Universitat de Lleida

Sense and Sensitivity: on the biology and neuroethology of two tortricid moths

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TESI DOCTORAL

Sense and Sensitivity: on the biology and neuroethology of two tortricid moths

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To whom it may concern

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ABSTRACT

Pheromone-mediated mating disruption (MD), a pest management tactic that prevents moth mating, has helped reduce insecticide use and crop damage in agriculture. The need to decrease the economic and labor costs associated with its application has encouraged the development of automated dispensers. These “active” dispensers disrupt mating behavior of moth pests by releasing pheromone puffs during the time when insects are active. However, the lack of a firm basis on the biology and behavior of moths, and how external factors can alter their periodicity, prevent the proper adjustment of the pheromone dispensers, and may result in a decrease of their efficacy in the field. Although automated traps can be used to determine these activity periods and adjust pheromone release by automatic devices, commercially automated traps do not offer enough temporal resolution to outline insect activity periods, which usually last one or a few hours.

In my thesis, we have designed and tested a cheap and easy to build high temporal resolution image-sensor insect trap. Based on captures in traps lured with synthetic pheromone or virgin females I have determined the daily and seasonal sexual responsiveness of *Grapholita molesta* (Busk) males. Most captures were registered between 3 hours before and 1 hour after sunset, indicating a diurnal to crepuscular activity of the species. Lure type (septum or female) did not influence the daily time of flight. While low temperatures led to an earlier flight in males, warmer temperatures resulted in flights closer to the sunset time. To reduce the cost of MD and avoid wasting pheromone, automated dispensers should be programmed to spray at a variable time throughout the season, following the curve of activity of the insects.

In order to explain the mechanism of pheromone-mediated MD in pest management, most studies have focused on the effect of high levels of synthetic pheromones on the behavior of males, whereas females were considered unresponsive to their own pheromone. However, a recent review sows that there is substantial behavioral evidence that female moths respond to their own sex pheromone. Nevertheless, the evidence for sex pheromone "autodetection" at the olfactory receptor neuron (ORN) level is limited. By means of electrophysiological methods I compared the responses of ORNs housed in antennal sensilla trichodea to an array of biologically relevant compounds of male and female *G. molesta*, a species with reported pheromone autodetection. Hierarchical cluster analysis (HCA) indicated a radically different peripheric olfactory system between sexes that could be related to their specific ecological roles. In males no cells responded to their own courtship pheromone ethyl *trans*-cinnamate, while most (63%) were tuned specifically to the major or minor pheromone compounds (*Z*8-12:Ac and *E*8-12:Ac, respectively), their relative abundance being similar to their ratio in the female pheromone. Plant volatile cells were relatively frequent in females (6%) and 3% of the female ORNs were also tuned to the male-produced

courtship pheromone, ethyl *trans*-cinnamate. Several female cells were excited by the female-produced sex pheromone, but their responses were generally lower than in males, and they responded broadly to the other compounds as well, so the HCA grouped them in a large cluster (91%) of "unspecific" female neurons. The lack of differential sex pheromone receptor neurons in females, and their relatively low sensitivity to sex pheromone would not allow them to detect variations in the pheromone ratios in nature. Thus, the alteration of their behavior after exposure to conspecific pheromone under laboratory conditions does not appear to be species-specific.

Female behavior can nonetheless be specifically altered by other cues more relevant to their biological needs. The headspace of different plants may induce a different effect on females stemming from different hosts. I analyzed electroantennogram (EAG) responses of male and female adults of the European grapevine moth *Lobesia botrana* (Denis et Schiffermüller) (Lepidoptera: Tortricidae) collected as larvae from grapevine (*Vitis vinifera* L.) and flax-leaved daphne (*Daphne gnidium* L.) to specific and shared plant volatiles of the two hosts, as well as to sex pheromone compounds. My results indicate that pheromone detection did not differ between the two populations. Furthermore, host-plant volatile detection was not affected by sex or the larval host plant. Polyphagous populations developing on a specific host seem therefore able to detect the plant volatiles of alternative hosts. However, lack of statistical differences in odor discrimination at the antennal level does not imply that insects from each host would show similar preference for the two host-specific odor blends, since behavioral responses to plant odors require brain integration of the antennal input of each individual odorant in a blend. Differences in plant preference could still occur if there are no differences at the EAG level. Understanding the potential ability of a polyphagous moth to reproduce or find food and shelter in alternative hosts is important to assess the topographical limitations of MD.

My thesis serves to understand a bit better the complex functioning of a technique like MD and the repercussions that some environmental factors can have in its proper functioning. Even though pesticide use has been reduced, we still rely on these chemicals to prevent pest outburst. A more deep and rigorous study of the variables affecting the efficacy of environmentally friendly alternatives should be conducted.

RESUMEN

La confusión sexual mediante feromonas es una táctica de control de plagas que, al impedir el apareamiento entre polillas, ha ayudado enormemente a reducir los daños en cultivos, así como el uso de pesticidas. La necesidad de disminuir los costes asociados a su aplicación ha fomentado el desarrollo de dispensadores automáticos. Estos dispensadores liberan una gran cantidad de feromona mientras los insectos están sexualmente activos. Sin embargo, la falta de información sobre la biología y el comportamiento de los insectos y del efecto que algunos factores externos tienen sobre su periodicidad diaria impiden un correcto ajuste de los dispensadores automáticos, lo que puede suponer una disminución de su eficacia en campo. A pesar de que el trampeo automático puede servir para determinar estos periodos de actividad y decidir cuándo liberar feromona, las trampas disponibles comercialmente no confieren una resolución temporal suficiente para bosquejar la actividad de los insectos, que puede estar reducida a unas pocas horas.

Para mi tesis hemos diseñado y puesto a prueba una trampa asequible y fácil de construir que me ha permitido determinar el vuelo sexual diario y estacional de *Grapholita molesta* (Busk) a partir de fotografías tomadas con una gran resolución temporal. Tanto en trampas cebadas con feromona sintética como en trampas cebadas con hembras vírgenes, la mayoría de las capturas tuvieron lugar entre 3 horas antes y una hora después de la puesta de sol, indicando una actividad diurna crepuscular en la especie. El tipo de cebo (septo de feromona o hembra virgen) no afectó el periodo de vuelo diario. Aun así, las bajas temperaturas avanzaron los vuelos, mientras que las temperaturas más altas retrasaban el vuelo hasta horas cercanas a la puesta de sol. Estos resultados destacan la necesidad de programar los dispensadores para que liberen la feromona a distintas horas a lo largo del año, siguiendo la curva de vuelo de los insectos. Hacerlo aumentaría la eficacia de este sistema en campo, al tiempo que reduciría el coste de su aplicación y evitaría un desperdicio de feromona.

Para explicar los mecanismos que subyacen al funcionamiento de la confusión sexual en el control de plagas, la mayoría de los estudios se han centrado en el efecto de grandes niveles de feromona sintética sobre los machos. Las hembras, por otro lado, se han considerado insensibles a su propia feromona. Sin embargo, una revisión reciente indica que hay una alteración notable en el comportamiento de las hembras al ser expuestas a su propia feromona, un fenómeno conocido como “autodetección”. A pesar de ello, no hay pruebas que confirmen que las hembras sean capaces de detectar su propia feromona a nivel de las neuronas olfativas. Mediante técnicas de electrofisiología, he comparado la respuesta de las neuronas olfativas alojadas en *sensilla trichodea* de machos y hembras a compuestos biológicamente relevantes para *G. molesta,* una especie en la que se ha descrito autodetección. Un análisis de agrupamiento jerárquico indica un sistema olfativo periférico radicalmente diferente en cada sexo, lo que podría estar relacionado con las diferentes

necesidades biológicas de cada uno. Mientras que en los machos no había células que respondieran a su propia feromona de cortejo, cinamato de etilo, la mayoría (63%) respondieron específicamente a los compuestos de feromona sexual mayoritario y minoritario (*Z*8-12:Ac y *E*8-12:Ac, respectivamente). La frecuencia de cada uno de estos tipos de células es similar al ratio de mezcla de feromonas de las hembras. En hembras, un 6% de las células respondían a volátiles de planta y un 3% son específicas para la feromona de cortejo de los machos. A pesar de que algunas células eran estimuladas por la feromona sexual de las hembras, estas respuestas eran siempre menores que las de machos y, además, respondían a más compuestos. De hecho, la agrupación jerárquica las incluye en un grupo (91%) de neuronas inespecíficas. La ausencia en hembras de receptores para la feromona sexual y su baja sensibilidad a la misma les impediría detectar variaciones en las ratios de feromona en la naturaleza. Por lo tanto, cualquier alteración del comportamiento tras una exposición a feromona conspecífica en el laboratorio no podría ser atribuida a un reconocimiento de la propia especie.

El comportamiento de las hembras sí que puede verse alterado por otras señales más relevantes para sus necesidades biológicas. El olor característico de una planta puede provocar un efecto diferente en hembras que provienen de huéspedes distintos. En mi tesis he analizado respuestas electroantenográficas de machos y hembras adultos de *Lobesia botrana* (Denis et Schiffermüller) (Lepidoptera: Tortricidae) recogidas en fase larvaria de la vid (*Vitis vinífera* L.) o del torvisco (*Daphne gnidium* L.) a volátiles específicos y compartidos de los huéspedes, así como a compuestos de feromona sexual. Mis resultados indican que la detección de feromona no difiere entre ambas poblaciones. Además, la detección de volátiles de planta no se ve afectada por el sexo o la planta de desarrollo larvario. Poblaciones polífagas desarrollándose en un huésped concreto parecen retener la capacidad para responder a los volátiles de otros huéspedes. Esta falta de diferencias estadísticas en la diferenciación de compuestos olfativos al nivel de la antena no implica, sin embargo, que los individuos que provienen de cada huésped muestren preferencias similares ante ambos conjuntos de volátiles, dado que las respuestas biológicas dependen en última instancia de la integración cerebral de cada individuo. Aun así, es importante comprender la capacidad de los insectos polífagos para reproducirse o buscar alimentos y refugios alternativos a la hora de determinar los límites topográficos de la confusión sexual.

Mi tesis sirve para entender mejor las repercusiones que algunos factores ambientales pueden tener sobre el funcionamiento de una técnica compleja como es la confusión sexual. A pesar de que el uso de pesticidas se ha reducido, aún nos valemos de estos químicos para evitar el descontrol de las plagas, y es necesario conocer las variables que afectan a la eficacia de las alternativas sostenibles.

RESUM

La confusió sexual mitjançant feromones és una tàctica de control de plagues que, en impedir l'aparellament entre arnes, ha ajudat enormement a reduir els danys en cultius, així com l'ús de pesticides. La necessitat de disminuir els costos associats a la seva aplicació ha fomentat el desenvolupament de dispensadors automàtics. Aquests dispensadors alliberen una gran quantitat de feromona mentre els insectes estan sexualment actius. No obstant això, la falta d'informació sobre la biologia i el comportament dels insectes i de l'efecte que alguns factors externs tenen sobre la seva periodicitat diària impedeixen un correcte ajust dels dispensadors automàtics, la qual cosa pot suposar una disminució de la seva eficàcia en camp. A pesar que el parany automàtic pot servir per a determinar aquests períodes d'activitat i decidir quan alliberar feromona, els paranys disponibles comercialment no confereixen una resolució temporal suficient per a esbossar l'activitat dels insectes, que pot estar reduïda a unes poques hores.

Per a la meva tesi hem dissenyat i posat a prova un parany assequible i fàcil de construir que m'ha permès determinar el vol sexual diari i estacional de *Grapholita molesta* (Busk) a partir de fotografies preses amb una gran resolució temporal. Tant en paranys encebats amb feromona sintètica com en paranys encebats amb femelles verges, la majoria de les captures van tenir lloc entre 3 hores abans i una hora després de la posta de sol, indicant una activitat diürna crepuscular en l'espècie. El tipus d'esquer (septe de feromona o femella verge) no va afectar al període de vol diari. Així i tot, les baixes temperatures van avançar els vols, mentre que les temperatures més altes retardaven el vol fins a hores pròximes a la posta de sol. Aquests resultats destaquen la necessitat de programar els dispensadors perquè alliberin la feromona en diferents hores al llarg de l'any, seguint la corba de vol dels insectes. Fent-t'ho així augmentaria l'eficàcia d'aquest sistema en camp, al mateix temps que reduiria el cost de la seva aplicació i evitaria un desaprofitament de feromona.

Per a explicar els mecanismes subjacents al funcionament de la confusió sexual en el control de plagues, la majoria dels estudis s'han centrat en l'efecte de grans nivells de feromona sintètica sobre els mascles. Les femelles, d'altra banda, s'han considerat insensibles a la seva pròpia feromona. No obstant això, una revisió recent indica que hi ha una alteració notable en el comportament de les femelles en ser exposades a la seva pròpia feromona, un fenomen conegut com a “autodetecció”. Malgrat això, no hi ha proves que confirmin que les femelles siguin capaces de detectar la seva pròpia feromona a nivell de les neurones olfactòries. Mitjançant tècniques d'electrofisiologia, he comparat la resposta de les neurones olfactòries allotjades en sensilla trichodea de mascles i femelles a compostos biològicament rellevants per a *G. molesta*, una espècie en la qual s'ha descrit autodetecció. Una anàlisi d'agrupament jeràrquic indica un sistema olfactori perifèric radicalment diferent en cada sexe, la qual cosa podria estar relacionat amb les

diferents necessitats biològiques de cadascun. Mentre que en els mascles no hi havia cèl·lules que responguessin a la seva pròpia feromona de festeig, cinamato d'etil, la majoria (63%) van respondre específicament als compostos de feromona sexual majoritari i minoritari (Z8-12:Ac i E8-12:Ac, respectivament). La freqüència de cadascun d'aquests tipus de cèl·lules és similar al ràtio de mescla de feromones de les femelles. En femelles, un 6% de les cèl·lules responien a volàtils de planta i un 3% són específiques per a la feromona de festeig dels mascles. A pesar que algunes cèl·lules eren estimulades per la feromona sexual de les femelles, aquestes respostes eren sempre menors que les de mascles i, a més, responien a més composts. De fet, l'agrupació jeràrquica les inclou en un grup (91%) de neurones inespecífiques. L'absència en femelles de receptors per a la feromona sexual i la seva baixa sensibilitat a la mateixa els impediria detectar variacions en les ràtios de feromona en la naturalesa. Per tant, qualsevol alteració del comportament després d'una exposició a feromona coespecífic en el laboratori no podria ser atribuïda a un reconeixement de la pròpia espècie.

El comportament de les femelles sí que es pot veure alterat per altres senyals més rellevants per a les seves necessitats biològiques. L'olor característica d'una planta pot provocar un efecte diferent en femelles que provenen d'hostes diferents. En la meva tesi he analitzat respostes electroantenogràfiques de mascles i femelles adults de *Lobesia botrana* (Denis et Schiffermüller) (Lepidoptera: Tortricidae) recollides en la fase larvària de la vinya (*Vitis vinífera* L.) o del torvisco (*Daphne gnidium* L.) a volàtils específics i compartits dels hostes, així com a compostos de feromona sexual. Els meus resultats indiquen que la detecció de feromona no difereix entre totes dues poblacions. A més, la detecció de volàtils de planta no es veu afectada pel sexe o la planta de desenvolupament larvari. Poblacions polífagues desenvolupant-se en un hoste concret semblen retenir la capacitat per a respondre als volàtils d'altres hostes. Aquesta falta de diferències estadístiques en la diferenciació de compostos olfactoris al nivell de l'antena no implica que els individus que provenen de cada hoste mostrin preferències similars davant tots dos conjunts de volàtils, atès que les respostes biològiques depenen en última instància de la integració cerebral de cada individu. Així i tot, és important comprendre la capacitat dels insectes polífags per a reproduir-se o buscar aliments i refugis alternatius a l'hora de determinar els límits topogràfics de la confusió sexual.

La meva tesi serveix per a entendre millor les repercussions que alguns factors ambientals poden tenir sobre el funcionament d'una tècnica complexa com és la confusió sexual. A pesar que l'ús de pesticides s'ha reduït, encara ens valem d'aquests químics per a evitar el descontrol de les plagues, i és necessari conèixer les variables que afecten l'eficàcia de les alternatives sostenibles.

GENERAL INTRODUCTION

"An apple a day keeps the doctor away". But what is beyond this proverb? The believe that we have to eat an apple every day to avoid health problems leads us to demand this and other products from the field. As demand has increased, production has made an effort to keep the pace and the cropping system has transformed from a landscape of diversity to sceneries of continuity of one crop (FAO, 2017). Within this homogeneity, pests find an oasis of food and shelter, and they have become quite reluctant to abandon their favorable position (Altieri et al., 2009; Paredes et al., 2020). In Spain, where grapevine and stone and pome fruit production are one of the major sources of income (MAPAMA, 2019) (**Fig. I**), economy is endangered by a number of important pest species, among which the European grapevine moth (EGVM) *Lobesia botrana* (Denis et Schiffermüller), and the Oriental fruit moth (OFM) *Grapholita molesta* (Busk), play a key role. These species are polyphagous (Pogue, 2009; CABI, 2019) and have the potential to produce multiple generations by season under favourable conditions. Major damage by these pests is caused by larval feeding on fruits, although they can consume other host organs, like flowers, leaves and shoots (Myers et al., 2007; Ioriatti et al., 2011).

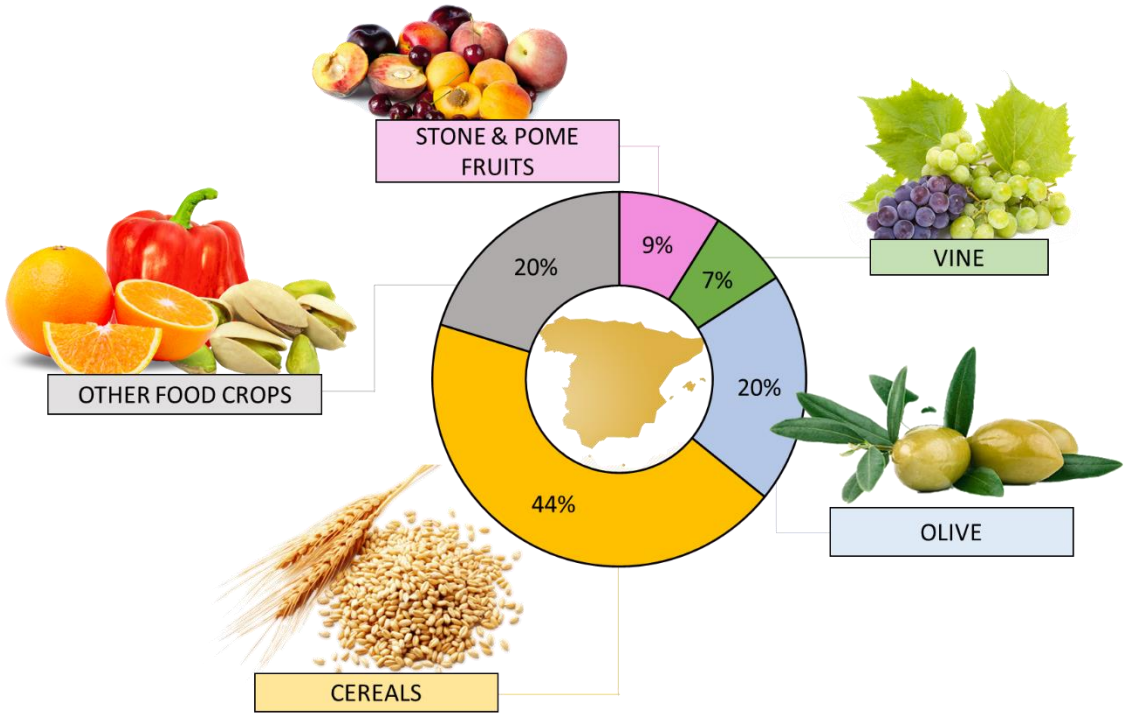

Figure I. Percentage of food crops in Spain of a total area of 16719931.37 ha (data from MAPAMA, 2019).

