	968.255 ± 55.746	1,519.071 ± 88.751	0.628 ± 0.058
		LC10	575.73 ± 9.924	3.133 ± 0.295	992.63 ± 34.254	1,637.483 ± 130.319	0.571 ± 0.113
		LC20	586.695 ± 10.131	2.545 ± 0.324	960.2 ± 44.345	1,728.317 ± 100.277	0.702 ± 0.108
	10^3	Acetone	503.55 ± 15.297	6.940 ± 0.515	1,470.18 ± 44.566	2,322.333 ± 172.129	0.443 ± 0.095
		LC0.001	507.67 ± 12.207	6.626 ± 0.463	1,557.01 ± 60.372	2,362.267 ± 155.371	0.466 ± 0.048
		LC1	500.39 ± 14.873	6.667 ± 0.580	1,543.845 ± 80.997	2,079.9 ± 104.789	0.635 ± 0.149
		LC10	511.795 ± 15.45	6.725 ± 0.468	1,487.09 ± 66.009	2,145.083 ± 128.579	0.582 ± 0.186
		LC20	522.75 ± 11.36	6.227 ± 0.568	1,490.485 ± 64.661	2,179.233 ± 166.112	0.572 ± 0.080
G. molesta	1	Acetone	562.39 ± 11.531	0.256 ± 0.075	601.235 ± 38.783	NA	NA
		LC0.001	559.04 ± 5.888	0.262 ± 0.091	599.225 ± 33.025	NA	NA
		LC1	581.885 ± 7.098	0.195 ± 0.059	555.61 ± 26.988	NA	NA
		LC10	565.415 ± 6.764	0.181 ± 0.041	569.845 ± 31.041	NA	NA
		LC20	566.425 ± 6.641	0.275 ± 0.083	610.855 ± 33.802	NA	NA
	10	Acetone	561.95 ± 9.607	0.911 ± 0.119	736.885 ± 36.02	NA	NA
		LC0.001	563.165 ± 5.495	0.984 ± 0.172	698.495 ± 31.724	NA	NA
		LC1	568.03 ± 7.921	0.767 ± 0.102	628.15 ± 32.6	NA	NA
		LC10	552.38 ± 8.505	0.715 ± 0.076	633.97 ± 31.884	NA	NA
		LC20	559.945 ± 6.527	0.913 ± 0.163	702.4 ± 29.629	NA	NA
	10^2	Acetone	501.175 ± 17.742	3.113 ± 0.271	824.47 ± 32.669	1,297.3 ± 55.169	0.679 ± 0.101
		LC0.001	519.185 ± 12.226	2.957 ± 0.337	803.24 ± 24.833	1,211.914 ± 122.972	0.502 ± 0.103
		LC1	519.98 ± 13.688	2.705 ± 0.314	789.14 ± 14.139	1,160.283 ± 31.415	0.515 ± 0.052
		LC10	527.01 ± 14.23	2.556 ± 0.211	741. ± 34.109	1,092.067 ± 65.705	0.465 ± 0.073
		LC20	521.28 ± 12.539	2.767 ± 0.462	826.445 ± 31.781	1,152.443 ± 75.048	0.425 ± 0.095
	10^3	Acetone	441.405 ± 18.128	7.289 ± 0.684	1,029.985 ± 45.184	1,346.214 ± 30.357	1.043 ± 0.181
		LC0.001	454.235 ± 18.923	7.703 ± 0.885	970.51 ± 29.363	1,334. ± 56.726	0.800 ± 0.181
		LC1	435.405 ± 14.876	6.828 ± 0.530	952.33 ± 37.551	1,413.6 ± 31.112	0.803 ± 0.114
		LC10	445.32 ± 19.39	7.102 ± 0.746	972.51 ± 38.354	1,388.25 ± 34.526	0.652 ± 0.177
		LC20	478.41 ± 18.615	6.951 ± 0.759	973.88 ± 27.146	1,413.214 ± 23.965	0.795 ± 0.177
L. botrana	1	Acetone	591.795 ± 8.756	0.339 ± 0.126	551.015 ± 36.531	NA	NA
		LC0.001	613.075 ± 18.886	0.228 ± 0.081	565.715 ± 29.191	NA	NA
		LC1	599.825 ± 10.415	0.424 ± 0.143	569.28 ± 33.992	NA	NA
		LC10	597.585 ± 8.61	0.238 ± 0.086	613.705 ± 21.158	NA	NA
		LC20	599.065 ± 8.117	0.230 ± 0.061	577.605 ± 17.036	NA	NA
	10	Acetone	580.74 ± 15.026	1.200 ± 0.319	656.18 ± 27.312	NA	NA
		LC0.001	590.905 ± 8.615	1.293 ± 0.203	666.69 ± 23.788	NA	NA
		LC1	598.1 ± 9.814	1.231 ± 0.229	617.29 ± 34.155	NA	NA
		LC10	581.22 ± 8.28	0.911 ± 0.173	672.79 ± 29.184	NA	NA
		LC20	577.33 ± 6.951	0.984 ± 0.128	603.79 ± 29.069	NA	NA
	10^2	Acetone	578.44 ± 11.412	3.542 ± 0.358	773. ± 22.428	1,272.45 ± 34.946	1.177 ± 0.157
		LC0.001	559.76 ± 7.628	4.288 ± 0.429	809.555 ± 17.382	1,294.886 ± 49.521	1.225 ± 0.068
		LC1	566.055 ± 7.864	3.574 ± 0.405	787.515 ± 26.347	1,216.414 ± 38.369	0.898 ± 0.106
		LC10	551.3 ± 12.293	3.938 ± 0.317	827.05 ± 22.252	1,323.057 ± 54.876	1.175 ± 0.108
		LC20	557.19 ± 5.723	3.930 ± 0.211	782.84 ± 17.718	1,309.843 ± 38.713	1.392 ± 0.112
	10^3	Acetone	541. ± 6.84	6.735 ± 0.474	1,017.105 ± 22.651	1,635.683 ± 115.445	1.563 ± 0.157
		LC0.001	519.315 ± 12.941	8.046 ± 0.630	1,061.93 ± 20.274	1,729.086 ± 50.901	1.747 ± 0.070
		LC1	528.92 ± 11.341	6.366 ± 0.506	1,063.53 ± 28.552	1,671.257 ± 63.959	1.126 ± 0.183
		LC10	539.51 ± 13.489	7.052 ± 0.537	1,053.545 ± 32.665	1,679.971 ± 110.897	1.382 ± 0.202
		LC20	518.885 ± 11.488	7.563 ± 0.439	1,005.025 ± 22.279	1,727.557 ± 59.253	1.736 ± 0.150

[a] Pheromone loaded on filter paper (ng).

Peak amplitude parameter is represented in Figure 3.

Figure S1. Diagram of wind tunnel section with main dimensions, recording area and coordinates plan representation.

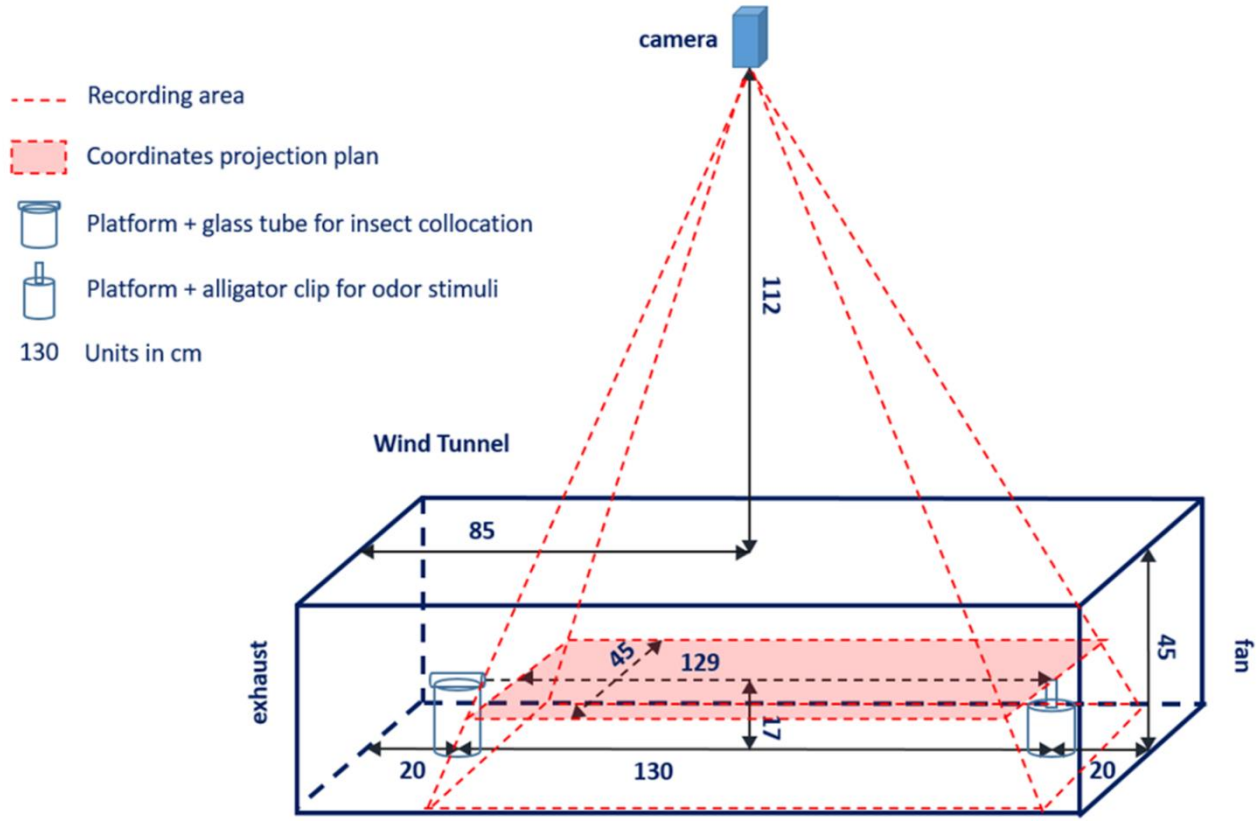


Figure S2. Representation of a flight track, including inter-turn reversals and parameters and terminology from the triangle of velocities (similar than shown in Kuenen 2013). Abbreviation meanings are shown in Table S1.