In consequence, science has had to invest uncountable resources to fight them. What in the beginning was an advantageous war where chemicals would be spread thoughtlessly all over the countryside, has currently become a dead end. Insects develop resistance towards pesticides (Borel, 2017; Sparks et al., 2020) and these products have a noxious effect on environmental and human health (Aktar et al., 2009; Saillenfait and Malard, 2020). Thus, science has to change the strategy and look for alternatives that are less harmful to our one and only planet. Integrated pest management (IPM) programs intend to reduce the use of pesticides by increasing the knowledge on biology and chemistry of the pests (McNeil, 1991; Damos et al., 2015; Knight et al., 2019). Thus, complementary practices to the use of pesticides are being investigated in order to achieve a more sustainable agriculture. Insect semiochemicals and particularly, attractants, have been used for pest controlling in different approaches (McNeil, 1991; Norin, 2007).

Biology of moths: mechanisms of odor perception

To find food, shelter or mates and avoid predators and other dangers, insects rely on a wide range of sensory systems. Through these senses, the insects' nervous system can generate a simplified representation of the complex external world, which allows them to decide how to respond behaviorally given the perceived situation (Hansson and Stensmyr, 2011). The sense of smell plays a fundamental role, emphasized by the elaborated antennal patterns, the functional equivalents of the human nose, found in many of them. Antennae are covered with sensory structures, the sensilla, which show a wide variety of shapes and forms (**Fig. II**). Regardless their capricious appearance, the olfactory sensilla all serve to encapsulate and

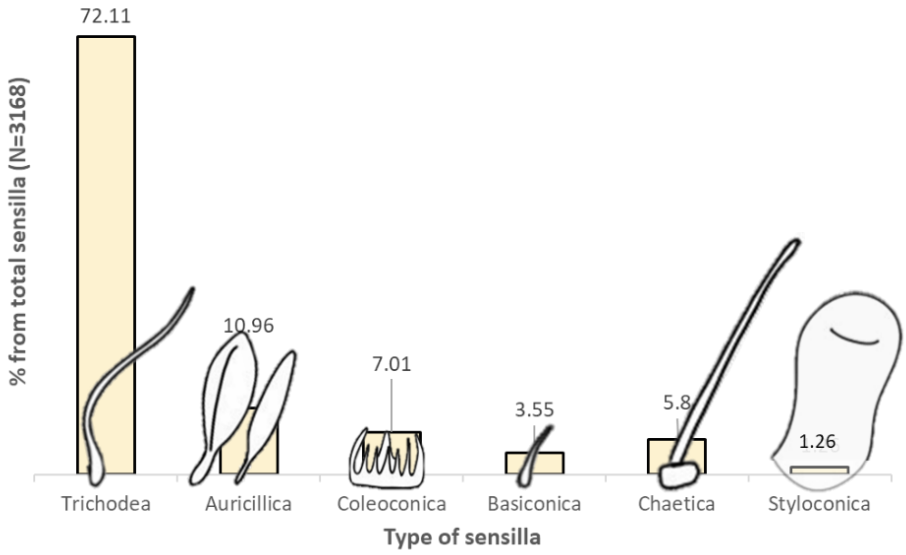

Figure II. Type of sensilla and proportion found on the antenna of male *Grapholita molesta*, proportions from Ammagarhalli and Gemeno (2014). Figure adapted from Ansebo (2014).

protect the sensitive dendrites of the olfactory sensory neurons (OSNs).

The most specialized OSNs are those detecting pheromones, where OSNs capable of separating two enantiomers with a specificity spanning over more than four decadic concentration steps have been found (Lassance, 2016; Kaissling, 2019). Pheromone receptor neurons (PRNs) are housed in long hair-like structures known as trichoid sensilla that appear in great quantities along the antennae to increase the surface of perception, onto which pheromone molecules attach to enter the lumen through their porous membrane (Hansson and Stensmyr, 2011; Kaissling, 2019). From there, they are transported along pheromone binding proteins to the odorant receptors present in the dendrites of the neurons. When the correct compound is detected and certain concentration thresholds are surpassed, the cells can be induced into a response (Kaissling, 2019; Si et al., 2019).

The adaptations at the antennal level correspond with further adaptations in the primary olfactory center of the insect brain, the antennal lobe (AL). The AL is composed by spheroid structures called glomeruli. All OSNs expressing the same receptor converge onto one out of these usually between 50 and 200 glomeruli (Hansson et al., 1992; Hansson and Stensmyr, 2011). The glomerulus also houses the branches of local interneurons and the dendrites of projection neurons that transmit the processed information to higher brain areas (Anton and Hansson, 1994). The male AL presents enlarged glomeruli that form the macroglomerular complex (MGC), each of which is targeted by OSNs tuned to different pheromone components (Hansson et al., 1992; Hansson and Stensmyr, 2011). Input regarding the main component of a sex pheromone mixture is usually processed by an enlarged glomerulus denominated the cumulus. This MGC part can then be surrounded by a number of smaller satellite-MGC-glomeruli receiving information regarding the presence of other pheromone components, or behavioral antagonists preventing interspecific attraction (Hansson and Stensmyr, 2011; Dekker and Kárpáti, 2020).

Mating disruption: implementation and constraints

This extremely sensitive olfactory system allows males to find females from great distances, following the patterns of release of pheromone plumes, that excite sensilla in different ways (Cardé, 2016). Mating disruption (MD) is a technique that takes advantage of the great specialization of the olfactory system of males to entangle with the olfactory communication of moths. Just as plants dupe insects into doing their bidding by imitating irresistible odors, we can confuse males so that they cannot locate females to reproduce by permeating the environment with synthetic pheromone (Miller and Gut, 2015). No negative effects on human

health and non-target organisms have been observed this far, allowing researchers to claim this method as fully compatible with modern IMP criteria (Miller and Gut, 2015). However, MD success is restricted to low population densities and isolate orchards where immigration of gravid females is precluded (Baker, 2008), and its efficacy can be reduced by multiple factors (Suckling, 2000). In Spain, more than 120.000 ha are treated via MD against *L. botrana* and *G. molesta*, Catalunya being the main user of this technique (CBC Iberia S.A., personal communication) (**Fig. III**). MD is traditionally implemented by installing hundreds of passive dispensers per hectare (Witzgall et al., 2008). While this approach is effective, it requires a high labor input and much of the pheromone is wasted during the period when insects are not sexually active, which is most of the daytime (McNeil, 1991). In the 1990s, a wide array of technological advances were proposed to boost the efficacy of MD by reducing the number of dispensers per hectare, and thus labor cost (Benelli et al., 2019). Automated sprayers release larger quantities of pheromone from fewer point sources and can be programmed to do so at selected time intervals. This way it is possible to optimize MD by concentrating the release of multiple synthetic plumes in the hours of the day when the insects are more active (Benelli et al., 2019). However, the exact periods of activity of most insects are still only known in the laboratory (Groot et al., 2014), and little is known about the effect of temperature and other environmental factors on flight periodicity, mainly due to monitoring limitations.

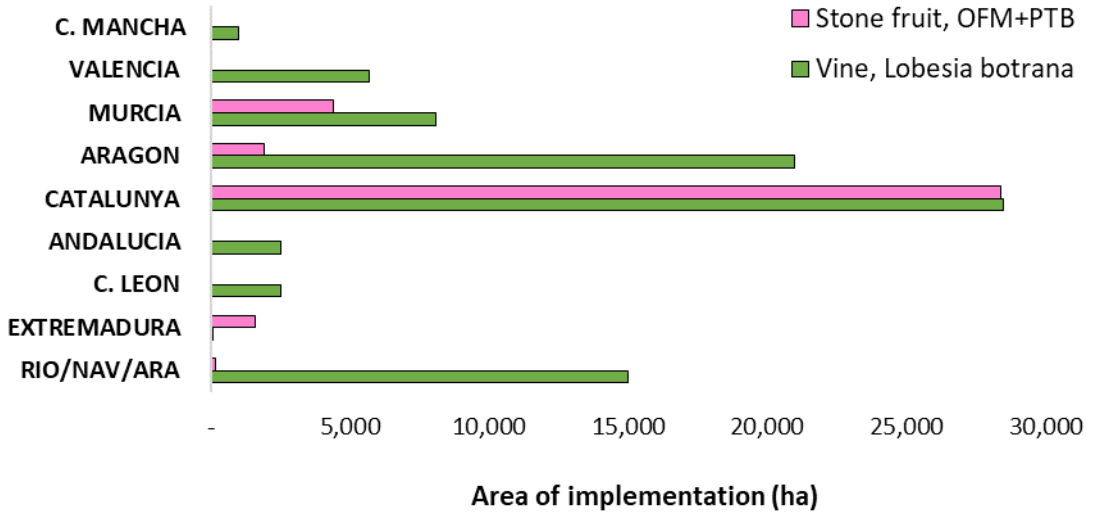

Figure III. Area of implementation (ha) of mating disruption in various regions of Spain to *Grapholita molesta* (pink) and *Lobesia botrana* (green) in stone fruit and vine orchards, respectively (CBC Iberia S.A., personal communication).

Pest monitoring and trap evolution

Pest monitoring using pheromone baited traps requires periodic visits to the field by a human operator to check and record male captures trapped in the sticky traps. This is a labour-intensive, time consuming and expensive monitoring process, and can become even more difficult when insects have nocturnal habits. Development of automated trapping systems can ease continual monitoring of many economically important pest species (Manoukis and Collier, 2019), but commercially available automated recording systems are scarce (e.g., Semios Technologies Inc., Vancouver, Canada; TrapView, Hruševje, Slovenia), and are designed to count captures remotely only once or twice a day in order to help growers take prompt management decisions (Preti et al., 2020). Although it is possible to increment the sampling frequency of commercial traps (Lucchi et al., 2018), the associated increase in price and energy requirements has led research to rely on self-made traps of increasing complexity (Beroza and Machan, 2006; Guarnieri et al., 2011; Kim et al., 2011; Doitsidis et al., 2017; Ünlü et al., 2019).

Figure IV. Commercially available traps. A) Semios Technologies Inc, B) TrapView, C) Spensa Technologies Inc.

My first aim is then to develop a low-cost and durable image-sensor insect trap, made with accessible components, that can be easily programmed with open-source software, and that allows both diurnal and nocturnal sampling with high time resolution. Automated monitoring equipment should prove an invaluable tool for making daylong field studies less labor intensive, increase accuracy, and possibly help in correlating adult catch to action thresholds. Moreover, the automated trap will allow the simultaneous processing of moth activity and exogenous variables, which may provide a means of documenting the effect of environmental factors.

Circadian rhythms and effect of environmental factors

Under natural conditions insects are exposed to one sunrise and one sunset each day. Moth species show specific daily circadian rhythms in many of their behaviors, including mating

activity, which are entrained to a 24-h periodicity by these light-dark cycles and can be further regulated by temperature (Danks, 2005; Beck, 2012; Saunders, 2013; Groot, 2014; Groot et al., 2014; Shiga, 2019). Phase regulations of rhythmic functions involve daily adjustments of a few minutes, because of the gradual nature of seasonal changes. These minor daily adjustments maintain the insect's rhythms in synchrony with the environmental photoperiod; that is, the endogenous rhythms are entrained by the exogenous photoperiodic rhythm (Beck, 2012). This serves to ensure that a great proportion of the population is active simultaneously (Saunders, 2014; Shiga, 2019), and the probability of contact between sexes is thus enhanced (Haynes and Birch, 1986; Byers, 2006; Beck, 2012; Groot, 2014).

Although it is clear that the circadian regulation of male response to sex pheromone complements circadian regulation of female pheromone biosynthesis and calling behavior (Groot, 2014; Allison and Cardé, 2016), most studies regarding male mating flight in the field rely on synthetic pheromone baited traps (Päts and Wiktelius, 1992; Quiring, 1994; Cardé et al., 1996; Ivanova et al., 2010; Ünlü et al., 2019). The release of the synthetic pheromone contained in the septum into the environment is not dependent upon female behaviour. Thus, the periodicity of male response as well as the threshold values of temperature could be different than the ones found for the response where females were used as the source of pheromone, as exposed by some authors (Shorey, 1966; Fatzinger and Asher, 1971; Haynes and Birch, 1984; Giebultowicz et al., 1992; Zhang et al., 1998; Stevenson and Harris, 2009). The differences in trap catches of synthetic versus natural female lures illustrate the necessity of determining the rhythms of male attraction and female attractiveness to evaluate the comparative efficacy of these lures. By means of the developed trap, I will determine male moth response towards synthetic and natural pheromone sources, highlighting the bias that the use of septa could have when understanding the action method of MD. Furthermore, I will assess the influence that environmental factors may have on their daily and seasonal periods.

Effect of pheromone on females: female autodetection

Many studies have centered on male pheromone perception and have helped to understand how their sensitive olfaction is hindered via MD. However, female response to its own sex pheromone remains relatively unexplored. Recent research has highlighted putative effects of conspecific pheromone on females (McNeil, 1991; Groot, 2014; reviewed in Holdcraft et al., 2016), and how the indiscriminate use of pheromones in the field could affect the control of the pest by fomenting their aggregation (Stelinski et al., 2014) or dispersion (Witzgall et al., 2008; Bakthavatsalam et al., 2016). Furthermore, some studies show that female onset of

calling can be advanced or delayed several hours when exposed to pheromone (Holdcraft et al., 2016). Altogether, these alterations could alter the efficacy of MD.

Nevertheless, electroantennographic studies regarding the response of females compared to that of males tend to show a lower to null response of the former towards pheromone (reviewed in Holdcraft et al., 2016). The scarce single sensillum recordings (SSR) studies concerning this question seem to indicate that, in general, most female ORNs responding to pheromone are less specific and less frequent than the ones found in males. This would account for the absence of a MGC in female brains and for the smaller size of the physiologically analogous glomeruli (Ochieng et al., 1995; Rospars and Hildebrand, 2000; Sadek et al., 2002; Masante-Roca, 2005; Hillier et al., 2006; Kárpáti et al., 2008; Trona et al., 2010). Although lacking an MGC, females present some glomeruli that are essentially different from those of males, indicating specialization in other compounds that are probably more relevant for their life cycle. I believe that a comparative analysis of the electroantennographic and cellular responses of OFM females and males to compounds relevant for the species will give us an idea of the analogies and differences in their olfactory system. In doing so, I aim to determine whether OFM female can detect conspecific female-produced sex pheromone to stablish a firm base onto which properly start assessing the matter of autodetection.

Host preference: steppingstones and reservoirs

Some chemical signals, like insect sex pheromones, target only conspecifics of the opposite sex, while a flower might aim to attract as many pollinators as possible with its odor bouquet. Polyphagous species rely to a large extent on olfaction to locate potential food sources and, in the case of females, to find suitable hosts to support larval development (Krieger and Breer, 1999; Bruce et al., 2005; Agosta, 2006; Bruce and Pickett, 2011). However, they live in environments of great complexity where they have to choose between a great number of different plant species of variable nutritional value, that will be ready to defend themselves from predation (Mithöfer and Boland, 2012). The presence of certain compounds in the headspace of a plant acts as cues that indicate females of the suitability of the host for larvae development. Although showing preference for a specific host, many species remain polyphagous and may perform better on other wild or cultivated hosts which contribute with a higher nutritional value, decreased natural enemy pressure or that enhance larval installation and survival (Thiéry and Moreau, 2005; Torres-Vila et al., 2012). By using different hosts with variable phonologies as steppingstones, females can ensure the survival of the species and disperse beyond their origin, invade different environments and hamper the control of the

pest species (Hughes and Dorn, 2002). Host preferences can be investigated at antennal and behavioral levels, comparing the response to compounds of the headspace of different hosts (Tasin et al., 2010; Conchou et al., 2017).

I wanted to explore sex differences in their response to pheromone and plant stimuli. Comparing the EAG response of moth populations stemming from different hosts to various plant volatiles may somehow reflect a preference of the species for the more suited host and help me understand how can these insects adapt and invade to different environments so rapidly.

Laboratory vs. field research

Most of the knowledge on biology of insects is obtained from laboratory experimentation, since mass rearing is a way of to ensure adequate number of insects. However, some studies seem to indicate that there exists a difference in the biology of wild and laboratory reared insects in their performance in the wild (Giebultowicz et al., 1992; Shelly and Edu, 2009). While wild insects develop under the influence of diverse agronomic practices (Ricci et al., 2009; Muneret et al., 2018) and environmental variability (Onstad, 2008), populations maintained in the laboratory experience a different selection pressure. This selection is based on parameters like the adaptation to constant abiotic factors, artificial diets, new oviposition substrates or the development under high moth densities, that can promote multiple mating (Franck et al., 2011). For this reason, it is important to check the fitness and population dynamics in the wild of laboratory reared insects, such comparison being especially important when laboratory-derived information is used to formulate and implement strategies for the control of insects in nature (Huettel, 1976; Huho et al., 2007). In my thesis, I have attempted to gain some understanding on the suitability of laboratory reared insects to extrapolate the results to the field.

As exposed here, there is an increasing need to understand how different factors can affect the efficacy of MD in the field. In my thesis I aim to explore the influence of some of these factors on the control of two pest species, *G. molesta* and *L. botrana*. My results can serve as a basis onto which explore their influence on many other important pest species.

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THESIS OUTLINE AND OBJECTIVES

Thesis outline

Through this thesis I aim to gain knowledge on the basic biology and behavior related to the efficacy of mating disruption of two moths of increasing importance as agricultural pest species. By doing so I intend to give some guidelines to implement current control strategies that have emerged to a achieve a sustainable agriculture. To accomplish this, I developed a trap that can register male mating activity and relate it to environmental conditions. It can further help compare male response towards different pheromone sources, gaining perspective on the true action of pheromone in the field. Furthermore, I wanted to obtain clear evidence of the possible effects mating disruption can have on female behavior and how this could relate to a reduced efficacy of this control technique. I explored male and female olfactory systems by means of electrophysiological techniques. These techniques were also pivotal to explore polyphagia in another tortricid moth, establishing the similitudes and differences between male and female moths of two different populations and highlighting the importance on odor detection to find alternative hosts. Altogether, my results serve to understand the effect of MD at different levels and to increase its efficacy.

This thesis consists of 4 experimental chapters. Chapter I, Chapter II, Chapter III and Chapter IV. Methodologies will be explained in detail within each respective chapter. Inevitably, there will be some repetition of the concepts presented in the General Introduction to make each chapter understandable by itself. In the section “General Discussion” I will be integrating important results of each experimental chapter and discussing them in a broader sense, remarking the scope of my thesis.

Objectives

Chapter I. *"Accessible image-sensor bait traps for recording insect activity periods in the field".*
Develop an affordable automated trap that can be programmed to register diurnal and nocturnal insect activity in the field with high time resolution. This trap will serve the purpose of increasing our knowledge on insect biology and behavior and apply that information to regulate pheromone release by new automated dispensers, in order to implement new IPM strategies in agriculture.

Chapter II. *"Oriental Fruit Moth: diel periodicity of catch in synthetic sex attractant vs. female-baited traps".*
Assess the effect of various environmental factors on the diel flight periodicity of *Grapholita molesta*, a pest species of great importance in Spain, to improve the adjustment of automatic pheromone dispensers in the field and boost mating disruption. Compare male flight towards natural and synthetic pheromone sources in the field to fill some lagoons of knowledge that could decrease the efficacy of mating disruption in the field regarding male mating periodicity.

Chapter III. "*Olfactory receptor neuron responses of male and female Oriental fruit moths to sex and courtship pheromones and host-plant volatiles*"
Assess the mater of autodetection in females from a comparative perspective with the previously stablished male olfactory system. The existence of several key aspects in males should be reflected in females to allow the recognition of its own pheromone and distinguish it from that of other closely related species. We intend to report differences from female antennae morphology to electrophysiological parameters, like electroantennograms and single sensillum recordings. Compare female and male response to conspecific pheromone compounds and other biologically relevant volatiles compounds to assess the veracity of autodetection. Report female antennae morphology and cell distribution compared to male's.

Chapter IV. *"EAG responses of adult* Lobesia botrana *males and females collected from* Vitis vinifera *and* Daphne gnidium *to larval host-plant volatiles and sex pheromone".*
I intend to understand if individuals developing on different hosts perceive host plant volatiles in a different way. This comparison at electroantennogram level will give us an insight on plausible preferences to one host or another and help shed light on the matter of polyphagia in a multivoltine species like *Lobesia botrana.* Likewise, we expect to find some differences in male and female olfactory perception that reflect their biological needs.

CHAPTER I. ACCESSIBLE IMAGE-SENSOR BAIT TRAPS FOR RECORDING INSECT ACTIVITY PERIODS IN THE FIELD

Abstract

Automated pheromone dispensers disrupt mating behavior of moth pests by releasing pheromone during their daily activity period, which is different for each target species. Automated traps are used to determine the activity period in order to adjust automated dispensers' activity time. Commercially available automated traps take few pictures per day (2-4) which is not enough temporal resolution to outline the activity periods which normally last one or a few hours. In addition, commercial traps are relatively expensive. We designed and tested a cheap and easy to build high temporal resolution image-sensor insect trap. It consisted of a Raspberry Pi computer board and an infrared camera operated with open-source software (Linux and Python), all mounted on a plastic box and powered with a solar panel and rechargeable battery. The set was mounted on a solid and weather-proof structure made with cheap hardware materials, and the pictures were downloaded wireless from the Raspberry´s SD card to the computer. Six traps baited with the sex pheromone or unmated females of *Grapholita molesta* (Busk), were tested in the field. The traps were sturdy, reliable and easy to use, taking pictures at 10 min intervals, 24 h a day during several months. Captures confirmed previous results regarding the sexual activity period of this species, which will aid in determining the best time to run mating-disruption automated pheromone dispensers.

Introduction

Mating disruption (MD) is a highly effective non-insecticide insect pest control method that reduces the probability of sexual encounters in species that rely strongly on the use of sex pheromones (Cardé and Minks, 1995). It consists in air permeation with synthetic sex pheromone which, in the case of moths, prevents males from detecting or finding pheromone releasing females (Miller and Gut, 2015).

The traditional method to apply pheromone for mating disruption is to manually install hundreds of passive dispensers per hectare to properly permeate an orchard with pheromone (Witzgall et al., 2008). This is labor intensive and wastes costly pheromone during the period when insects are not sexually active, which is most of the daytime (McNeil, 1991; Groot, 2014). A more cost-effective technique consists in releasing larger quantities of pheromone from fewer point sources only during the time that the insects are active. Such automated sprayers were developed in the 1990s, and their use is expanding (Benelli et al., 2019).

Although the sex pheromone of several hundred moth species has been identified (El-Sayed, 2019), the exact period of sexual activity has been established for just some of them (McNeil, 1991; Quartey and Coaker, 1996; Cardé et al., 2012; Pellegrino et al., 2013; Broadhead et al., 2017). The period when females release sex pheromone (i.e., calling period) is known for several moths mainly under laboratory conditions (Groot, 2014; Harari et al., 2015). Conversely, there are very few reports on the sexual periodicity of male moths, either in the laboratory or in the field (e.g., Batiste et al., 1973a; Rothschild and Minks, 1974; Quiring, 1994; Cardé et al., 1996; Zhang et al., 1998; Kim et al., 2011; Lucchi et al., 2018; Yang et al., 2019). One possible explanation for the scarcity of periodicity studies in the field, not only with moths but also with most other insects, is that they require frequent (<1h) observations since activity periods are usually short. Likewise, they often take place in the dark because many insects have nocturnal habits, which further difficult the data collection. Automated recording methods would ease observations considerably, but commercial automated recording systems were not available in the early days (Manoukis and Collier, 2019; Preti et al., 2020). Therefore, the majority of studies of insect periodicity in the field have relied on self-made traps (e.g., Beroza and Machan, 2006; Guarnieri et al., 2011; Kim et al., 2011; Doitsidis et al., 2017; Ünlü et al., 2019). In the early days these traps were relatively involved because they used mechanical methods to segregate captures on fixed time periods (e.g., Batiste, 1970; Schouest and Miller, 1994; Liu and Haynes, 1994; Stevenson and Harris, 2009). With the advent of cheaper, smaller, and more sophisticated and accessible electronic components and sensors, several automated

traps have been developed. Image capture systems can take pictures and allow species identification, either manually (Guarnieri et al., 2011; Ünlü et al., 2019) or with the aid of digital-image processing (Zhao et al., 2016; Doitsidis et al., 2017). However, running the sensor for prolonged time periods requires a relatively high-power supply that should be further increased if images are sent remotely. High digital storage capacity would otherwise be required when pictures are stored *in situ*. Event sensors count each passing of an individual across a point detector, and they are based on the interruption of a light beam (Kim et al., 2011; Jung et al., 2013; Goldshtein et al., 2017), or some other methods (e.g., Tobin et al., 2009), but they do not provide species identity. Finally, as different insects flap their wings at specific frequencies, wing-beat detectors have been developed to detect the approach of insects. Early devices used microphones (reviewed in Chen et al., 2014) but more recent ones use light interference detectors (Moraes, 1998; Chen et al., 2014; Potamitis et al., 2014). Wing-beat detectors can determine species identity by means of learning algorithms (Chen et al., 2014).

Although companies have been tracking these technical developments, there are relatively few commercial automated moth traps in the market (e.g., Semios Technologies Inc., Vancouver, Canada; TrapView, Hruševje, Slovenia). These traps have been designed to count captures remotely once or twice a day so that growers can take prompt pest management decisions in response to daily or seasonal changes in population numbers. For that goal they only need to take one or two pictures per day, but a higher temporal resolution is needed to outline the short circadian activity periods of most insect species. Although it is possible to increment sample frequency of commercial traps on demand (Lucchi et al., 2018), this requires increasing the power supply, which rises the cost of a product which is not especially cheap in the first place.

In this study we present a low-cost and durable image-sensor insect trap, made with accessible components, that can be easily programmed with open-source software, and that allows both diurnal and nocturnal sampling with high time resolution. To test it, we sampled the activity of males of the Oriental fruit moth (OFM), *Grapholita molesta* (Busk), comparing their response towards sex pheromone or live-female baited traps in the field every 10 min for three months.

Material and methods

The trap

The insect trap (from here on "trap box") consists of two plastic boxes (lunchboxes), one of them serving as a cover of the other one to protect the electronic components from adverse

weather conditions (**Fig. 1.1A**). The electronic components were attached to the ceiling of the inner box. A hole drilled at its center allows the camera to capture the glue card lining on the lunch-box original lid, which is the actual floor of the trap. The sticky card attaches to the lid with hook-and-loop fastener for easy replacement. Holes are drilled on each corner of the floor lid to drain water accumulation from the rain. Flying males enter the trap box through 11.5 x 5 cm windows cut on two opposing walls of the inner plastic box. The top box slides over the bottom one and they are fixed in place with two small screws.

System power supply is provided by a solar panel, lead-acid battery, and charge controller (**Table 1.1**). A structure made of aluminum and wood (35.5 x 27 x 1.5 cm) holds and protects the battery and the charge controller from weather (**Fig. 1.1B**). The solar panel roofs the structure and is hinged to the wooden frame for easy access to the battery and charge controller. An extra metal plate on top provides further weather proofing. The power-supply unit is relatively heavy (frame = 3.47 Kg, solar panel + battery = 4 Kg) and is attached to a galvanized steel bar which has to be relatively stiff to hold that weight (4.5 x 2.5 cm cross section, 150-cm long, 2.43 kg). Therefore, the final weight of the solar unit + metal bar is 9.9 Kg. The bar is mounted on site with two large (8 mm diameter, 9 cm length) bolts that can be hand-tightened to metals brackets screwed to the wooden power supply structure with wing nuts (**Fig. 1.1B**).

The electronic components of the trap consist of a Raspberry Pi Zero Wifi computer board connected to an infrared camera (Raspberry Pi Camera NoIR v.2.1) and an infrared LED for night vision (**Fig. 1.2**). Infrared camera has a maximum resolution of 3280x2464 px.

Table 1 lists the specifications of the trap components. The infrared LED is equipped with a photoresistor to switch it on and off at user-defined ambient light level. A heat sink was attached to the Raspberry´s microprocessor to prevent overheating under field conditions. The operating system GNU/Linux (Full NOOBS 2.3) was installed in a micro-SD card and image acquisition is configured (image resolution, picture frequency, etc.) with a script written in Python 2.7 code. Acquired pictures are also stored in the micro-SD card. Initial installation and configuration of the operating system requires USB mouse, USB keyboard and HDMI display. Afterwards, communication with the Raspberry board for programing and picture download is made via Wi-Fi connection with a computer using a VNC server (RealVNC for linux 6.7.2).

The height of the plastic box is critical because it determines the camera field of view. If the camera is too close to the sticky card it will capture only a portion of sticky bottom and captures outside of this area will be missed. If the camera is too far from the sticky card the

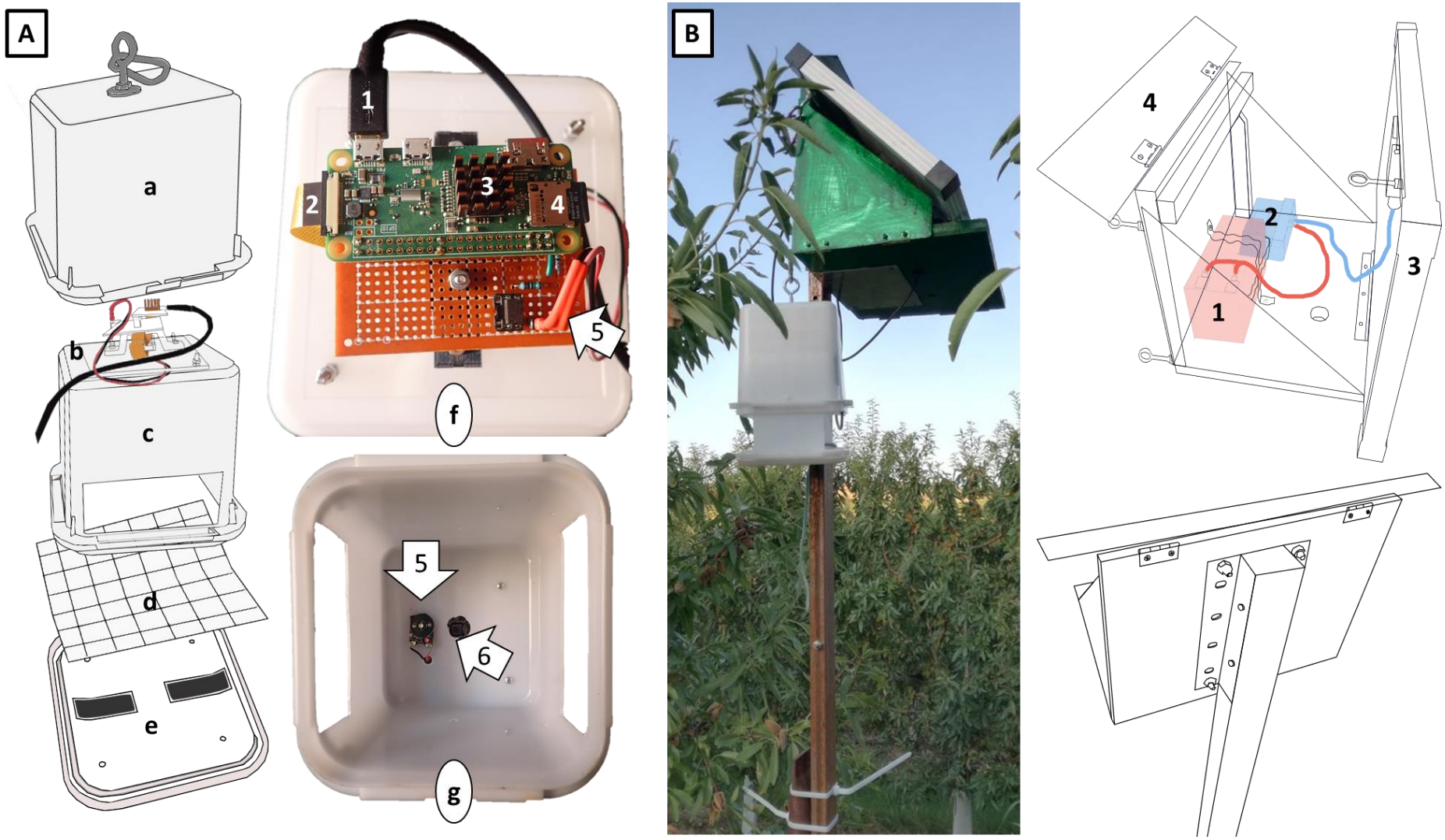

Figure 1.1. Trap and power supply diagrams. The trap-box (A) consists of an outer (a) and an inner (c) plastic box that slide one inside the other to protect the electronic components (b and f) from weather. The lid of the bottom plastic box (e) is lined with an insect sticky card (d) attached with hook-and-loop fasteners for easy replacement. Close up of the electronic components around the Raspberry Pi zero microcomputer (f) showing the charge port (1), camera cable (2), heat disperser (3), micro-SD card (4) and infrared LED and connector (5). Underneath (g) the infrared LED (5) is placed near the IR camera (6). The camera´s angle can be tilted by adjusting the screws of the plastic plate on which it is mounted (7). The power supply unit (B) consists of a wooden and aluminum structure (a) and a metal post to hold the system in place (b and picture on the left). The structure holds a battery (1), a charge controller (2) and the solar panel (3). A lid made from metal sheet (4) provides further protection of the electronic components from weather.

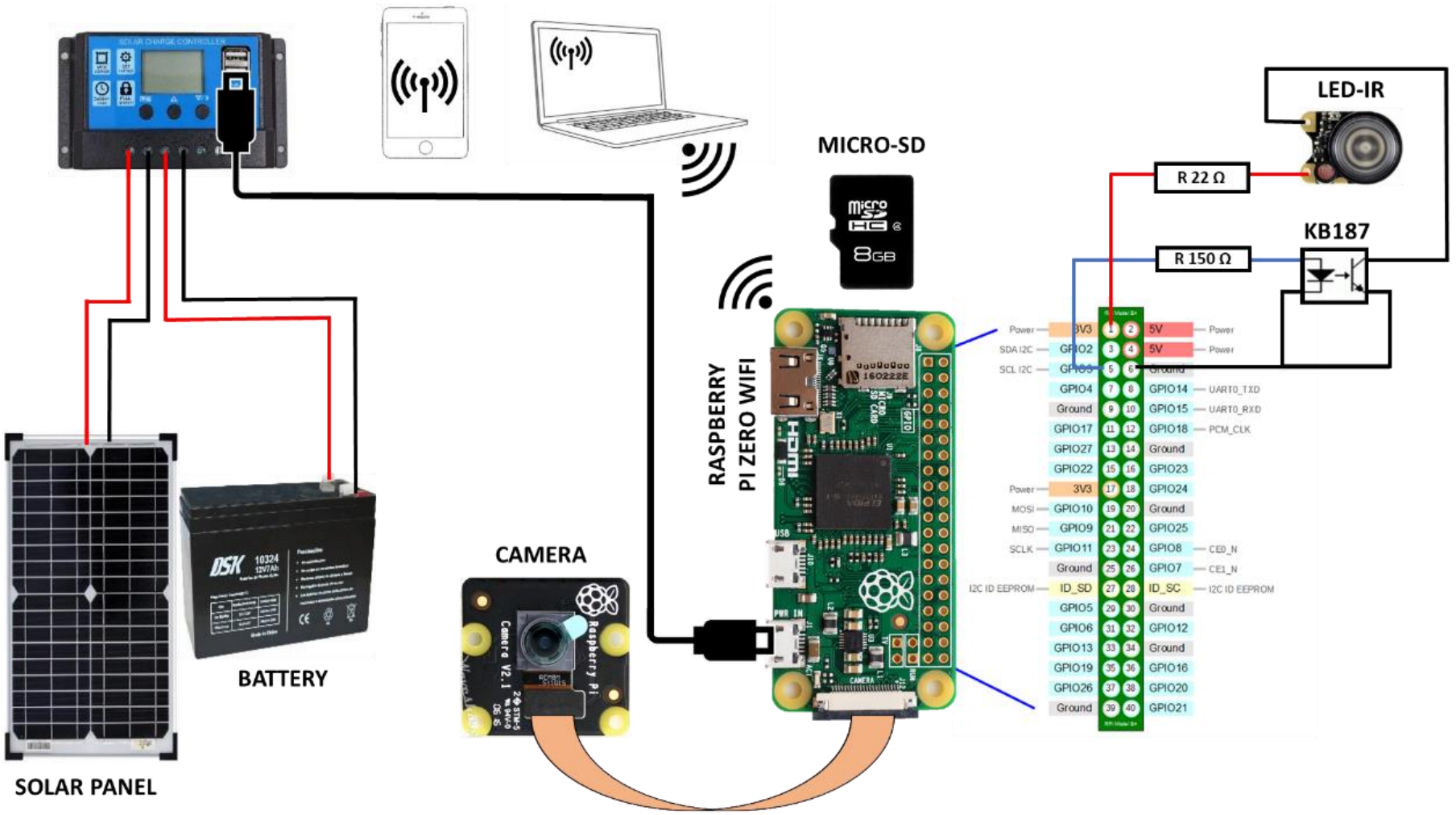

Figure 1.2. Schematic representation of the electronic components of the Raspberry-based automated vision trap. Cable connections are represented with wires and wireless connections are represented with symbols. KB187 refers to the solid-state relay that activates LED onset when ambient light goes below a user stablished level.