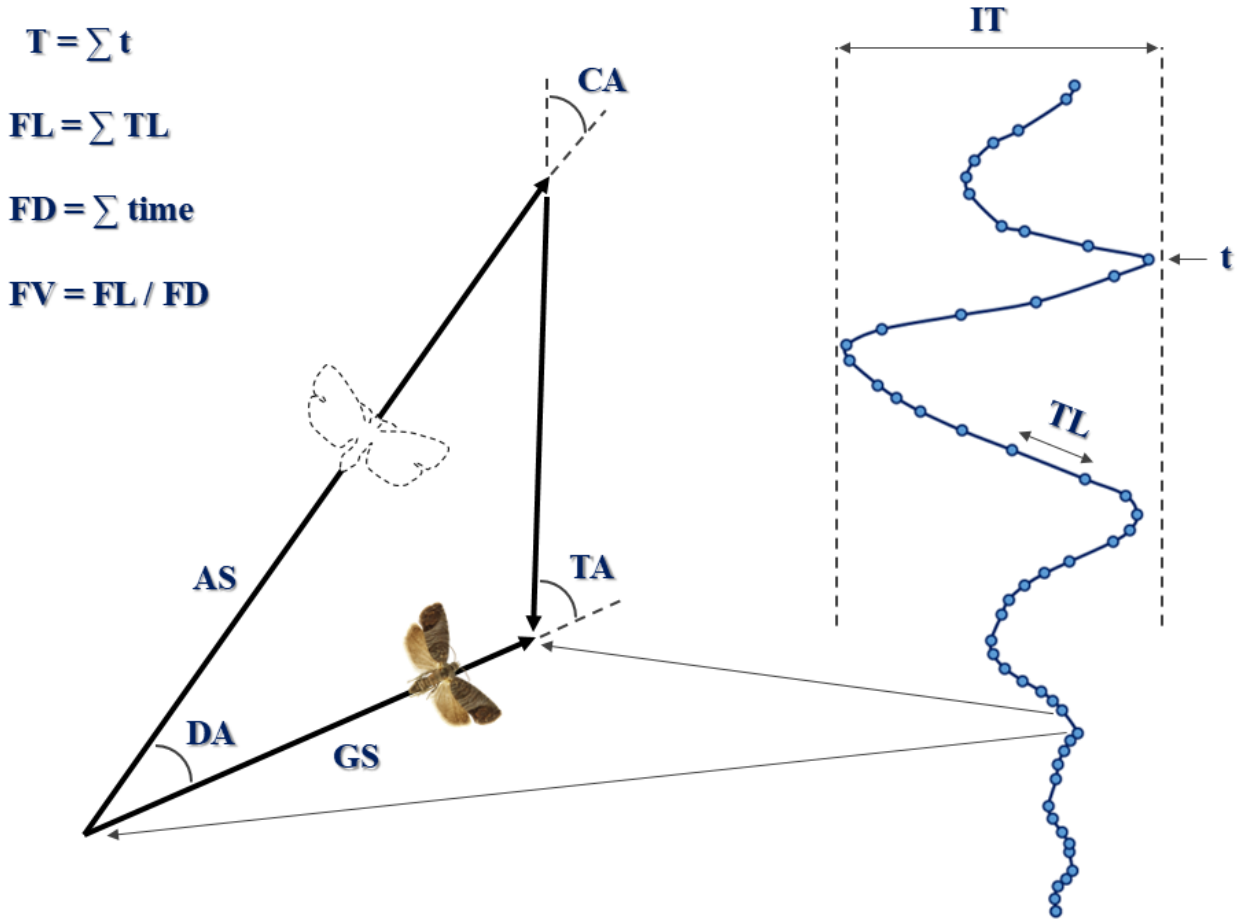
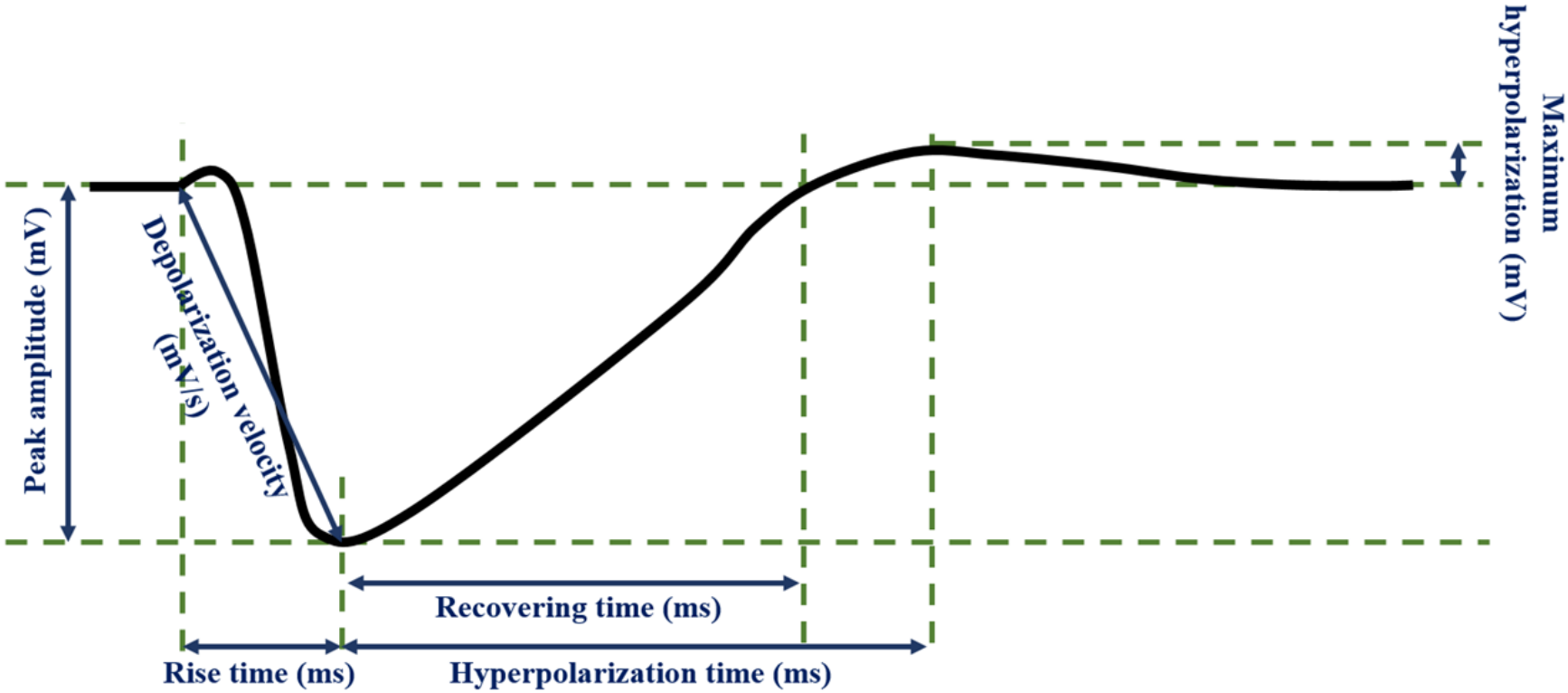


Figure S3. Diagram of EAG output an parameters analysed.



GENERAL DISCUSSION AND CONCLUSIONS

General discussion

Since the widespread use of insecticides, problems in pest control were becoming apparent, including target pest resurgence and secondary pest outbreaks, among others (Gill and Garg 2014). There can be many reasons for such failures and one is indubitably insecticide-resistance (Insecticide Resistance Action Committee [IRAC] 2016), but other problems associated with insecticide use are becoming relevant, like the effect of sublethal doses of insecticides, as much in pests (Haynes 1988, Tricoire-Leignel *et al.* 2012) as in beneficial arthropods (Desneux *et al.* 2007). Thus, basic knowledge on lethal and sublethal insecticide effects on pest insects is particularly important in the optimization and continuous improvement of IPM strategies, especially when insecticides are still used as the main crop protection strategy. The work presented in thesis reports differences in susceptibility and detoxification mechanisms against several neurotoxic insecticides in several pest species, and in both sexes. In addition, this thesis shows differences among species on sex-pheromone chemical communication of males and females treated with sublethal doses of the neonicotinoid-insecticide thiacloprid. All the work was done with adult individuals, which are poorly represented in the scientific toxicological literature of Lepidoptera, probably because most insecticides are mainly designed to kill egg or larval stages. However, neurotoxic insecticides that act by ingestion and contact could affect all life stages of the species tested (Reyes and Sauphanor 2008, Ioriatti *et al.* 2009, Magalhaes and Walgenbach 2011, Rodríguez *et al.* 2011). On other hand, sublethal effects of neurotoxic insecticides act on physiological and behavioral aspects that have their major expression on adult insects (Haynes 1988). To reach the objectives set out in this work, different methods and techniques were used like: topical mortality bioassays, biochemical analysis of enzymatic activities, calling behavior analysis under controlled conditions, quantification of female gland

contents by GC-FID, sex-pheromone stimuli perception by males using EAG and male response behavior and flight track analysis in response to a sex-pheromone stimuli in wind tunnel conditions.