Table 1.1. Trap component specifics

Electronic components	
Component (Model, Brand)	**Specifications**
Computer board (Raspberry Pi Zero W, Raspberry Pi, UK)	Broadcom BCM2835 microprocessor, 512MB RAM, VideoCore IV, microSD
Camera (NoIR V2.1, Raspberry Pi, UK)	8 MP, Sony IMX219 sensor, focal length 3.04 mm
Infrared LED (Waveshare Electronics, China)	850 nm wavelength, photosensitive resistor
Micro SD Card (SDC4, Kingston, USA)	8GB
Camera cable (Raspberry Pi Zero cable, Raspberry Pi, UK)	15 x 1.6 x 0.02 cm
USB 2.0 cable (AmazonBasics, USA)	male A to micro B, 1.83 m
Heatsink (RoHS 750-0888 27K/W, ABL Components, UK)	13 x 13.5 x 10 mm, Aluminum
Power unit	
Component (Model, Brand)	**Specifications**
Solar panel (Enjoysolar, Germany)	20W, 51 x 30 x 2.5, 2.4 kg
Charge regulator (JZK, China)	20A, 0.195 kg
Lead Battery (10324, DSK, India)	12V 7A/h, 151 x 65 x 94 cm, 1.95 kg
Plastic box	
Component (Model, Brand)	**Specifications**
Lunch box (GASRONORM, GreatPlastic, Spain)	15 x 14.5 x 15 cm, 1.5L
Sticky cards (Pherocon®, Trecé Incorporated)	White, 8.5 x 18.5 cm
Commercial pheromone lure (rubber septa) [Pherocon® OFM, Lot Number: 84350758, Trecé Incorporated, USA]	186 µg of *Z*8-12:Ac and 11.8 µg of *E*8-12:Ac
Software	
Function (Sofware)	**Version**
Operating system (Linux)	Raspbian GNU/Linux NOOBS 2.3
Image acquisition (Python)	Python 2.7.13
Remote access (RealVNC, Cambridge)	VNC Viewer/VNC Server 6.7.2 (Linux)
Video streaming (VLC, VideoLAN)	3.0.11 Vetinari

area around the sticky card will be captured, which is unnecessary and will reduce image resolution. The angle of the camera's lens axis to the object is also critical and should be kept as perpendicular as possible in order to center the sticky liner in the field of view. To this end, the camera was mounted on a plexyglass platform with 3 bolts that allowed coarse adjustment of the angle (**Fig. 1.1A**). Focus is adjusted manually by turning the camera's lens. The open-source video program VLC enabled real-time video on the computer screen to keep track of the focusing process.

The IR LED is attached to the ceiling of the trap box, beside the camera, (**Fig. 1.1A**). A 1.5-m-long USB cable connects the Raspberry Pi to the power source (5VDC). The ceiling of the top plastic box is fitted with an eye bolt screw to hang the trap box with a carabineer shackle from another eye bolt screwed on the power supply structure or elsewhere. The trap box is tied to the post to prevent it from swinging with the wind.

Field tests

Synthetic pheromone and life-female baits were tested. The synthetic pheromone was a commercial lure (Pherocon® OFM, Lot Number: 84350758, Trecé Incorporated, Adair, Oklahoma, USA) that is the standard bait to monitor adult populations of *G. molesta* in the field. It contains the two main pheromone ingredients components of *G. molesta* (186 µg of Z8-12:Ac and 11.8 µg of E8-12:Ac) loaded on a red-rubber septum. Insects were reared in the laboratory following standard procedures (Navarro-Roldán and Gemeno, 2017). Female pupae were placed outdoors on a windowsill protected from direct sun and rain and they were provided with 10% sugar water drinkers. Emerged females were collected every 1-5 days and placed in groups of 4 in wire cages. These were made from one half of a spherical tea strainer, 1.5 cm diameter, 1.8 cm high and 5.5 mesh screen and were fixed with double-sided tape to a 5.5 diameter Petri dish. A 0.5 mL Eppendorf tube filled with 10% sugar water and fitted with a cotton plug served as a drinker. The female cages were placed directly on the sticky card in the center of the trap box (**Fig. 1.3E**). Male pupae were placed in ventilated 600-ml plastic boxes provided with a 1.5 mL Eppendorf drinker.

The field experiment was carried out between August 15th and October 4th, 2019 in two locations near Lleida (Spain). The first location was a 10-year-old almond orchard in Lleida, Spain (41.675181N, 0.509680E) with 4 m high trees laid on a trellis 1.6 m apart from each other on row distance of 3.5 m. Mating disruption towards *Anarsia lineatella* was implemented in the orchard, but it was not sprayed with insecticides against *G. molesta*. Six traps were fixed to the trellis metal posts, just above the tree canopy (at approximately 3 m heigh), and at

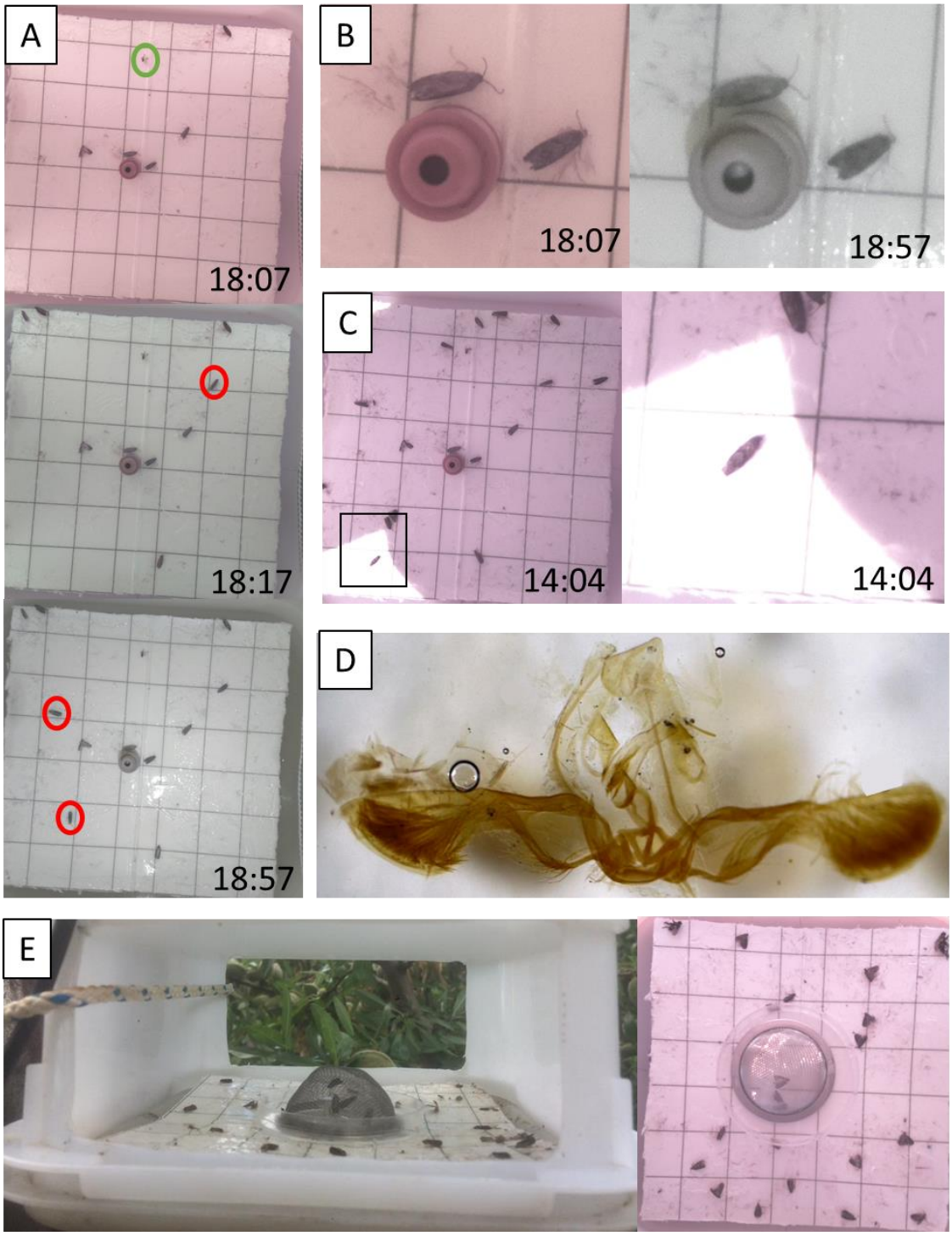

Figure 1.3. A. Three consecutive 10-min interval shots from the IR camera, the first two before the IR LED went off and the last one showing the reflection of the IR LED on the glue card. B. Close-up of the first and last captures from A to illustrate image resolution with daylight and with the IR LED light. C. Effect of sunlight and shade on the resolution of moth pictures over the sticky liner (right is a close-up from left). D. Genitalia of one of the males captured showing. E. Left: View of a trap box baited with live females from the outside. Right: camera picture of a sticky liner with females in the wire cage and males captured on the sticky liner.

least 30 m of distance from each other. In the first sampling period (August 15th to September 9th) the traps were baited with sex pheromone. In the second sampling period (September 23rd to October 4th) two traps were baited with sex pheromone and four were baited with life females.

The second field site was a yard amidst apple and pear orchards and therefore with minimal *G. molesta* populations, in Lleida, Spain (41.613529N, 0.566422E). The metal bar holding the power-supply set was placed directly on the ground beside a wooden fence post to which it was tied with common household rope. Six traps were placed 5 m apart from each other forming a circle. The trap boxes hung from the power supply sets 1.2 m high from the ground. Males were placed in the shade at the center of the trap arrangement, inside a screen cage that prevented predation (mainly from ants) and allowed free exit. Four traps were baited with virgin females and two with synthetic pheromone from September 12thh to 23rd.

Traps were visited every 3-4 days to download the pictures and free SD card space, to replace females, and to change sticky cards as needed. When only septa were being tested, we reduced the visits to every 10 days. Captures were registered manually in each 10-min picture. Occasionally individuals changed position on the sticky card which we could track in most cases (**Fig. 1.3A**). To confirm that the captured moths were *G. molesta*, the genitalia of 30 individuals randomly selected from among all the tests was dissected under the stereomicroscope after 1-h digestion in saturated KOH (Dickler, 1991) (**Fig. 1.3D**).

Results and discussion

Field study

A total of 571 males were captured during the entire experiment, on average 11.3 per trap per week, which is not too different from what is usually captured in delta traps commonly used to sample this species (Kovanci and Walgenbach, 2005; Knight et al., 2011, 2014; Cichon et al., 2013; Özpinar et al., 2014; Kutinkova et al., 2018), but whether our traps are as good as delta traps remains to be tested.

Diel male *G. molesta* captures concentrated between 2 or 3 hours before sunset (i.e., civil twilight) (**Figure 1.4**). The capture period relative to sunset varied slightly depending on whether synthetic pheromone or live females were used as lure or whether feral or laboratory males were sampled (**Figure 1.4**). The small sample size and the variability introduced by sampling at two different locations during a 3-month period prevent performing a statistical comparison, but the distributions of the four groups practically overlapped and all peaked

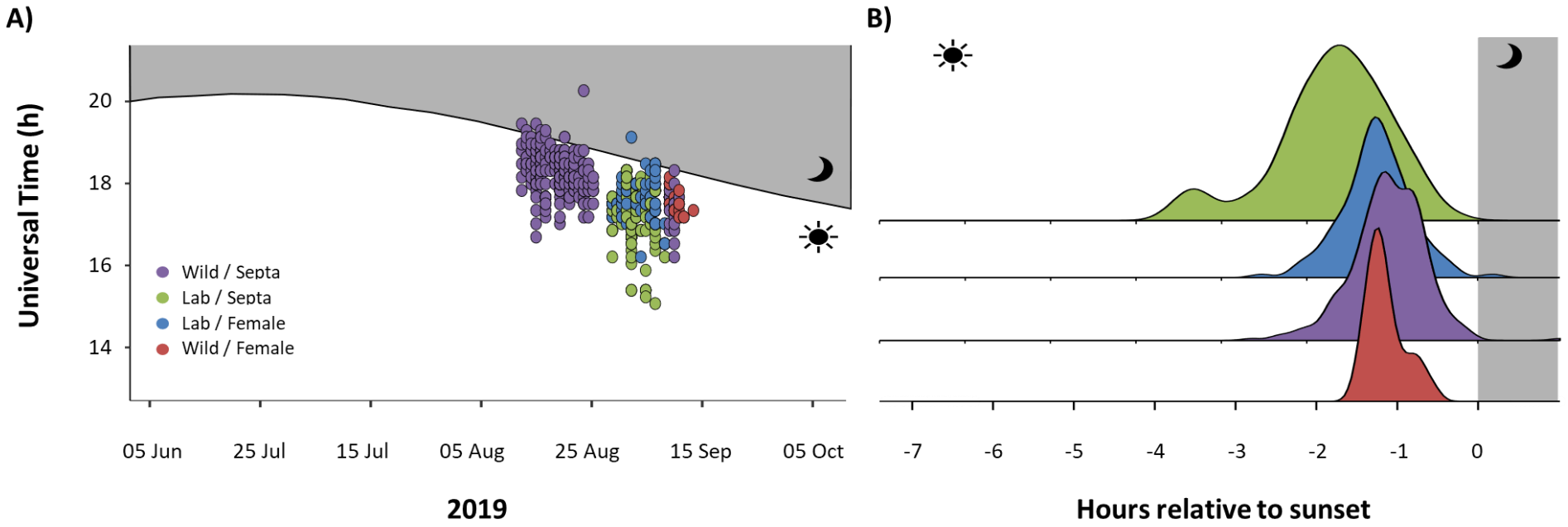

Figure 1.4. Time of male *G. molesta* daily captures relative to sunset between August 15th and October 4th, 2019. B. Kernel density distribution of captures in each treatment relative to sunset time.

within a 1 h period between 1 and 2 h before sunset so there does not appear to be a strong effect of lure type of laboratory versus wild males (**Figure 1.4**). This period of response is similar to that reported in previous studies in Australia, USA and Korea (Rothschild and Minks, 1974; Gentry et al., 1975; Kim et al., 2011).

Environmental factors, mainly temperature, affect the time of sexual activity of both males and females (Batiste et al., 1973b, 1973a; McNeil, 1991). The wide overlap in the period of male capture in pheromone and female traps suggests that this effect is similar in both sexes. In addition, overlap in the period of capture between laboratory and wild males also indicates that a long-term (>5 years) laboratory colony maintained with introduction of feral individuals maintains retains the reproductive potential of wild populations. These data could help producers choose an optimal time window to release pheromone from automated dispensers. However, an extended study throughout the entire flight season would be needed to provide a more accurate picture, since the flight period relative to sunset varies somewhat through the seasons (Kim et al., 2011).

Trap performance

The aim of this study was to build and test high-sample-resolution, durable and affordable automated traps to outline insect daily periods throughout the season. At a price of around 150 euros each, our traps are cheap, accessible and perform their task uninterruptedly. Considering our results, we believe this trap to be a tool of interest specially for research, especially when higher time resolution than that provided by commercial traps is required. Here we discuss its strengths and limitations and provide suggestions to make up for the latter. Our traps managed to record insect activity every 10 minutes for 2 months. In order to preserve the batteries, it is important to fully charge them in the electric grid before taking them to the field, both when new and after prolonged storage, and orient the solar panel southward unobstructed from leaves and branches. While this can be easily achieved by one person they are placed on the ground, it requires two people to place the 10kg traps high on the tree canopy. Future designs should consider making the traps more manageable, for example by redistributing the heavier power system components (solar panel and battery).

Once installed, the traps resisted standard weather, including relatively heavy rains and strong winds. After completing the tests, nonetheless, we noticed that the plastic boxes had degraded in various places, making some of them non-reusable. We attribute this degradation to the UV radiation from the sun. Replacing the boxes would involve reinstalling the electronic components and adjusting the camera position. Using UV-resistant plastic or painting the

boxes with UV-resistant paint would help decrease deterioration through weather and prolong the longevity of the trap.

After the initial configuration of the Raspberry Pi, wireless connection with the trap is easy using a smart phone as hotspot. After one week of capturing at 10 minutes intervals the micro-SD memory card was almost full, and the traps had to be visited at least every 10 days to download and posteriorly erase the images to liberate card space. A larger capacity SD card would lengthen download time, which is at 10-20 minutes/1 week capturing at 10 min intervals. To avoid these long waiting periods in the field, traps could be implemented with a GPRS system that would make the trap accessible via internet from any computer, but this would increase the price of the traps. Moreover, visits should still be needed during population peaks to change the sticky papers.

The Raspberry Pi can be fitted with sensors that would provide useful meteorological information for a minor cost and without a compromise in card space. Although it could capture pictures at video speed (25 frames · s^{-1}) the SD memory card would fill very quickly, but it could be used for a several weeks if set to capture at 1 h or lower time intervals, making it a useful tool for laboratory tests as well.

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CHAPTER II. ORIENTAL FRUIT MOTH: DIEL PERIODICITY OF CATCH IN SYNTHETIC SEX ATTRACTANT VS. FEMALE-BAITED TRAPS

Abstract

Knowing the diel sexual activity period of moths can optimize the use of active pheromone dispensers in mating disruption. Diel activity periods are usually estimated using traps baited with synthetic pheromone lures. However, the temporal pattern of male response to synthetic pheromone and to live females does not necessarily overlap. Here we report diel and seasonal patterns of *Grapholita molesta* male sexual activity based on captures in traps baited with synthetic pheromone or virgin females. Six automated pest monitoring systems located in an almond field took pictures every 10 minutes throughout the 2020 flight period (February-October). Two traps were baited with a synthetic sex pheromone and four traps were baited with live females from a laboratory colony. Traps were visited twice a week to download pictures and replace females. Five flights were predicted according to a standard degree-days model. The mean diel activity period of males relative to sunset was similar for synthetic pheromone traps and traps baited with live females, with 95% of the captures occurring between 2.5 h before to 30 min after civil twilight. However, the period of activity occurred slightly earlier relative to sunset in the spring than in the summer or fall flights. Regression analysis indicates that this effect may be accounted for by lower spring temperatures preceding the daily flight. The diel flight curve was asymmetric as it raised slowly towards the scotophase and ended more abruptly at around the time of sunset. To reduce the cost of mating disruption, sprayers should be adjusted throughout the season following the curve of activity of the insects, which may vary in different latitudes and according to local temperatures.

Introduction

Mating disruption (MD) has been traditionally implemented in the control many moth species by manually installing hundreds of passive dispensers per hectare that emit synthetic pheromone constantly throughout the day (Witzgall et al., 2010; Miller and Gut, 2015). However, this is a highly time-consuming activity, and much pheromone is wasted during the period when insects are not sexually active, which is most of the daytime. The growing demand for more cost-effective techniques has resulted in the development of automated sprayers in the 1990s (Benelli et al., 2019). These devices release large quantities of pheromone from few point sources and can be programmed to do so during the time when the insects are active. Knowing the daily flight period of male moths is thus crucial to program the time intervals at which the automated dispensers should release synthetic pheromones.

Moths, like most animals and plants, show specific daily rhythms in many of their behaviors, which are run by an internal clock which is entrained to the actual 24-h light-dark period, and can be further regulated by temperature (Danks, 2005). Although it is clear that the circadian regulation of male moth response to sex pheromone complements circadian regulation of female pheromone biosynthesis and calling behavior (Groot, 2014; Allison and Cardé, 2016), most studies regarding male sexual activity in the field rely on synthetic pheromone lures and not on live females (e.g.: Päts and Wiktelius, 1992; Quiring, 1994; Cardé et al., 1996; Ivanova et al., 2010; Ünlü et al., 2019). Since the release of the synthetic pheromone by passive emitters is constant whereas female release it at discrete diel periods, the periodicity of male response to pheromone as well as the effecto of temperature on the periodicity of male flight may be different to each of the two sources (Shorey, 1966; Fatzinger and Asher, 1971; Haynes and Birch, 1984; Giebultowicz et al., 1992; Zhang et al., 1998; Stevenson and Harris, 2009). The differences in trap catches of synthetic lures versus female lures illustrate the necessity to determine both, the rhythms of male attraction and female attractiveness.