Mortality bioassays on adults of both sexes belonging to several pest species, and using different neurotoxic insecticides with different mode of action (MoA) at different concentrations, allowed us to create dose-mortality curves. Mathematical modeling and comparisons among these curves revealed large mortality differences among insecticides (maximum 7,800-fold for LD_{50}), followed by much lower, yet important, differences among species (115-fold), and between sexes (41.5-fold), and showed that each of these three factors were not independent from each other, and all of them have a critical effect on adult mortality. Insecticide showed the stronger effect, where the pyrethroid was significantly more effective than the other two insecticide types. Previous studies have shown that pyrethroids are more toxic at low concentrations than other insecticides on *G. molesta* and *C. pomonella* (Linn and Roelofs 1984, Pasquier and Charmillot 2003, Mota-Sánchez *et al.* 2008, Rodríguez *et al.* 2012, Wu *et al.* 2015). In the case of our test species, there was not one species that was the most susceptible or tolerant to all tree insecticides; the susceptibility of each species depended on the insecticide. The three species are phytophagous insects which use different food sources containing secondary plant metabolites which, when acquired through ingestion, are environmental toxins that need to be metabolically detoxified (Terriere 1984). It is plausible, then, that species using different food sources may have different detoxifying-enzyme activity levels (Yu 1982), and this may explain why they show different tolerance to insecticides. However, one of the most striking findings of Chapter 1 is the relatively large difference in susceptibility between males and females, and the higher tolerance of males to chlorpyrifos, in all three species. Higher male tolerance to organophosphates in *G. molesta* was previously reported (Shearer and Usmani 2001). The expected trend of higher male susceptibility was observed with thiacloprid, and was similarly reported in *G. molesta* with carbamate insecticides (Kanga *et al.* 2001, Shearer and Usmani 2001), however it could not be explained by larger females body weight either, because after body mass correction, i.e., transformation LC into LD, females were still less susceptible than males. This suggests that additional factors, such as differences in enzymatic activity families involved in detoxification mechanisms and/or their quantities, might be playing a role in both cases.

Analysis of major metabolic detoxification mechanisms in Chapter 2, by determination of mortality of individuals simultaneously treated with enzyme-inhibitors and insecticides, indicated that phase I enzymatic activities (EST and MFO) were involved in detoxification in the

three species and in both sexes, whereas phase II enzymes (GST) were important only in *G. molesta*. EST plays a general role in detoxification, acting on the three insecticides and in both sexes of the three moth species. This non-specific action of the EST enzymatic family has been reported in other pest species (Devonshire *et al.* 1982, Hemingway 2000, Faucon *et al.* 2015). GST enzymatic family appears to be less relevant in insecticide detoxification than the other two enzyme families (Rane *et al.* 2016, Appendix 1). In my study, GST was only relevant for insecticide detoxification in *G. molesta*. MFO showed more diverse effects than the other two enzymatic families. It had a high impact in detoxification of thiacloprid and increased the activity of chlorpyrifos, a phenomenon known as bio-activation which has been observed in organophosphates (Feyereisen 1999). In addition, MFO showed non-specificity for sex or species but specificity for thiacloprid. Indeed, a positive correlation (R^2 = 0.90) between thiacloprid LD_{50} (for the combination of each species and sex group) and MFO activity revealed that MFO is the main mechanism in thiacloprid detoxification, so that MFO quantities could explain the species differences found in Chapter 1 for this insecticide. A significant role of MFO in detoxification of thiacloprid has also been reported in *C. pomonella* (Reyes *et al.* 2007, İşci and Ay 2017). Nevertheless, the lack of correlation between enzymatic activity and mortality for the other insecticides and enzyme families may be due to the non-specificity observed for EST among insecticides and species, and for GST among insecticides. The enzymatic activity results in Chapter 2 did not explain the species, sex and insecticide differences in susceptibility of Chapter 1, or they did it but only partially. Because no inhibition effect was observed in MFO activity after 1 h PBO-pretreatment, we explored if time after inhibitor application had an effect on the kinetic action of PBO on MFO, as has been shown in *H. armigera* (Young *et al*. 2005, 2006) and *B. tabaci* (Young *et al.* 2006). I found limited MFO inhibition 4 h post-exposure in *G. molesta*, but I observed induction after 12 h of PBO application, which was more significant on males than on females, thus further investigations on enzyme activity kinetics could help explain the differences in susceptibility among sexes found in Chapter 1. Additionally, these results could explain the lack of inhibitor effect in our first enzyme inhibition test at 1 h for PBO and 24 h for DEM, in which test I also found enhanced (i.e., induction) of GST activity for *G. molesta* males. Furthermore, the induction of detoxification enzymes could be involved in the 48-h intoxication recovery observed for thiacloprid, whereas the increased mortality of chlorpyrifos at 48h (Figure S3 - Chapter 1), may be related to bio-activation by MFO of P=S compounds into the AChE-inhibitory P=O analogs, which would increase the toxicity of this insecticide over time (Yu 2008).

Sublethal effects are described as effects on physiology and behavior of an individual that has been exposed to an insecticide without directly causing death (Haynes 1988, Desneux *et al.* 2007, Tricoire-Leignel *et al.* 2012). Under field conditions, adult moths could be exposed to sublethal doses of insecticide even though the application is not aimed at them but to other life stages, or even at other pest species, or from drift by blast sprayers in neighbor fields. Thiacloprid, for example, persists as surface residue on fruit and leaves (Wise *et al.* 2006), and has a half-life in the soil of 10 to16 days (Krohn 2001). Determination of sublethal doses of thiacloprid using dose-mortality curves of Chapter 1, allowed me to test the effect of these sublethal doses of insecticides on different physiological and behavioral aspects of sex-pheromone chemical communication in the test species. Thiacloprid-sublethal doses selected for all test were under 20 % of mortality: $LD_{0.001}$, LD_1, LD_{10} and LD_{20}. The expected levels of mortality in Chapters 3 and 4 were similar than the estimated in Chapter 1. Sublethal doses of thiacloprid producing as low as 0.001 % mortality ($LD_{0.001}$) significantly modified sex-pheromone communication, but the effect was not the same on the three tortricid species. The most dramatic phenotypic effect of sublethal thiacloprid doses in our test species was the significant reduction in the amount of calling in *C. pomonella* females treated with $LC_{0.001}$ and in the percentage of response to sex-pheromone stimuli in *G. molesta* males treated with LC_1, whereas no sublethal effects were found in pheromone gland content in *G. molesta* and *L. botrana* females and in male antennae perception of sex-pheromone to the major pheromone component in the three species.

A detrimental effect in calling behavior was observed in female of the three species under sublethal doses of thiacloprid, being less obvious in *L. botrana*. Sublethal insecticide effects on calling behavior has been also observed in other moth species with pyrethroid (Haynes and Baker 1985, Clark and Haynes 1992a, Yang and Du 2003, Shen *et al.* 2013, Quan *et al.* 2016) and organophosphate insecticides (Trimble *et al.* 2004). With regard to the time of calling behavior relative to the onset of the scotophase, sublethal effects were observed for LC_1 and higher lethal doses in *C. pomonella* and *G. molesta*. In these species, the calling curves were more compacted compared with control treatments, and no effect was found for *L. botrana* females. Shortened calling behaviors have been reported too in *P. gossypiella* (Haynes and Baker 1985), and in *O. furnacalis* (Wei and Du 2004) and *C. fumiferana* (Dallaire *et al.* 2004) larvae treated with deltamethrin and tebufenocide, respectively. Pheromone production, on the other hand, was only affected by thiacloprid in one of the three species, *C. pomonella*, and it required higher doses than what was needed to affect calling behavior. The quantity of the major compound, codlemone, and one of the three minor compounds, 12:OH, were approximately

halved compared to the acetone control at LC_{10} or LC_{20}, and the ratio with respect to codlemone of two minor compounds, E9-12:OH and 14:OH, increased 4 and 2.5-fold, respectively, at LC_{20}. Detrimental effects on pheromone production and changes in component ratios under sublethal doses of insecticide were also observed in other species (Delisle and Vincent 2002, Yang and Du 2003, Trimble *et al.* 2004, Wei *et al.* 2004, Shen *et al.* 2013). It is interesting that thiacloprid affected calling behavior and pheromone production in *C. pomonella* but only calling behavior in *G. molesta* and *L. botrana*. In other species there is also a differential effect of insecticide on calling behavior and pheromone production (Clark and Haynes 1992a,b, Yang and Du 2003, Trimble et *al.* 2004, Wei and Du 2004, Shen et *al.* 2013). It remains to be determined why similar sublethal doses of thiacloprid resulted in differential effects in pheromone production and calling behavior among the three moth species. It could be argued that the neurohormone PBAN, a brain-released neuropeptide that induces pheromone biosynthesis (Jurenka and Rafaeli 2011, Groot 2014), or juvenile hormone (JH), which is involved in the regulation of calling behavior (Rafaeli 2009), are implicated on these differences. However, both mechanisms are probably very similar in the three tortricid species (Roelofs and Rooney 2003, Jurenka and Rafaeli 2011).

In the wind tunnel tests, male's flight behaviour was affected in the three species tested, but the effect was not the same in all of them. The time to respond and the percentage of males responding were significantly affected in *G. molesta*, even with low concentrations ($LC_{0.001}$), whereas in *C. pomonella* and *L. botrana* the effects were only observed at the highest concentration (LC_{20}). Several studies have already shown insecticide effects on, at least, one part of males flight behaviour of pest insects when were exposed to insecticide as larva (Dallaire *et al.* 2004, Wei and Du 2004, Shen *et al.* 2013), or as adult (Linn and Roelofs 1984, Haynes and Baker 1985, Haynes *et al.* 1986, Hoelscher and Barret 2003, Zhou *et al.* 2005). Although 2D track analysis illustrates insect flight in fine detail (Marsh *et al.* 1978), there are no reported studies of insecticide sublethal effects on flight track parameters. In Chapter 4 I report the effect of sublethal doses of thiacloprid on flight track parameters. In general, velocities [air speed (AS) and ground speed (GS)] were reduced and drift angle (DA) increased with insecticide dose in *C. pomonella* and *G. molesta*, whereas in *L. botrana* the insecticide resulted in a narrowed course angle (CA). Reduced AS during flights enhances the effect of wind-induced drift (Witzgall 1997), which means wider DA, as we observed for two of the species. However, a reduced AS could also imply that insects perceive a higher than real pheromone concentration (Kuenen and Baker 1982, Charlton *et al.* 1993, Kuenen and Cardé 1994), or that the perception of visual cues is altered (Charlton *et al.* 1993, Kuenen 2013, Kuenen *et al.* 2014). A wider DA denotes a lower capability to oppose resistance to the wind, but males counteracted drift by modifying their CA

and AS and so maintained the same GS despite the insecticide application (Willis and Baker 1988, Willis *et al.* 1991, Kuenen and Cardé 1994, Bau *et al.* 1999, Riba *et al.* 2001, Rutkowski *et al.* 2009). Witzgall (1997) suggests that better stimulus generates straighter upwind flights in *G. molesta* and *L. botrana*. In my study LC_{20} generated straight CA (i.e., straight upwind) in *L. botrana* males, which suggests that males treated with sublethal doses perceived the stimulus as being of better quality than those treated with acetone. Unlike flight behaviour, thiacloprid did not affected EAG response in any of the three species. A similar result has been reported in other moth species (Lucas and Renou 1992, Wang *et al.* 2011, Barret *et al.* 2013). However, slight changes were reported in other species, like sensitivity reductions (Barret *et al.* 2013), spontaneous activity firing increasing (Lucas and Renou 1992), or lower EAG responses (Zhou *et al.* 2005).

Because sublethal doses of thiacloprid upset several elements of sex-pheromone communication, a decrease in reproductive success is possible, as it has been shown in other moth species (Nansen and Phillips 2004, Wei *et al.* 2004, Knight and Flexner 2007, Reinke and Barret 2007, Quan *et al.* 2016).

Conclusions

Activity of an insecticide against a pest includes both its direct toxicity to one or more life stages, as I assessed in Chapter 1 for adult stage, and the potential interaction with different parts of insect physiology and behavior, when the insecticide dose it is not enough to kill the insect, which I evaluated in Chapters 2-4. The main conclusions for those chapters are:

1) Insecticide, species and sex are not independent variables that influence results on adult mortality, and all of them must be considered in mortality bioassays.