The Oriental fruit moth (OFM), *Grapholita molesta* (Busk), is a widely distributed tortricid moth that attacks stone (*Prunus* spp.) and pome (*Malus, Cydonia* and *Pyrus* spp.) fruit trees (Myers et al., 2007; Piñero and Dorn, 2009; Najar-Rodriguez et al., 2013), causing important economic loses worldwide (Kirk et al., 2013; Knight et al., 2015). Although sprayable microencapsulated sex pheromone formulations have been tested in the field, control of this species by MD is based in hand-applied dispensers (K. Kim et al., 2018; Preti et al., 2020) Under favorable conditions, *G. molesta* undergoes up to six generations per year, each developing under

distinct environmental conditions. Even though it has been reported that in the laboratory *G. molesta* females call during the hours previous to the scotophase (Stelinski et al., 2014; Navarro-Roldán and Gemeno, 2017), and that males fly in the last hours of the photophase (Rothschild and Minks, 1974; Gentry et al., 1975; Y. Kim et al., 2011), precise field data including the effect of environmental factors on flight and daily activity on *G. molesta* are scarce, and no comparison of attraction between live females and synthetic lure has been conducted. Understanding the effect of these variables on male flight would help to adjust the timing of pheromone released by the automated pheromone dispensers. The aim of the present study is to compare the diel periodicity of captures of *G. molesta* males in traps lured with synthetic pheromone or live females. We used a camera-assisted pheromone trap that took pictures every 10 min, 24 h a day throughout the flight season (Chapter I).

Materials and methods

Lures

The synthetic pheromone that we used is a standard bait to monitor adult populations of *G. molesta* in the field (Pherocon® OFM, Lot Number: 84350758, Trecé Incorporated, Adair, Oklahoma, USA). It contains the two main pheromone components of *G. molesta* (186 µg of *Z*8-12:Ac and 11.8 µg of *E*8-12:Ac) loaded on a red-rubber septum. Unmated females were obtained from a laboratory colony reared on artificial diet following standard procedures (Navarro-Roldán and Gemeno, 2017). Female pupae were placed outdoors on a windowsill protected from direct sun and rain. Emerged adults were collected every 1 to 5-days and during that time they were provided with 10% sugar water drinkers. Female cages were made with one half of a tea infuser 4.5 cm diameter x 1.8 cm high fabricated with stainless-steel mesh screen. The strainer was fixed with double-sided tape on the bottom side of a 5.5-cm diameter plastic Petri dish. Four 1 to 5-day-old females were introduced in the cage and initially they were provided with a 1.5 µl Eppendorf tube filled with 10% sugar water and a cotton plug, but this drinker dried out during the hot summer days. For this reason, from July 17th the Eppendorf drinker was replaced by a 5.5-cm diameter Petri dish filled with 10% sugar water sealed with Parafilm®. The tea strained was fixed to the Petri dish and females drank from a piece of microfiber dish cloth inserted through a hole practiced on the lid of the dish. Female cages and rubber septa were placed directly on the sticky card in the center of the trap box.

Field experiment

The field experiment was carried out between February 28th and October 20th, 2020 in a 11-year-old almond orchard in northeast Spain (41.675181N, 0.509680E). The orchard consisted of 4-m-high trees laid on a trellis, ca. 160 apart from each other on rows 350 cm aside, and, although it was not sprayed with insecticides for *G. molesta*, mating disruption towards *Anarsia lineatella* was implemented. Seasonal and daily flight were recorded by six automated pest monitoring systems developed at the University of Lleida (Chapter I). Each trap consisted of a Raspberry Pi computer board and an infrared camera operated with open-source software (Linux and Python), all mounted on a plastic lunch box (15 x 14.5 x 15 cm) and powered with a solar panel and rechargeable battery. A sticky card (8.5 x 18.5 cm, Pherocon®, Trecé Incorporated) was placed at the bottom of the trap to capture males. The set was mounted on a solid and weather-proof structure made with cheap hardware materials. Pictures were taken every 10 min, 24 h a day for the entire flight season. Pictures were downloaded on a laptop through wireless connection. Traps were fixed to the trellis metal posts at approximately 3m height within and above the tree canopy, and at least 20-m apart from each other. On February 28th two sentinel traps baited with synthetic pheromone were placed in the field to indicate first captures. The first male was captured on February 29th. The remaining four traps baited with live females were placed in the field on March 11th, and this arrangement (2 synthetic lure and 4 live female traps) was maintained until the end of the experiment. Occasionally some traps did not capture images between the weekly visits, but there was at least one septum and one female trap at all times. Traps had to be removed for one day during pruning (May 27th) and for 17 days during harvest (September 14th-30th). The last capture occurred a few days later (October 7th) and the traps were removed on October 20th.

Traps were visited every 3-4 days to download the pictures and to replace females, and also to change sticky cards if needed. To confirm that the captured moths were *G. molesta*, the genitalia of 100 randomly individuals was dissected under the stereomicroscope after 72 h digestion in saturated room temperature KOH. Hourly mean temperatures were obtained from a weather stations located near the experimental field site (RuralCat, 2021). Statistical analyses were performed with R software (R Core Team, 2020).

Results

The two traps baited with synthetic pheromone captured significantly more males per trap per week [1864 total, 7.60 ± 0.9 (mean ± SEM)] than the 4 traps baited with live females [2216 total, 7.96 ± 0.6 (mean ± SEM)] ($df = 1$, $F = 5.68$, $p = 0.018$).

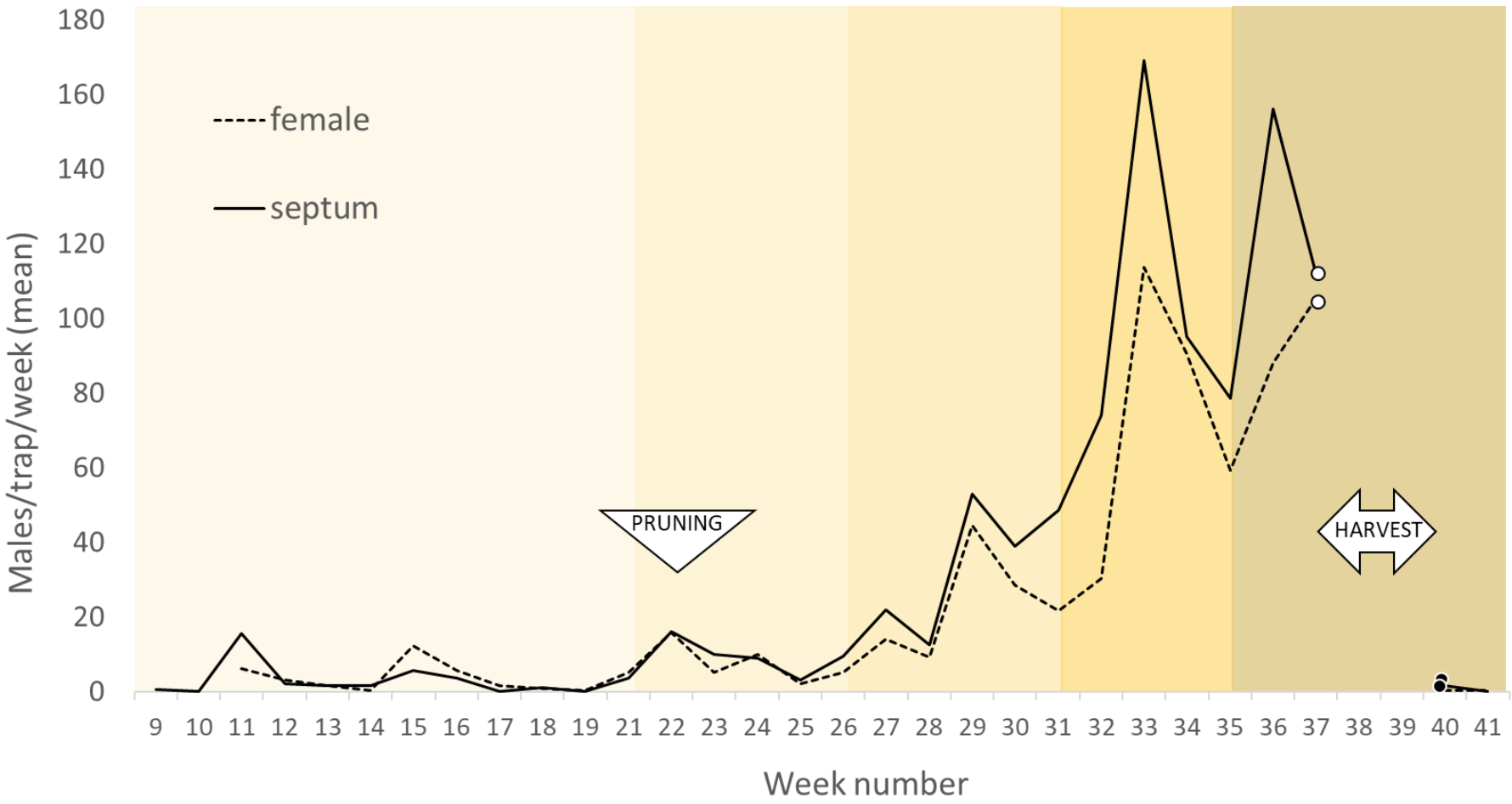

Figure 2.1. Captures of *G. molesta* males in traps baited with life females or synthetic pheromone septum. Different shadows in the background represent the flight periods predicted with a degree-days phenological model.

A degree days (DD) phenological model was used to predict the flights (Croft et al., 1980). Degree-days were calculated using the single sine method (Zalom et al., 1983). The lower threshold was set at 7.2ºC, a horizontal cut was applied when temperatures exceeded the upper threshold (32.2ºC), and biofix was set at January 1st. The model predicted first moth captures at 126.1ºC DD and a generation time of 535 DD. Using mean daily temperature from a local weather station the model predicted five flights starting on February 29th, May 19th, June 28th, July 29th, and August 27th, and the last flight was predicted to end in October 16th. In the first predicted flight there were very few captures, the second and third predicted flights consisted of slightly larger peaks, and the fourth and fifth predicted flights consisted of two very large peaks (**Fig. 2.1**).

When daily captures are plotted against hour of the day it is apparent that most of the captures occur prior or close to the civil twilight irrespective of the duration of the light and dark phases of the day, which change with the season (**Figure 2.2**). Therefore, male and female *G. molesta* appear to adjust their sexual activity period relative to sunset. Two second-order polynomial equations were fitted individually to the captures on traps baited with synthetic pheromone and live females. The two curves had similar estimated model parameters (slope, and first and second order constants) and practically overlapped for most of the season (**Figure 2.2**; **Table S2.1**). To determine if lure type (female or septum) had an effect on the time of capture, a simpler model testing the effect of date on time of capture including all the data was compared with a more complex model including lure as an interaction factor (**Table S2.1**). The comparison was significant (df =3, F = 5.28, P = 0.001) but none of the interaction terms of the more complex model was significant, which indicates that lure had no effect on the time of capture. The time of capture relative to sunset time varied throughout the year. During the spring (28th February – 31st May), early summer (1st – 30th June) and late summer (1st July- 20th October) the peak kernel distribution of captures peaked at 2:02, 1:10 and 0:45 h before civil twilight, respectively (**Figure 2.3**).

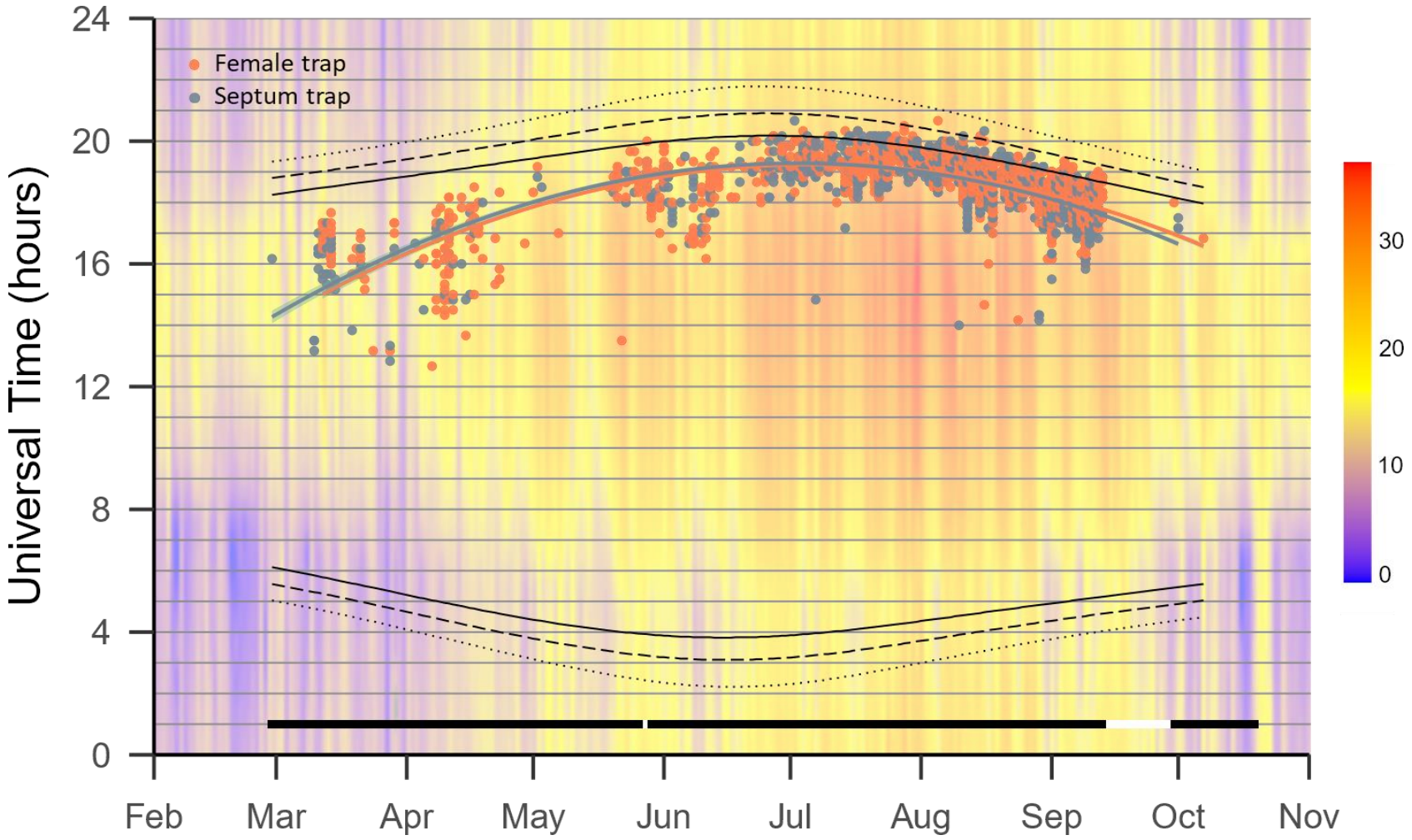

Figure 2.2. Daily captures of *G. molesta* males in traps baited with live females or synthetic sex pheromone. The lines represent second order polynomial regression fitted for each type of lure ± 95% C.I. (**Table S2.1**). Background colors represent mean hourly temperature as indicated in the scale. Bottom bar indicates periods sampling (black) and periods when traps were removed (white). Onset of civil, nautical and astronomical twilights are indicated by solid, dashed and pointed lines, respectively.

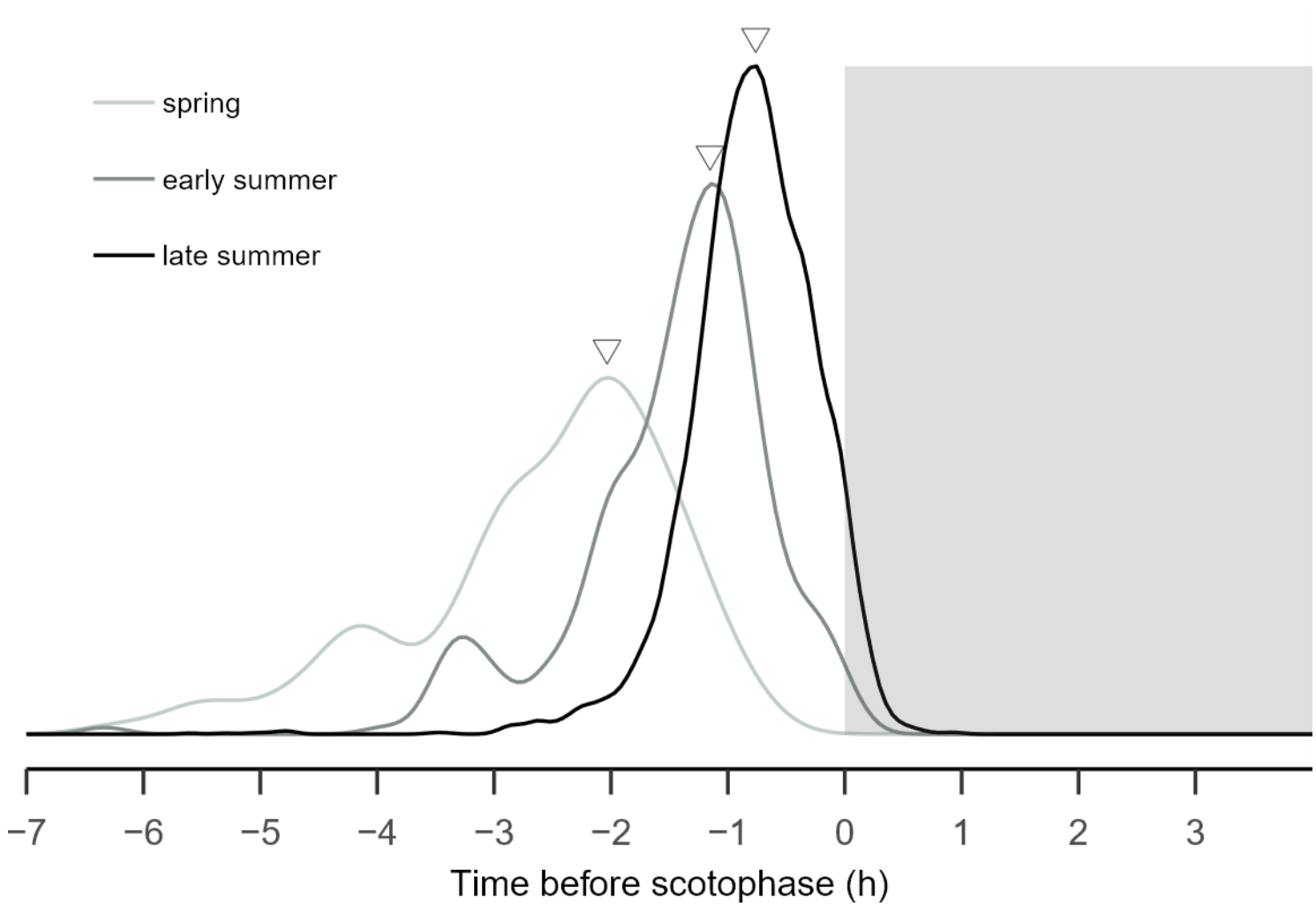

Figure 2.3. Kernel density distribution of the time of capture of *G. molesta* males relative to the civil twilight (i.e., scotophase, grey shadowed area). The combined captures of traps baited with synthetic pheromone and live females are divided in three periods: spring (28th February – 31st May), early summer (1st – 30th June) and late summer (1st July- 20th October). Vertical lines indicate the highest point of each distribution.