2) Differential species and sex response to different insecticides showed that: (i) pyrethroids are more toxic at lower concentrations than other insecticides; (ii) comparisons among species vary for each insecticide, and no single species was most susceptible or tolerant to all insecticides; (iii) females of the three moth species were less susceptible to the neonicotinoid thiacloprid, whereas males of three species were less susceptible to the organophosphate chlorpyrifos.

3) Differential species and sex response to different insecticides should be considered in IPM programs, and bring up several issues: (i) the importance to alternate insecticide active ingredients; (ii) not all pest species are equally well controlled with the same insecticide

treatments; and (iii) an inadequate dose of insecticide treatment could select one sex over the other.

4) The dose-mortality curves on the susceptible strains used in this study provide a diagnostic baseline to test possible resistance cases in field populations of the tested species and insecticides, and possible cross-resistance with others insecticides.

5) Different levels of specificity of detoxification enzymes were found: GST was specific of *G. molesta*; MFO played a main role in the detoxification of thiacloprid and the bio-activation of chlorpyrifos; and EST did not have specificity for insecticide or species.

6) The positive correlation between MFO activity and LD_{50} explains species-specific differences in susceptibility to thiacloprid. However, sex differences in susceptibility cannot be explained with the enzymatic-activity results of the present study.

7) Sex differences in enzymatic inhibition and induction observed in the kinetic experiment could help explain sex differences of insecticide susceptibility, but further kinetic investigations are needed.

8) If enzymatic-inhibition wants to be considered as an insecticide synergist pre-treatment under field conditions, careful considerations must be given, because the presence of many "metabolic enzyme-inducers" could influence the insect's metabolic enzyme status in susceptible strains.

9) Observed calling behavior under laboratory conditions may be different under more-natural light conditions, because our laboratory photoregime did not provide the smooth light:dark transition that occurs at dawn and sunrise in the field.

10) Sublethal doses of thiacloprid producing as low as 0.001 % mortality significantly modified sex-pheromone chemical communication, but the effect was not the same on the three tortricid species. The main sublethal effects of thiacloprid on males and females were:

- Significantly reduced the amount of calling behavior in *C. pomonella* and *G. molesta* females, the effect was significant but relative small in *L. botrana*.

- Altered the female calling period of *C. pomonella* and *G. molesta*, but no of *L. botrana*.

- Modified sex pheromone production only in *C. pomonella*.

- Decreased the response of males to sex pheromone stimuli in the wind tunnel. The effects were stronger in *G. molesta* than in *C. pomonella*, and less important in *L. botrana*.

- Produced weaker and slower flights in *C. pomonella* and *G. molesta* males, and straighter upwind flights in *L. botrana*.

- Did not influence EAG responses to the major sex pheromone compound.

11) Our results on male's flight behaviour and perception suggest that thiacloprid affected centres involved in sex pheromone stimulus integration and the guidance mechanism rather than specific motor pathways of flight or the peripheral perception.

12) *L. botrana* had the highest tolerance to thiacloprid, and both males and females of this species were the least affected by sublethal doses of this insecticide.

13) If thiacloprid is detrimental to these elements of mating behavior (i.e., female calling behavior and male flight response), its effect on reproduction may be even larger than what our results suggest, with a possible enhancement of semiochemical IPM control. For this reason, basic knowledge of insecticide effects on insect behavior, physiology, and reproductive success could be a critical issue if we want to optimize IPM strategies.

14) In addition, further investigations are needed in order to determine the impact of our findings in IPM control. We need to know if thiacloprid: (i) alters the composition of the pheromone blend emitted by females because males respond not to the pheromone in the gland but to the volatiles released by calling females; (ii) treated females are as attractive to males as untreated ones, or less active at mating than untreated ones; (iii) affects contact chemical cues and short-range pheromones associated with male hair pencil displays, both used in the courtship that precedes mating in *G. molesta*.

15) Male pheromone trap catches may be unfit to monitor threshold population levels if: (i) males are less susceptible than females to insecticide, (ii) males are under effect of sublethal doses of neonicotinoids, at least at thiacloprid doses of 20 % mortality or lower.

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APPENDIX 1

Table 1. List of metabolic mechanisms involved in detoxification of insecticides for important worldwide Lepidopteran pest with described cases of metabolic resistance to insecticides, including our Chapter 2 results.

Species	Family [a]	Strain [b]	Stage [c]	Metabolic mechanism [d]	In detoxification of [e]	Reference [f]
Agrotis ipsilon (Hufnagle)	Noctuidae	Susceptible	Larva	MFO + EST	Cypermethrin (Py)	Usmani and Knowles 2001*
		Susceptible	Adult (♀)	EST	Cypermethrin (Py)	
		Susceptible	Adult (♂)	MFO + EST	Cypermethrin (Py)	
Choristoneura rosaceana (Harris)	Tortricidae	Susceptible	Larva	MFO + GST	Tebufenocide (IGR)	Waldstein and Reissing 2000
		Resistant	Larva	MFO + GST	Tebufenocide (IGR)	
		Resistant	Larva	EST	OP and Car	Pree *et al.* 2002
		Susceptible	Larva	MFO	Indoxacarb (Ox)	Ahmad and Hollingworth 2004
		Resistant	Larva	**MFO** + GST + EST	Indoxacarb (Ox)	
		Susceptible	Larva	MFO + GST + EST	Cypermethrin (Py)	
		Resistant	Larva	**MFO** + GST + **EST**	Cypermethrin (Py)	
		Susceptible	Larva	MFO*	Chlorpyrifos (OP)	
		Resistant	Larva	MFO + GST + EST	Chlorpyrifos (OP)	
		Susceptible	Larva	GST	Azinphos-methyl (OP)	
		Resistant	Larva	MFO + GST + EST	Azinphos-methyl (OP)	
		Susceptible	Larva	MFO + GST	Tebufenocide (IGR)	
		Resistant	Larva	MFO + GST + **EST**	Tebufenocide (IGR)	
		Susceptible	Larva	**MFO** + **GST** + EST	Chlorfenapyr (Ch)	
		Resistant	Larva	**MFO** + **GST** + EST	Chlorfenapyr (Ch)	
		Susceptible	Larva	MFO	Spinetoram (Sp)	Sial and Brunner 2011

Continue

		Resistant	Larva	MFO	Spinetoram (Sp)	
		Resistant	Larva	EST	Chlorantraniliprole (Di)	
Cydia pomonella (Linnaeus)	Tortricidae	Susceptible	Adult (♀+♂)	EST + MFO*	Chlorpyrifos (OP)	Chapter 2
		Susceptible	Adult (♀+♂)	EST	lambda-cyhalothrin (Py)	
		Susceptible	Adult (♀)	MFO + EST	Thiacloprid (Ne)	
		Susceptible	Adult (♂)	MFO	Thiacloprid (Ne)	
		Susceptible	Larva	EST	Deltamethrin (Py)	Sauphanor *et al.* 1997
		Resistant	Larva	MFO + EST	Deltamethrin (Py)	
		Susceptible	Adult (♀+♂)	EST	Azinphos-methyl (OP)	Reuveny and Cohen 2004
		Resistant	Adult (♀+♂)	EST + **AChE**	Azinphos-methyl (OP)	
		Resistant	Adult	I.AChE	Azinphos-methyl (OP)	Cassanelli et al. 2006
		Resistant	Adult	MFO	Tebufenocide (IGR)	Ioriatti *et al.* 2007
		Resistant	Adult	MFO + GST	Azinphos-methyl (OP)	Reyes *et al.* 2007
		Resistant	Adult	MFO	Diflubenzuron (IGR)	
		Resistant	Adult	MFO	Spinosad (Sp)	
		Resistant	Adult	MFO + ↓EST	Thiacloprid (Ne)	
		Resistant	Larva	EST	Azinphos-methyl (OP)	Soleño *et al.* 2008
		Resistant	Adult	MFO + GST	Azinphos-methyl (OP)	Reyes *et al.* 2009
		Resistant	Larva (pd)	MFO + GST + EST	OP	Rodriguez *et al.* 2010
		Resistant	Adult	MFO + GST	OP	
		Resistant	Larva	MFO + EST(m)	Azinphos-methyl (OP)	Reyes *et al.* 2011
		Resistant	Larva	MFO + GST + EST(m)	Diflubenzuron (IGR)	
		Resistant	Larva	**MFO** + GST + EST	-	Voudouris *et al.* 2011
		Susceptible	Adult	GST + ?	lambda-cyhalothrin (Py)	Liu *et al.* 2014
		Resistant	Adult	MFO + GST + ↓EST	OP	Reyes *et al.* 2015
		Resistant	Larva	MFO + EST	Thiacloprid (Ne)	İşci and Ay 2017
Epiphyas postvittana (Walker)	Tortricidae	Resistant	Adult	GST + EST	Azinphos-methyl (OP)	Armstrong and Suckling 1988
		Resistant	Adult	GST + EST	Azinphos-methyl (OP)	Armstrong and Suckling 1990
		Resistant	Larva	MFO	Azinphos-methyl (OP)	
Grapholita molesta (Busk)	Tortricidae	Susceptible	Adult (♀+♂)	GST + EST + MFO*	Chlorpyrifos (OP)	Chapter 2
		Susceptible	Adult (♀)	MFO+ EST	lambda-cyhalothrin (Py)	
		Susceptible	Adult (♂)	GST + EST	lambda-cyhalothrin (Py)	
		Susceptible	Adult (♀+♂)	MFO + GST + EST	Thiacloprid (Ne)	
		Resistant	Adult (♂)	EST	Azinphos-methyl (OP)	Usmani and Shearer 2001*
		Resistant	Adult	EST + I.AChE	Car	Kanga *et al.* 2001
		Resistant	Adult (♀)	I.AChE	OP	de Lame *et al.* 2001
		Resistant	Adult (♂)	EST	OP	
		Resistant	Adult	MFO + GST + EST	Chlorpyrifos (OP)	Siegwart *et al.* 2011
		Susceptible	Adult	MFO + GST + EST	-	Guo *et al.* 2017

Continue

Helicoverpa armigera (Hübner)	Noctuidae	Resistant	Larva	MFO	Py	Ahmad and McCaffery 1991*
		Resistant	Adult	MFO	Fenvalerate (Py)	Daly and Fisk 1993*
		Resistant	Larva	MFO + EST	Py	Kranthi *et al.* 1997
		Resistant	Larva	EST	Py	Gunning *et al.* 1999*
		Resistant	Larva	EST	Fenvalerate (Py)	Young *et al.* 2005*
		Resistant	Larva	EST	Cypermethrin (Py)	
		Susceptible	Larva	GST + EST	Indoxacarb (Ox)	Vojoudi *et al.* 2017
		Susceptible	Larva	GST + EST	Hexaflumuron (IGR)	
Helicoverpa zea (Boddie)	Noctuidae	Susceptible	Larva	MFO + EST	Cypermethrin (Py)	Usmani and Knowles 2001*
		Susceptible	Adult (♀)	EST	Cypermethrin (Py)	
		Susceptible	Adult (♂)	EST	Cypermethrin (Py)	
Heliothis virescens (F.)	Noctuidae	Resistant	Larva	MFO	Cypermethrin (Py)	McCaffery et *al.* 1991*
		Resistant	Larva	MFO	Cypermethrin (Py)	Martin *et al.* 1997
		Susceptible	Larva	EST + I.AChE	OP	Hamadain and Chambers 2001*
Lobesia botrana (Denis & Schiffermüller)	Tortricidae	Susceptible	Adult (♀+♂)	EST + MFO*	Chlorpyrifos (OP)	Chapter 2
		Susceptible	Adult (♀)	EST	lambda-cyhalothrin (Py)	
		Susceptible	Adult (♂)	MFO + EST	lambda-cyhalothrin (Py)	
		Susceptible	Adult (♀)	MFO	Thiacloprid (Ne)	
		Susceptible	Adult (♂)	MFO + EST	Thiacloprid (Ne)	
Ostrinia nubilalis (Hübner)	Crambidae	Resistant	Adult (♀)	GST	Py	*Siegwart et al. 2011*
		Resistant	Adult (♂)	MFO	Py	
Platynota idaeusalis (Walker)	Tortricidae	Resistant	Larva	EST	Azinphos-methyl (OP)	Biddinger *et al.* 1996
		Resistant	Larva	EST	Diflubenzuron (IGR)	
		Resistant	Larva	GST + EST	Azinphos-methyl (OP)	Karoly *et al.* 1996
		Resistant	Adult (♀+♂)	EST	Azinphos-methyl (OP)	
Plutella xylostella (L.)	Plutellidae	Susceptible	Larva	MFO + GST	Diazinon (OP)	Takeda *et al.* 2006*
		Susceptible	Larva	MFO + EST	Chlorantraniliprole (Di)	Wang *et al.* 2010
Spodoptera frugiperda (J. E. Smith)	Noctuidae	Susceptible	Larva	MFO	Cypermethrin (Py)	Usmani and Knowles 2001*
		Susceptible	Adult (♀)	MFO + EST	Cypermethrin (Py)	
		Susceptible	Adult (♂)	MFO	Cypermethrin (Py)	
		Resistant	Larva	MFO + GST + EST	Carbaryl (Car)	Yu *et al.* 2003
		Resistant	Adult	MFO + EST	Carbaryl (Car)	
Spodoptera littoralis (Boised)	Noctuidae	Resistant	Larva	EST	Py	Riskallah 1983*
		Resistant	Larva	MFO	Methoxyfenozide (IGR)	Mosallanejad and Smagghe 2009