Regression analysis suggests that the time of flight relative to sunset is related to ambient temperatures before the diel flight period (**Fig. 2.4**). This means that, when temperatures are low, males tend to fly earlier in the day, while high temperatures often delay flight during the latter half of the season. No male was captured at temperatures below 13°C or over 34°C in any generation.

Discussion

The economic impact of *G. molesta* in almond is usually negligible, so there are relatively few studies of *G. molesta* in this crop (Rothschild and Vickers, 1991; Zalom, 1994). Our experimental field site was surrounded by very extensive grapevine plantations, in an semi-arid area tens of kilometers away from the nearest peach and apple orchards. It is then likely that the insects captured in our study bred mainly in the almond orchard itself. The seasonal flight curve of *G. molesta* in this almond plantation agreed with the flight periods predicted by a DD model tested in peach and apple orchards in California (Croft et al., 1980). The seasonality of *G. molesta* in the almond orchard was also similar to flight curves reported in peach and apple orchards in other locations (Y. Kim et al., 2011; Zhai et al., 2019).

The diel timing of *G. molesta* male captures in traps baited with synthetic pheromones has generally demonstrated a late afternoon or crepuscular pattern with higher levels of activity just a few hours before dusk, and extending to shortly after dark (Rothschild and Minks, 1974; Gentry et al., 1975; Y. Kim et al., 2011). Our observations using camera traps confirm these results, and as in the other studies the circadian rhythmicity of daily flight varied throughout the year following the annual oscillation in sunset time. Insects, like most other organisms, have internal clocks that regulate their diel (circadian) and seasonal (photoperiodism) activity periods (Danks, 2005; Saunders, 2014). The internally generated circadian clock is relatively persistent under continuous light and dark conditions and adapts to the gradual changes in duration of the light and dark phases of the day that occur throughout the year as the earth axis tilts on its translation around the sun (Dolezel, 2015). Photoreceptors detect changes in light intensity and allow measurement of daylength so that the clock is synchronized to the actual light-dark cycle (Tomioka and Matsumoto, 2010). Insects can also measure the speed of transition between day and night to adjust their activity (Dreisig, 1980). The calling period of many moth species has been characterized in order to sample sex pheromone for chemical and behavioral analyses (Groot, 2014). This periodicity is often recorded indoors but it may be different outdoors where temperature and other environmental variables oscillate daily and seasonally.

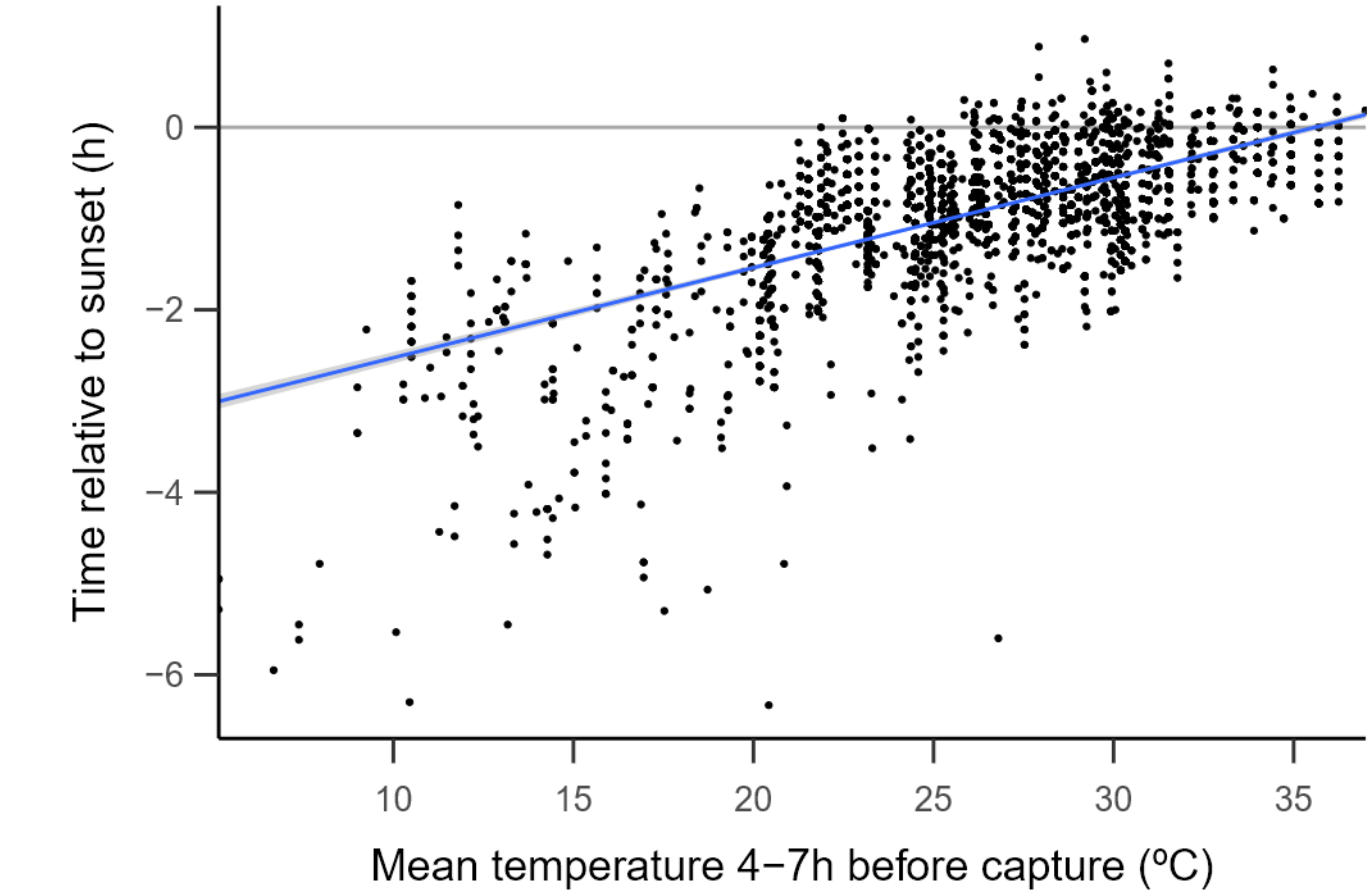

Figure 2.4. Male *G. molesta* time of capture relative to the temperatures experienced by each individual 4 to 7 hours before flying. Grey line indicates time of sunset.

G. molesta males preferentially responded to sex pheromone and female-baited traps during a relatively narrow time period that adjusted closely to the time of sunset. However, there was a significant advance from sunset time during the spring flight. Several crepuscular and nocturnal moth species' activities can occur earlier under cold temperatures than warmer ones, presumably to minimize the energetic costs associated with being active under cooler temperatures (Cardé et al., 1975; Comeau et al., 1976; McNeil, 1991). In our experiment, male OFM flight was restricted to temperatures between 13 and 34°C. Indeed, OFM female calling is restricted to 15-32°C, and temperatures experienced the previous calling period have been demonstrated to alter current calling behaviour (Baker and Cardé, 1979). Our results indicate that the temperatures experienced some hours before flight onset often partially and sometimes completely, limited male daily activity. The lower temperatures experienced by the males of *G. molesta* during spring seem to account for the unexpected shifts in flight relative to sunset, as it appears to be the case for the tortricid moths *Cydia pomonella* (Batiste et al., 1973a) and *Lobesia botrana* (Lucchi et al., 2018). Likewise, the high temperatures registered in summer favored the accumulation of captures in the last hour of light before sunset.

That male *G. molesta* captures occurred earlier relative to sunset in the spring than in the summer months in both synthetic pheromone- and female-baited traps suggests that whatever factor caused this gradual shift (probably temperature as we have shown), it affected females and males in a similar way. If it would have modified only one sex, or each of them in opposite ways (e.g., delaying males and advancing females, or the other way around), then the capture period of the two trap bait types would have been different, which was not the case. This is to be expected because photoperiodically regulated rhythms serve as cues for individuals of the same species to adopt similar reactions to light, temperature and other environmental factors, and by doing so, the probability of contact between sexes is increased (Beck, 2012), while reducing the chances of mating with closely related species that have similar pheromone blends (Haynes and Birch, 1986; Byers, 2006; Groot, 2014).

In the laboratory, male moths can respond to the pheromone before and after female calling period (Shorey, 1966; Furlong et al., 1995), although most field studies indicate that, under natural conditions, males respond similarly to synthetic and natural sources of pheromone (Batiste et al., 1973b; Cardé et al., 1974; Klun et al., 1981; Zhang et al., 1998), as we have reported for *G. molesta*. Although we cannot know for certain if females of *G. molesta* were active before or after males were captured, a close observation of female position in the wire cages suggests that males began to fall in female baited traps slightly after females started to walk around (which is an indication that they are starting to release pheromone) and stopped

shortly before they became quiescent again. The registered period of activity towards pheromone septa represents the time at which males are programed to respond. The fact that there exists no clear difference in responsiveness towards this lure and virgin females indicates that females are calling at the time males would expect them to, i.e. they are synchronized.

The quality of individuals from long-term insect colonies and their suitability to represent wild populations is sometimes questioned (Teixeira et al., 2016). The marked agreement in the catches of male wild *G. molesta* in traps baited with sex pheromone and traps baited with laboratory-reared females suggests that the laboratory colony maintains the sexual periodicity of the wild population. Similarly, the calling period of laboratory females under laboratory conditions (Navarro-Roldán and Gemeno, 2017) peaked at about the same time relative to sunset than the peak of captures in female-baited traps (**Fig. S2.1**). So even in the absence of a gradual onset of light and darkness through twilight, as happens in nature, laboratory females show a period of sexual activity that is very similar to the activity period of wild males. In *Drosophila melanogaster* the periodicity of locomotor activity is dramatically affected by the transition between light and dark (Vanin et al., 2012). When this transition is sharp, as in most laboratory experiments, there are two peaks of activity, but when the natural sunrise and sunset periods are simulated in the laboratory, a completely different pattern of activity arises (Vanin et al., 2012). Our laboratory population of *G. molesta* - which has been kept under laboratory conditions for over a decade without the introduction of wild individuals - has retained the ability to rapidly adapt to natural cues. Indeed, a cross-attraction test between laboratory and field individuals showed similar levels of attraction (Knight et al., 2014). Hence, the use of laboratory reared individuals for future experiments in the field should serve as a reliable means to broaden our understanding of insect biology.

Programming dispensers to adjust pheromone release to the shifts in sunset as the year advances would help save pheromone and increase the efficacy of mating disruption against OFM. As exposed, mating disruption is a complex phenomenon involving multiple sciences like physics, chemistry, meteorology, biochemistry, physiology and behaviour. From an applied point of view, the findings presented here can be useful to optimize MD technique, adjusting time intervals to include the effects of environmental variables like time of sunset and temperatures on OFM biology. This aerosol formulations can provide a cost-effective alternative to hand-applied dispensers in the control of this and other species in the close future.

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Table S 2.1. Summary parameters of second order polynomial equations fitting daily captures of *G. molesta* males relative to sunset time. A) Captures in traps baited with live females. B) Captures in traps baited with rubber septum loaded with synthetic sex pheromone. C) Captures in both types of traps. D) As in D but with lure as interaction term. Notice how none of the interaction parameters in D are significant, suggesting that lure did not have an effect on time of capture. A and B predicted values shown in **Figure 2.2**.

A. Females; $y = a + b*x + c*x2$				
Parameter	Estimate	Std. Error	t value	Pr(>|t|)
a	8.17	$2.07*10^{-1}$	39.56	<0.0001
b	0.17	$2.34*10^{-3}$	50.04	<0.0001
c	$-3.11*10^{-4}$	$6.38*10^{-6}$	-48.71	<0.0001
R2 = 0.64, df = 2, 2249, F = 1267, P < 0.0001				
B. Septa; $y = a + b*x + c*x2$				
Parameter	Estimate	Std. Error	t value	Pr(>|t|)
a	8.28	$2.23*10^{-1}$	37.03	<0.0001
b	0.19	$2.47*10^{-3}$	48.11	<0.0001
c	$-3.22*10^{-4}$	$6.69*10^{-6}$	-48.15	<0.0001
R2 = 0.55, df = 2, 1873, F = 1166, P < 0.0001				
C. Females and septa, no interaction; $y = a + b*x + c*x2$				
Parameter	Estimate	Std. Error	t value	Pr(>|t|)
a	8.18	$1.52*10^{-1}$	53.87	<0.0001
b	0.18	$1.71*10^{-3}$	69.25	<0.0001
c	$-3.16*10^{-4}$	$4.64*10^{-6}$	-68.20	<0.0001
R2 = 0.54, df = 2, 4125, F = 2399, P < 0.0001				
D. Females and septa, with interaction; $y = (a + b*x + c*x^2) * lure$				
Parameter	Estimate	Std. Error	t value	Pr(>|t|)
a	8.17	$2.03*10^{-1}$	40.24	<0.0001
b	0.17	$2.30*10^{-3}$	50.91	<0.0001
c	$-3.11*10^{-4}$	$6.27*10^{-6}$	-49.56	<0.0001
lure	0.10	0.30	0.34	0.732
b*lure	$1.75*10^{-1}$	$3.42*10^{-3}$	0.51	0.609
c*lure	$-1.12*10^{-5}$	$9.28*10^{-6}$	-1.21	0.226
R2 = 0.63, df = 5, 4122, F = 973.3, P < 0.0001				

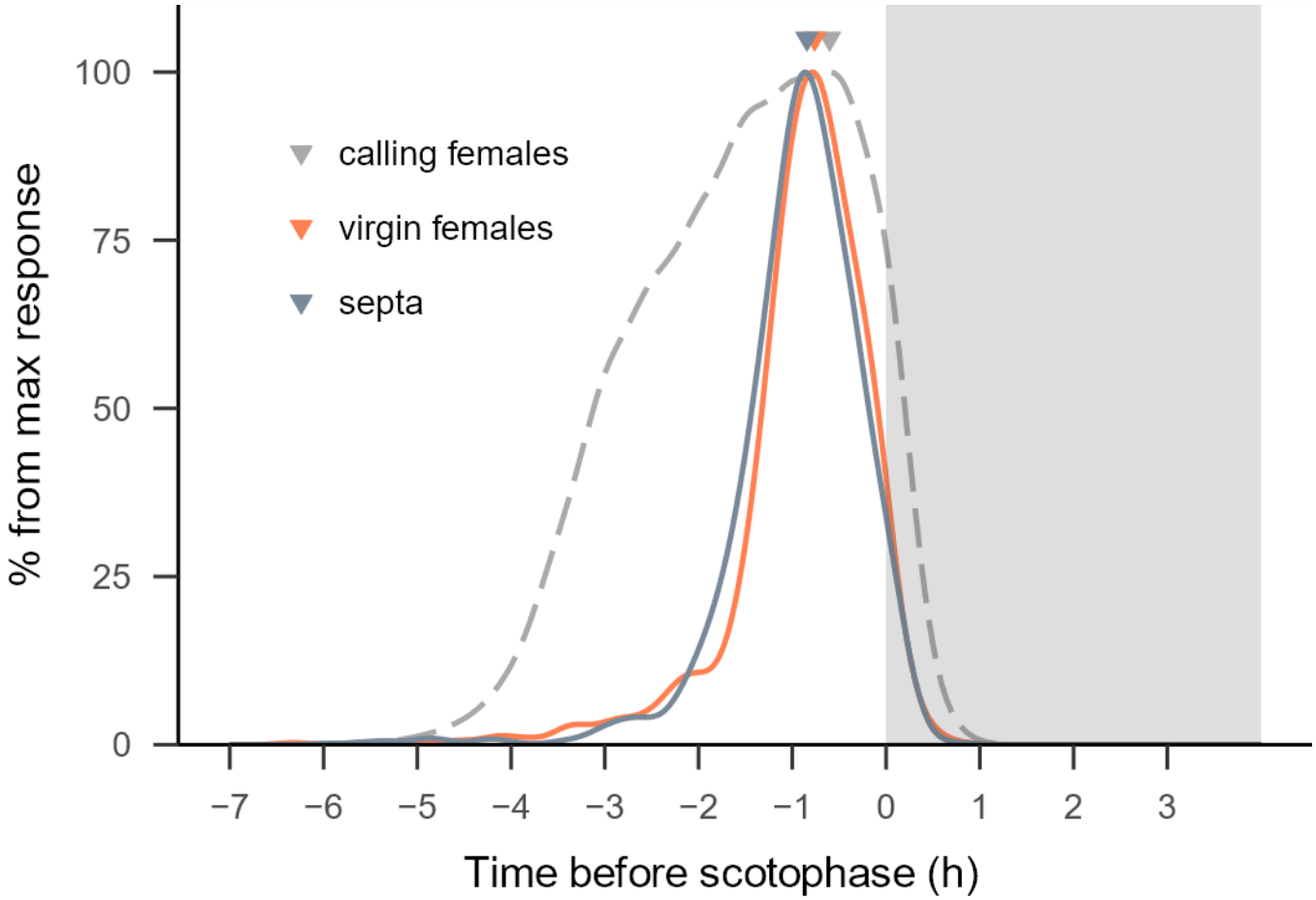

Figure S 2.1. Male flight (solid) towards synthetic pheromone (blue) and virgin females (red), and female calling (dashed) of *Grapholita molesta* relative to sunset. Mean peaks for each curve are indicated by an arrowhead. Shadowed area represents scotophase.

CHAPTER III. THE ANTENNA OF *GRAPHOLITA MOLESTA* FEMALES IS AN INNEFFICIENT SEX PHEROMONE DETECTOR

Abstract

The olfactory sensory system of male moths is highly specialized in detecting the female-produced sex pheromone to find potential mates, while females rely on plant volatiles to locate host plants for oviposition. While there is substantial behavioral evidence that female moths respond to their own sex pheromone, the evidence for sex pheromone "autodetection" at the olfactory receptor neuron (ORN) level is very limited. We compared the responses of ORNs housed in antennal sensilla trichodea, where males have sex pheromone sensitive cells, in both sexes of *Grapholita molesta*, a species with reported pheromone autodetection. Two concentrations of the major (*Z*8-12:Ac) and minor (*E*8-12:Ac) sex pheromone compounds selected from dose-response curves, a 3-compound plant blend, and the male-produced hairpencil courtship pheromone, ethyl trans-cinnamate, were used to evaluate the differential specificity and sensitivity of 45 male and 305 female ORNs. Hierarchical cluster analysis (HCA) indicated radically different peripheric olfactory systems between sexes that could be related to their specific ecological roles. In males no cells responded to their own courtship pheromone, but 4% of the male cells were tuned to the plant volatile blend. Most male cells (63%) were tuned specifically to the major or minor pheromone compounds and their relative abundance was similar to their ratio in the female pheromone blend. Plant volatile cells were relatively frequent (6%) in females, and 3% of the female ORNs were specifically tuned to the male-produced courtship pheromone. Several female cells were excited by female-produced sex pheromone, but their responses were generally lower than in males, and they responded broadly to other compounds as well, so the HCA grouped them in a large cluster (90%) of "unspecific" female neurons. The lack of differential pheromone receptor neurons in females, and their relatively low sensitivity to pheromone, would not allow females to detect variations in the pheromone ratios in nature. Thus, the alteration of their behavior after exposure to conspecific pheromone under laboratory conditions does not appear to be species-specific.

Introduction

Female moths produce a mixture of related fatty acid derivatives that is liberated from their protruded abdomen tip during the calling period and serves as an attractant for males of the same species. Although some species use only one pheromone compound to communicate, they generally synthetize a more complex blend that includes major behavior inducing compounds and minor compounds that, in the proper amount, can increase the attraction of males to the source (Allison & Cardé, 2016a). Female moth pheromones are of variable nature, and slight differences in carbon chain length, double bond location and isomer blend favor discrimination not only between species, but among populations or strains of the same species (Dekker & Kárpáti, 2020). The European corn borer (ECB) *Ostrinia nubilalis*, for instance, exists as two separate sex pheromone races. ECB(Z) females produce a 97:3 blend of *Z*11- and *E*11-tetradecenyl acetate, whereas ECB(E) females produce an opposite 1:99 ratio of the Z and E isomers. Males of each race respond specifically to their conspecific female's blend (Lassance, 2016; Dekker & Kárpáti, 2020).

We are therefore presented with a highly specific communication system, where the qualitative nature of the compounds is entwined with a quantitative proportion of each of the components of the pheromone blend. Male moths rely on the precise blend of species-specific compounds to locate and arrive at potential mates from relatively large distances by flight, and they have evolved an olfactory system that detects pheromone compounds with great sensitivity and specificity over a wide range of concentrations (Baker et al., 2012; Kaissling, 2019). Males present long trichoid sensilla along the antennae that house olfactory neurons specifically tuned to components of the female pheromone blend. Pheromone molecules attach to the surface of the sensilla and enter the lumen through their porous membrane. There, they are transported by pheromone binding proteins to the odorant receptors (ORs) present in the dendrites of the neurons. When the correct compound is detected and certain concentration thresholds are surpassed, the cells can be induced into a response (Leal, 2016; Kaissling, 2019).

Olfactory receptor neurons are generally tuned to one or a few biologically significant compounds to which they respond strongly, but they will also respond to several other compounds if present at sufficiently high concentrations (Si et al., 2019). Moth pheromone receptors are at an extreme of the specificity spectrum, since they generally respond maximally to just one pheromone compound at the concentration that they encounter it in the wild, but they will also respond to other pheromone compounds when stimulated with

abnormally high concentrations not normally occurring in nature. The composition of a pheromone blend is then represented across the existing types of specialist receptor cells on the antennae (Dekker & Kárpáti, 2020). The distribution of pheromone receptor neurons (PRN) on the male moth antenna follows two distinct patterns. In many species each pheromone receptor neuron is housed in a different sensillum trichodeum. In some other species the different PRNs, usually just 2, share the same sensillum. In the first case, the proportion of PRNs of major and minor compounds corresponds to the proportion of these compounds in the pheromone blend, whereas in the second case the different PRNs types occur in similar numbers on the antenna regardless of their proportion in the pheromone blend. In addition, when to PRNs cooccur in the same sensillum, the one tuned to the major pheromone compound usually has larger dendrite surface. These two arrangements appear to confer males with an efficient mechanism to continuously track the chemical identity of the pheromone plume along the widely variable concentration range they experience when flying towards females (Baker et al., 2012). The axons of the PRNs arborize in the antennal lobe, where glomeruli organize in relation to function, a bigger glomerulus indicating a higher number or PRN inputs or larger dendrite diameters. In males, a specific structure called the macroglomerular complex usually consists of a large glomerulus receiving the input of the major pheromone compound and smaller ones receiving the input of the minor pheromone compounds (Dekker & Kárpáti, 2020).

Although females are not known to orient to their own sex pheromone like males do, there is increasing evidence that they do respond to conspecific sex pheromone with changes in reproductive behaviors such as pheromone emission, oviposition or mating (Holdcraft et al., 2016). To the extent that pheromone “autodetection” implies spotting the presence of conspecific females in the area in order to maximize reproductive fitness by changing pheromone emission behavior (Holdcraft et al., 2016), females should not only be able to detect the pheromone compounds, but they should also be able to discriminate their conspecific blend from similar blends released by closely related species. For this to happen, females should possess an olfactory system having some of the characteristic described in males of the same species. At the very least, they should be able to discriminate their own species pheromone blend from similar ones. While the sensory system of males has been widely studied, little is known about female responses to sex pheromone, specifically at the ORN level.

Electroantennogram (EAG) responses to sex pheromone are typically much smaller in females than in males (Holdcraft et al., 2016). While the weak EAG response of female antenna to sex

pheromone could imply fewer pheromone receptors than males, it could also mean that females have a similar number of receptors as males but of lower sensitivity, or a combination of both. The scarce single sensillum recording (SSR) studies available indicate that, in general, females lack sex pheromone-specific receptor neurons (Holdcraft et al., 2016). This would account for the absence of a MGC in female brains and for the smaller size of the physiologically analogous glomeruli of females (Ochieng et al., 1995; Rospars & Hildebrand, 2000; Sadek et al., 2002; Masante-Roca, 2005; Hillier et al., 2006; Kárpáti et al., 2008; Trona et al., 2010). This is also the case in the oriental fruit moth, *Grapholita molesta* Busck (OFM) (Varela et al., 2011b).

Pheromone communication in *G. molesta* depends on a specific ratio of the major cis-8-dodecenyl acetate (*Z*8-12:Ac) and minor trans-8-dodecenylacetate (*E*8-12:Ac) pheromone compounds to achieve the best attraction level possible (Baker et al., 1981a; Knight et al., 2015). In males 64% and 7% of the olfactory receptor neurons housed in sensilla trichodea respond specifically to *Z*8-12:Ac and *E*8-12:Ac, respectively (Ammagarahalli & Gemeno, 2014), these numbers being roughly proportional to the ratio of the acetate compounds in the female pheromone blend (Han et al., 2001). The remaining 29% of the sensilla do not respond to pheromone compounds, although some are excited by plant volatiles (Ammagarahalli & Gemeno, 2015).

Stelinski et al. (2006) observed alterations in the calling periods of females previously exposed to conspecific pheromone and Kuhns et al. (2012) reported a diminished reproductive success under similar conditions. Furthermore, EAGs of females at various concentrations showed a positive response, which suggests the existence of pheromone receptors in females (Stelinski et al., 2006). These studies suggest that female OFMs have ORNs that respond to their own sex pheromone. Previous studies have highlighted the fundamental role of plant volatiles for females, since they are the ones that have to locate oviposition places (Varela et al., 2011a; Barros-Parada et al., 2018). Likewise, *G. molesta* males produce a pheromone from their everted hairpencils during courtship that is an additional mechanism of conspecific recognition and is fundamental for females to accept mating (Löfstedt et al., 1989). Specialized ORNs for detecting hairpencil volatiles, with axons that innervate glomeruli located in the base of the antenna, have been described in *Heliothis virescens* (F.) (Hillier et al., 2006). Hence, we would expect OFM females to present similar cells specialized in the detection of some of the hairpencil components and plant volatiles

The objective of our study is to determine how female *G. molesta* detect the female-produced sex pheromone at the ORN level. Single-sensillum electrophysiological recordings were made on male and female antenna to the two main sex pheromone compounds *Z*8-12:Ac and *E*8-12:Ac (Ammagarahalli & Gemeno, 2014), the main male-produced hairpencil compound ethyl *trans*-cinnamate (Löfstedt et al., 1989), and a blend of plant compounds (terpinyl acetate, *E*-β-farnesene, and methyl salicylate) active for several tortricid species that stimulate ORNs in male OFM (Ammagarahalli & Gemeno, 2015).

Material and methods

Insects

The colony of *G. molesta* was established with insects collected in peach orchards in Piacenza, Italy, and has been maintained at the University of Lleida, Spain, since 2005. Larvae were reared on semisynthetic diet modified from Ivaldi-Sender (1974) under a L16:D8 photoperiod at 23 ± 1°C. Male and female pupae were placed in separate environmentally controlled chambers inside 1L polypropylene containers provided with 10% sucrose solution drinkers. Adults were collected regularly after hatching and used when 2-4 days old.

Scanning electron microscopy

The number and distribution of sensilla types is known in *G. molesta* males (Ammagarahalli and Gemeno, 2015), but not in females. Female antennae were analyzed by scanning electron microscopy following the procedure described in Ammagarahalli and Gemeno (2014). Briefly, antennae were dissected from live individuals and the scales were removed mechanically. The antennae were mounted on SEM stubs lined with conductive tape and let dry at room temperature before gold sputter coating. The scale-free (ventral) area of 9 antennae and the scaled (dorsal) area of 4 antennae, each from a different individual, were examined. Sensilla counts were made every 5th flagellomere, starting on the proximal one. Total sensilla count per antennae was estimated by extrapolating these counts to the skipped flagellomeres. The scale-free area, which covers one third of the perimeter of each flagellomere, was fully visible, but the scaled area, which covers the remaining of the flagellomere surface, was always partially obstructed from vision. Using characteristic landmark structures that indicated the sagittal axis on the scaled area we could extrapolate sensilla counts from the visible section of the scaled area to the section hidden from view. Abundance and pattern of distribution of all types of sensilla are reported. Length and basal and tip widths of all types of sensilla (N = 20 sensilla from four different antennae) were measured.

Odorant stimuli

The female sex-pheromone compounds *Z*8-12:Ac (CAS 28079-04-1) and *E*8-12:Ac (CAS 40642-40-8) were provided by Pherobank (Wijk bij Duurstede, The Netherlands) with an initial purity 99%. GC analysis revealed a Z:E isomer content of 100:0.301 in *Z*8-12:Ac and of 0.633:100 in *E*8-12:Ac. The male produced courtship pheromone, ethyl *trans*-cinnamate (CAS 18794-84-8, Fluka product number 96350, lot and filling 1105301 and 13407214), methyl salicylate (CAS 119-36-8, a present from Ashraf El-Sayed, New Zealand), and terpinyl acetate (CAS 80-26-2, Sigma-Aldrich product number W20470-0-K, lot number 06703D407, ≥ 95% pure), did not show significant contamination peaks by GC-MSD.

Stimulation

Chemicals were loaded in 1-µl aliquots onto *n*-hexane pre-cleaned and folded over filter paper pieces (0.5 x 1 cm, Whatman #1, Sigma-Aldrich, Spain). After solvent evaporation (5 min), the filter paper dispensers were introduced into 1-ml disposable plastic pipettes (73-mm long x 7-mm i.d. at the wide end, 1-mm exit hole). A 40-mm section of silicone plastic tubing (5-mm i.d. and 8-mm o.d., Sigma-Aldrich product number BR143358) was inserted into the larger opening of the pipette tip and the odor cartridges were stored in glass test tubes sealed with PTFE-coated screw to be used within the day. A given cartridge was not used for more than 20 stimulations. Filter papers formulated with solvent (*n*-hexane) controlled for puffing effects in the EAG test. The test tubes that kept the stimulus cartridges were rinsed with acetone and heated at 250ºC overnight before being reused. A 0.5 l/min flow of charcoal-filtered and humidified air blew continuously over the insect preparation through a 8-mm i.d. PTFE tube placed 15-20 mm from the preparation (air velocity at exit = 0.16 m/sec). The tip of the odor cartridge bearing the filter paper was positioned 8 cm down from the recording point and perpendicular to the direction of the continuous air flow. Charcoal-filtered room air was puffed at 0.2 l/m through the odor cartridge to the continuous flow tube for 200 ms (air velocity at exit = 4.24 m/sec). Time interval between puffs was at least 30 s, but longer if needed to let the spike activity return to pre-stimulation levels. A maximum of 10 cells were recorded per insect, and at least 5 min elapsed between recordings from two different cells. The air around the preparation was constantly exhausted to minimize contamination.

Electrophysiology

Moths were immobilized with CO_2 and fixed in an aluminum block. The protruding head was restrained with tape and the antennae either remained free (for EAG) or were fixed to a double-sided tape lining using minute strips of smoking paper (for SSR). Two types of

electrodes were used: tungsten and glass. Tungsten wire (0.125-mm diameter, 99.98% purity, Advent Research Materials Ltd, England) was electrolytically sharpened with 10% KOH. Pulled glass-capillary electrodes were filled with physiological saline solution (1mM KCL in distilled water) and housed 0.5 mm diameter platinum wires.

A glass electrode inserted through the mouthparts and connected to ground served as the reference electrode. For EAG the recording glass electrode contacted the cut tip of one antenna. For SSR, the recording tungsten electrode was placed near the base of a randomly chosen sensillum trichodeum and pushed gently inward with the help of a manual micromanipulator (NMN-25, Narishige, Japan) until action potentials were observed.

Sensilla were randomly sampled along the antennae, including the scaled region, where scales were removed with the help of a tungsten electrode. The signal from the recording electrode was pre-amplified (10x gain, Universal Single Ended Probe, Syntech, Germany), further amplified and band-pass filtered at 0.1 to 3KHz for SSR and 0.1-100 Hz for EAG (Model 300 AC/DC Differential Amplifier, AM-Systems, Sequim, Whasington, USA) and digitalized (MICRO4 CED 1401, CED Systems, NJ, USA). Spikes were sorted with Spike2 (CED Systems, Cambridge, UK).

Dose-response curves

Dose-response curves for both EAG and SSR used six concentrations of *E*8-12:Ac and *Z*8-12:Ac (10^0 to 10^5 ng). The order of the stimuli was first the negative control (*n*-hexane), followed by low to high doses of *E*8-12:Ac, and then low to high doses of *Z*8-12:Ac. For SSR an initial puff of a 1:1 blend of *Z*8-12:Ac and *E*8-12:Ac (10^3 ng of each) served to determine whether the cell was pheromone-responsive. Responding cells were then stimulated with a high dose of *E*8-12:Ac (10^3 ng) to determine if the cell was of the *Z*8-12:Ac type or of the much less abundant *E*8-12:Ac type (Ammagarahalli & Gemeno, 2014) before proceeding with the dose treatments. For EAG 21 male and 20 female antennae were tested. For SSR 11 sensilla from 5 different males were sampled. In total 6 Z-cells and 5 E-cells were used in the SSR dose-response curves.

ORN characterization

In total, six stimuli were used for ORN classification: 10^2 ng ("low") and 10^4 ng ("high") of the pheromone compounds *E*8-12:Ac and *Z*8-12:Ac; 100 µg of the plant blend (1:1:1 of FAR:MS:TA), and 10 µg of the hairpencil pheromone. Each contacted sensillum was stimulated with each of the six stimuli in random order (305 cells from 50 female antennae, 45 cells from 12 male antennae).

Statistical analyses

For EAG we measured the maximum potential generated by the antenna during the 2-sec immediately following a puff. For SSR we recorded the number of spikes pre-stimulation (from 2 sec to 1.5 sec before the puff), and the number of spikes during post-stimulation (500 ms from puff onset). Spike response was estimated as the number of spikes post-stimulation minus the number of spikes pre-stimulation. The reason for not including the spikes immediately before the puff in the pre-stimulation period was that, when the puff was loaded with the highest pheromone doses, some cells responded as far as 1.2 sec before puff onset, and the stimulus was pulled from the pipette by the continuous air flow (**Fig. S3.1**).

Dose-response curves (EAG and SSR) were fitted to non-linear regression models to estimate the expected half-dose (ED_{50}) (library *drc* in R, Ritz et al., 2015). Comparison between a model assuming equal parameters with one assuming different slope, maximum and ED50s were used to determine whether two curves were significantly different from each other. In order to classify cells according to their response to the odor pannel a hierarchical cluster analysis based on the maximum distance method was applied (library *superheat* in R, Barter and Yu, 2018). Analyses were run in R (R Core Team, 2020).

Results

Morphology

Scanning electron microscope (SEM) images of female antennae revealed 6 types of sensilla (**Fig. 3.1**). In all segments 4 sensilla chaetica can be found, and in the scale-free region it is frequent to encounter 1 sensillum styloconicum on each flagellomere. The most abundant sensilla type is sensilla trichoidea (54%), followed by auricillica (27%), coeloconica (8%) and basiconica (4%) (**Table S3.1, Fig. S3.2**).

Dose-response curves

The antenna of both males and females, and the Z- and E-ORNs of males produced dose-response curves to the two pheromone isomers (**Fig. 3.2A**). Antenna and ORN sensitivities were assessed using the ED_{50} estimated from the curves (**Table 3.1**). The antenna of males was more sensitive than the antenna of females to both sex-pheromone compounds. The antenna of males was more sensitive to the Z-isomer than to the E-isomer, whereas the antenna of females did not discriminate between them (**Fig. 3.2A**). Z- and E-ORNs were more sensitive to their respective isomers than to the other isomer (**Fig. 3.2B**). Furthermore, Z- and E-ORNs were equally sensitive to their respective isomer, and equally insensitive to the opposite isomer.

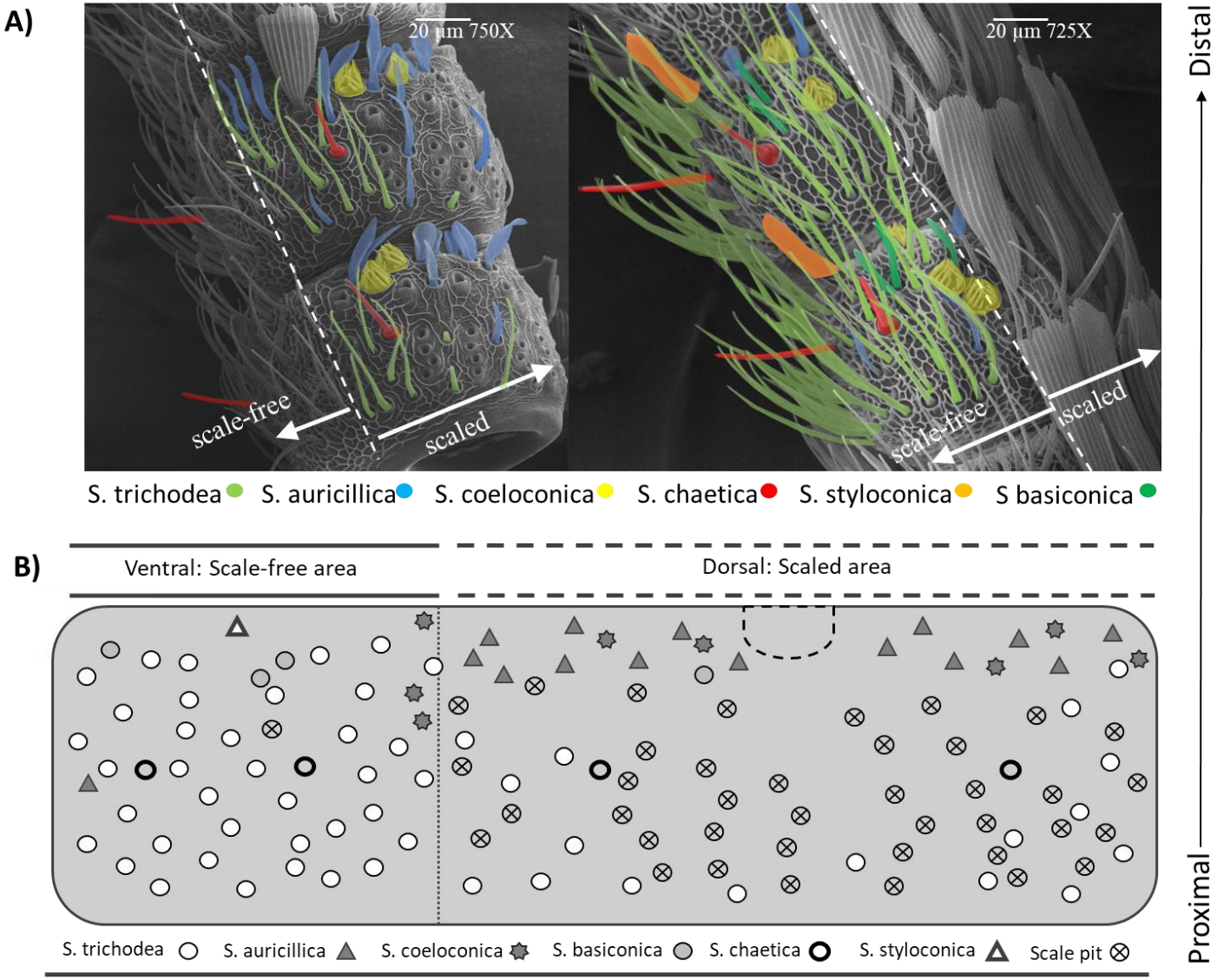

Figure 3.1. Distribution of sensilla types on the antenna of *G. molesta* females. A) Sensilla types are shown with different colors in the SEM picture of the 25th and 26th flageromeres. B) Representative distribution of sensilla and scales on a typical flagellomere towards the middle of the antenna.