[a] In Lepidoptera Order

[b] If no specification the resistance is to the same insecticide tested (Normally at LC_{50}).

Continue

[c] Noted as (♀+♂) when differences between sexes were tested in the study

[d] MFO = Multifunction oxidases; GST = Glutathione S-transferase; EST = Total esterases; I.AChE = Insensitive acetylcholinesterase. Normally, resistance cases were associated with an enhancement of the metabolic mechanism described. Bold letters indicate most important mechanism involved based on authors discussion. Mechanisms marked with (*) indicates activation of insecticide to the active compound, i.e., chlorpyrifos to chlorpyrifos oxon; (↓) = reduced; (m) = modified affinities to enzymatic substrates;

[e] (IGR) = Insect growth regulator; (OP) = Organophosphates; (Car) = Carbamates; (Ox) = Oxadiazines (voltage-dependent sodium channel blockers); (Py) = Pyrethroids; (Ch) = disruptors of the proton gradient; (Sp) = Spinosyns; (Di) = Diamides; (Ne) = Neonicotinoid.

[f] References marked with (*) indicates that the study didn't test all enzymatic mechanisms (MFO, GST and EST)

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LIST OF ABBREVIATIONS

7-HC	7-hydroxycoumarin
α-NA	α-naphthyl acetate
ACh	acetylcholine
AChE	acetylcholinesterase
AIC	akaike information criterion
AL	antennal lobe
ANOVA	analysis of variance
AS	air speed
CAP	common agricultural policy
CCTV	closed-circuit television
CDNB	4-dinitro-chlorobenzene
CA	course angle
CI	confidence interval
CNS	central nervous system
DA	drift angle
DEF	S,S,S, tributyl phosphorotrithioate
DEM	diethyl maleate

ECOD	7-ethoxycoumarin O-deethylation
EPA	environmental protection agency
EAG	electroantennogram or electroantennography
EST	carboxylesterases
FD	flight duration
FL	flight length
FV	flight velocity
GABAA	γ-aminobutyric acid
GLM	generalized linear models
GS	ground speed
GST	glutathione-S-transferases
HF	heterogeneity factor
IPM	integrated pest management
IRAC	insecticide resistance action committee
IT	intern turns
JH	juvenile hormone
LC	lethal concentration
LD	lethal dose
LRT	likelihood ratio test
MAPAMA	ministerio de agricultura y pesca, alimentación y medio ambiente
MFO	mixed-function oxidases
MGC	macroglomerular complex
MoA	mode of action
MS	muscular system
nAChR	nicotinic-acetylcholine receptor

OBP	odorant-binding proteins
ORN	olfactory receptor neuron
PBAN	pheromone biosynthesis activating neuropeptide
PBCR	plant biosecurity cooperative research centre
PBO	piperonyl butoxide
PBP	pheromone-binding proteins
PNS	peripheral nervous system
SEM	standard error of the mean
T	turns
TA	track angle
WS	wind speed

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Chapter 4: Sublethal effects on males

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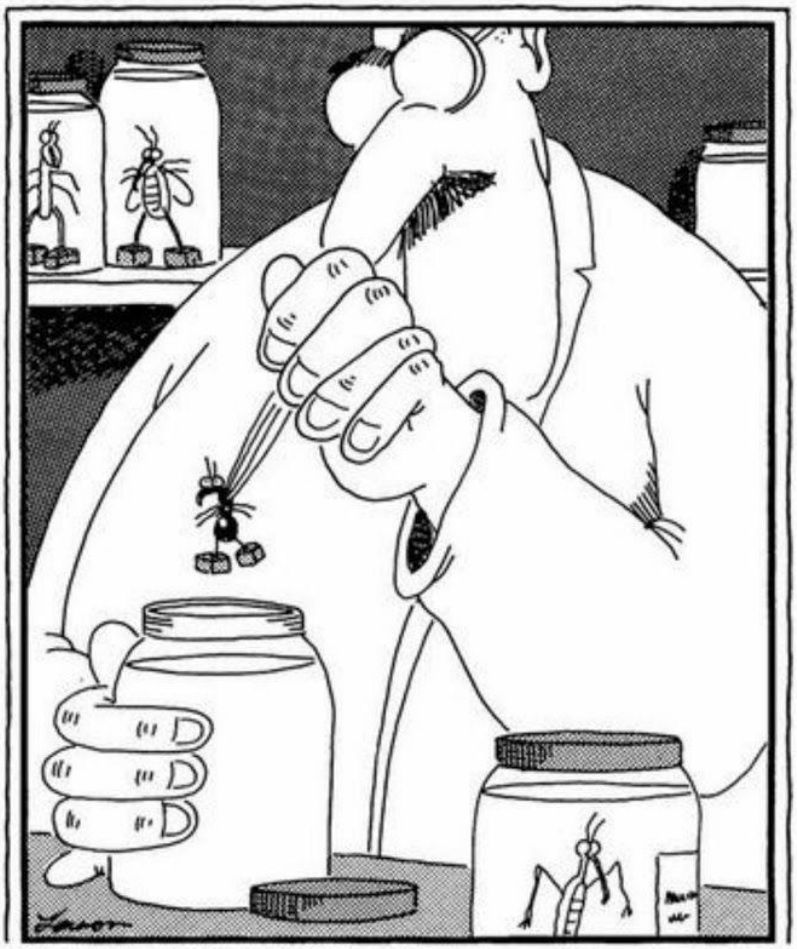
… GRACIAS!



Scenes from the entomology underworld

"The far side" by Gary Larson