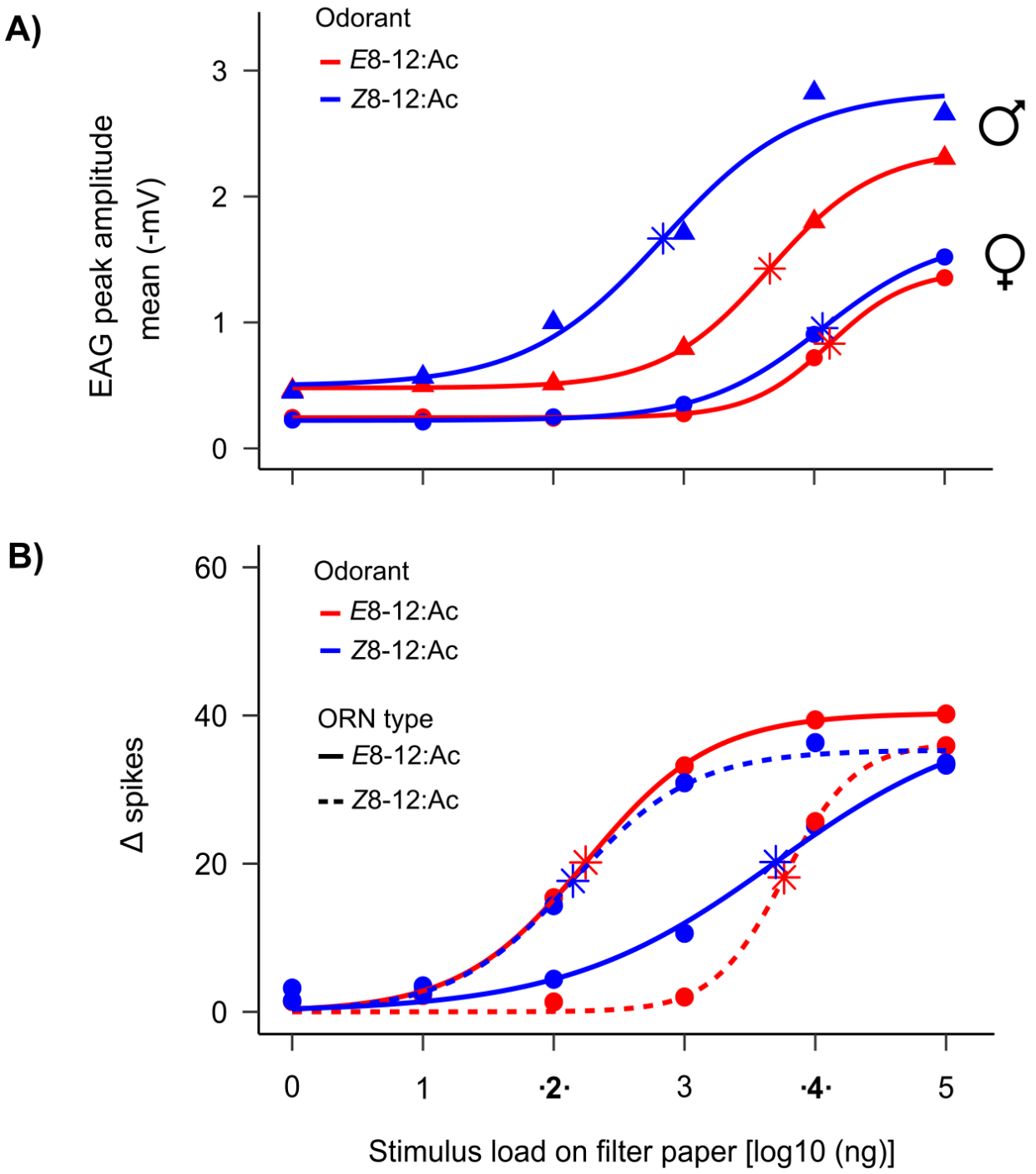

Figure 3.2. Dose-response curves of antennae (A) and ORNs (B) of *G. molesta* to the major (*Z*8-12:Ac) and minor (*E*8-12:Ac) pheromone compounds. Symbols represent the average response and curves are the predicted values from the fitted logistic functions. Asterisks indicate estimated dose 50 (ED50).

Based on the results of these curves, we chose the two concentrations (10^2 and 10^4 ng) of female pheromone compounds that would serve us to characterize ORN types in males and females. With the low dose, Z- and E-ORNs could be discriminated in males, but the female antenna was insensitive to this concentration. With the high dose the female antenna started to respond, indicating some ORN activity, and the male Z- and E-ORNs could still be discriminated.

Table 3.1. Estimated dose 50 (ED50) of EAG and SSR curves. Different letters indicate significant differences among stimuli within each sex (EAG) or each male ORN type (SSR) (ANOVA, $p < 0.05$).

EAG				**SSR**			
Antenna	Stimulus	ED_{50} (mean ± SEM, ng)		**ORN type**	Stimulus	ED_{50} (mean ± SEM, ng)	
Male	*Z*8-12:Ac	495.30 ± 190.65	a	**Z-ORN**	*Z*8-12:Ac	144 ± 44.78	a
	*E*8-12:Ac	4174.5 ± 1665.2	b		*E*8-12:Ac	5616.77 ± 1582.15	b
Female	*Z*8-12:Ac	10545.7 ± 3488.3	c	E-**ORN**	*Z*8-12:Ac	4931.43 ± 2315.7	b
	*E*8-12:Ac	20726.8 ± 8236.8	c		*E*8-12:ac	175.4 ± 62.59	a

ORN classification

Single sensillum recordings were made on 239 and 42 sensilla trichoidea from a single antenna of each 50 female and 12 male antennae, respectively. Most male ORNs exhibited background firing rates of 0-10 spikes·s^{-1}, while the distribution of spontaneous activity in female cells was broader, between 10 and 20 spikes·s^{-1}. Most female sensilla contained 1-3 active ORNs, while in males only 3 recordings presented two cells. Hierarchical cluster analysis grouped the 350 male and female ORNs in 5 distinct clusters (**Fig. 3.3A**) showing specific response patterns to the selected stimuli (**Fig. S3.2**). In females, the largest group comprised the so called "unspecific" ORNs (90 %) (**Fig. 3.3B**). In these neurons responses were weak in general and none of the test stimuli was clearly better than the others. Unspecific ORNs comprised 27% of the total in males. A second group of ORNs responded strongly to the plant blend and comprised 6.5% of the total in females and 4% in males. A third group that responded specifically to the hairpencil male pheromone (ethyl *trans*-cinnamate) contained 3.6% of the female cells and no male cells (**Fig. 3.3B**). The last two groups of cells responded strongly and preferentially to either the major or the minor female sex pheromone compounds. This group contained 54% and 8%, of the male cells respectively, and no female cells (**Fig. 3.3B**). The responses of the Z- and E-ORNs identified by HCA with the low (100 ng) and high (10 µg) doses of *Z*8-12:Ac and *E*8-12:Ac corresponded with the responses of the Z- and E-ORNs of the dose-response curves at the same two concentrations (**Fig. 3.2B**, **Fig. 3.3B**).

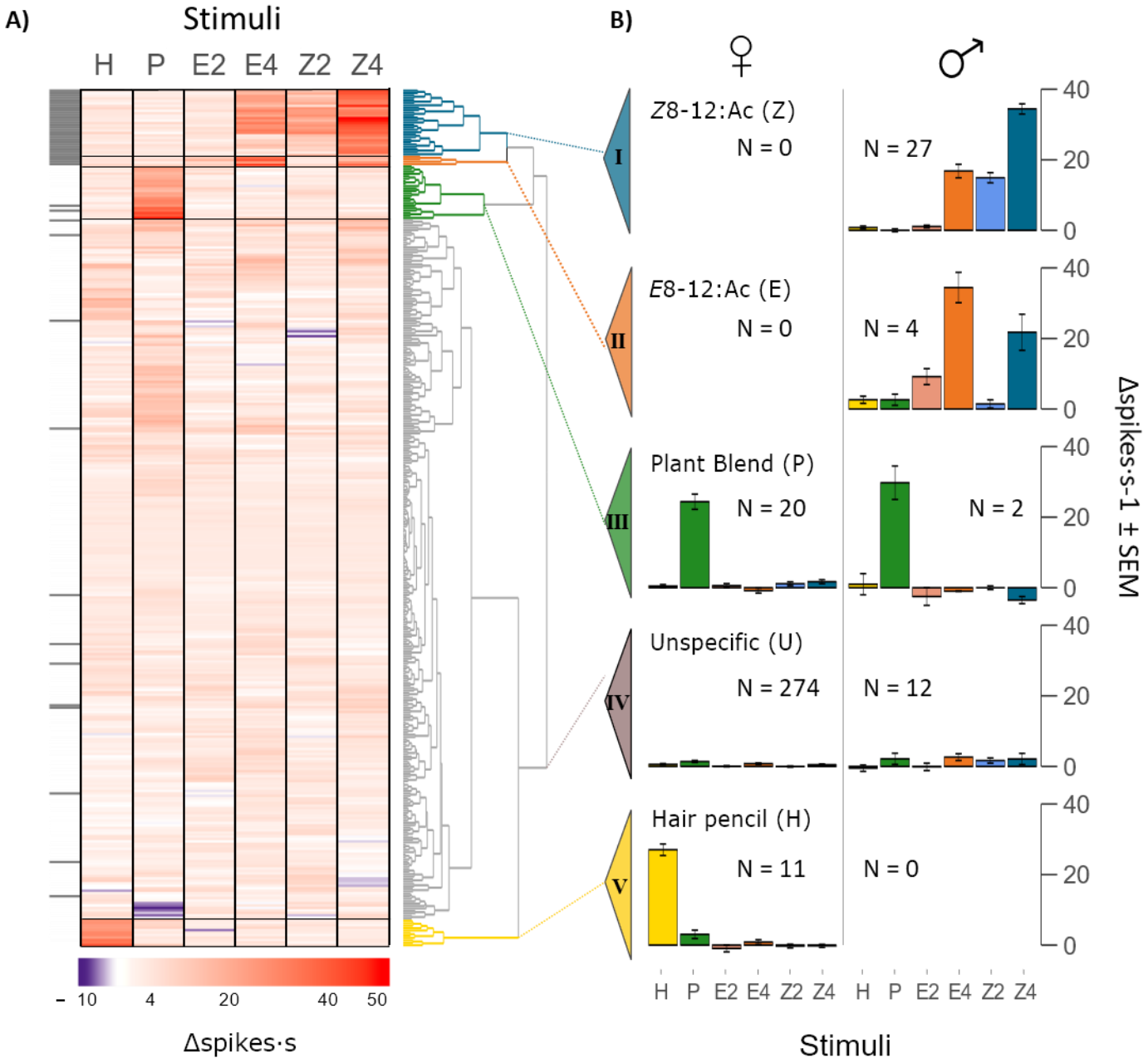

Figure 3.3. A) Hierarchical cluster analysis grouping *G. molesta* male and female ORNs according to their responses to 6 odorant stimuli [H: hair pencil pheromone at 10 µg, P: plant blend at 100 µg, E: minor pheromone compound *E*8-12:Ac, Z: major pheromone compound *Z*8-12:Ac, each at 2 (100 ng) and 4 (10 µg)]. Each entry in the y-axis indicates a different ORN. The first column on the left indicates sex (grey for male, white for female). B) Five distinct clusters were obtained, and the average responses of the cells in each cluster are represented by sex and compound.

Discussion

In order to discriminate odor blends composed of the same odorants but in slightly different ratios it may be crucial for receivers to bear sensory cells specifically tuned to each individual component in the blend. Male moths follow this pattern since they have different pheromone receptor neurons specifically tuned to each one of the two or three compounds that typically comprise the species-specific sex pheromone blend released by conspecific females (Andersson et al., 2015). Presumably, if female moths had to detect their own sex pheromone and discriminate it from similar ones, they would require a pheromone detection system similar to that of males. However, since males use sex pheromone to arrive at females whereas females appear to use it to detect other nearby females (Holdcraft et al., 2016), their sensory systems are likely to differ. We tested this hypothesis in the oriental fruit moth *G. molesta* by means of elecroantennography and single-sensillum electrophysiology.

At the EAG level, which represents the combined activity of receptor potentials across the antenna (Nagai, 1983; Jacob, 2018; Kaissling, 2019), we found that males were approximately 20 and 5 times more sensitive than females to the major (*Z*8-12:Ac) and minor (*E*8-12:Ac) pheromone compounds, respectively. This indicated that either the female antenna was equipped with a lower proportion of pheromone-responding cells, that these cells were not as sensitive as in males, or both. Furthermore, the male antenna was about 10-fold more sensitive to *Z*8-12:Ac than to *E*8-12:Ac, whereas the female antenna had similar sensitivity to the two isomers, suggesting additional differences in sensory specificity between males and females.

In order to uncover the cellular mechanisms that underlie sex differences in antennal reception, we compared the response of 305-female and 45-male ORNs housed in sensilla trichodea to an odorant panel consisting of the female pheromone compounds (*Z*8-12:Ac and *E*8-12:Ac), the main male courtship pheromone compound (ethyl trans-cinnamate) and a blend of host-plant volatiles ((*E*)-β-farnesene, terpenilacetate and methyl salicylate). To further discriminate between the two sex-pheromone ORN types we tested both high and low female pheromone doses, the higher above and the lower below the ED50s of male cells. Hierarchical cluster analysis (HCA) grouped the 350 ORNs in 5 neatly separated response classes, two of which responded strongly to sex pheromone and included male cells only. The more numerous of these two classes matched the described dose-response curve of cells tuned to the major compound, while the less numerous class matched the dose-response curve of cells specific to the minor-compound. Thus, they were classified as *Z*8-12:Ac and *E*8-12:Ac-specific ORNs,

respectively. The relative abundance of each cell type corresponded with their ratio in the female pheromone blend (Ammagarahalli & Gemeno, 2014; Knight et al., 2015). A third group composed exclusively of female cells responded very specifically to the male-produced hairpencil pheromone, and a fourth group composed primarily of female cells responded specifically to the plant-volatile blend, so they were identified as receptors for these two stimuli classes. The remaining ORNs, a vast majority in females, did not respond strongly or specifically to any particular odorant in the stimulus panel, and thus they were termed "unspecific". ORN responses explained EAG differences because in males most of the cells sampled were major-compound specialists, and correspondingly the male antenna was about 10-fold more sensitive to the major compound than to the minor compound. In contrast, female ORNs responded to sex pheromone weakly and unspecifically, and consequently the female antenna was much less sensitive to pheromone than the male antenna and it did not discriminate between the two sex pheromone isomers.

Pheromone-responsive ORNs have been reported in females of several moth species (Holdcraft et al., 2016), and in two of them, *Spodoptera littoralis* (Boisduval) and *Heliothis virescens* (F.), they have been studied in substantial detail. *S. littoralis* have long and short sensilla trichodea, but while the former occur abundantly (N=80) throughout each flagеromere in males, in females they are less numerous (N=12) and are located exclusively on the lateral edges, six on each side (Ljungberg et al., 1993). In males about 80% (N = 125) of these long sensilla trichodea house a single ORN that responds specifically to the major pheromone compound (*Z*9,*E*11-14:Ac), and the remaining 20% respond to minor pheromone compounds (Ljungberg et al., 1993). In females, 52 to 98% (N= 40-120) of their few long sensilla trichodea house one cell that responds mainly to the major pheromone compound, and dose-response curves show that this cell is as sensitive to this compound as males' (Ljungberg et al., 1993; Binyameen et al., 2012). Molecular analysis supports physiological observations, showing a 50-fold higher expression of the receptor protein of the major pheromone compound SlitOR5 in males than in females (Bastin-Héline et al., 2019), and also higher expression of receptor genes for minor pheromone compounds in males than in females (Bastin-Héline et al., 2019; Walker et al., 2019). By comparison, *H. virescens* males have many more long than short sensilla trichodea, and females only bear the short type (Almaas & Mustaparta, 1990). The majority of the male long trichoid sensilla (80% in a sample of 403) house a neuron that responds specifically to the major pheromone compound (*Z*11-16:Ald), whereas 3% of the sampled neurons respond specifically to the minor compound (*Z*9-16:Ald). The remaining 16% respond to heterospecific pheromone compounds with somewhat lower specificity than the

pheromone cells (Baker et al., 2004; Berg et al., 2014; Hillier & Baker, 2016). A small percentage of male ORNs in short sensilla trichodea (15% in a sample of 202) are major compound specialists, and 38% respond to minor pheromone compounds with diverse specificity (Hillier & Vickers, 2007). In females, however, only 2% of the cells out of 184 recordings from the short sensilla trichodea responded to the major compound and 37% of the cells responded to the minor compound or heterospecific sex pheromone compounds. In both cases the responses were weak and not too specific (Hillier et al., 2006). Corresponding with the physiological data the expression of the olfactory receptor genes HvirOR13 and HvirOR6 associated with sex pheromone compounds is significantly larger in males than in females (Vásquez et al., 2011).

The evidence for female pheromone specificity at the sensory neuron level is not better in the other moth species investigated. Hansson et al. (1989) did not find pheromone responses in a sample of 75 female *Agrotis segetum* (Denis & Schiffermüller) ORNs, and Kalinová et al. (2001) reported cells of *Manduca sexta* (L) with similar pheromone dose-response curves in both sexes, but it was to a minor compound and only 8 cells out of 200 belonged to this category. Pophof et al. (2005) reported weak responses to the major pheromone component 9*Z*,12*E*-14:Ac in one out of 36 female neurons of *Cactoblastis cactorum* (Berg), and Todd and Baker (1993) located five *Trichoplusia ni* (Hübner) female sensilla harbouring a neuron with similar threshold responses to the major pheromone compound as male neurons, but the number of cells sampled was not specified.

S. littoralis, and perhaps *T. ni*, stand out as the species where females appear to have a sensory neuron type that responds to the main sex pheromone component with similar sensitivity as males, but they occur in much smaller numbers than in males and their specificity is largely unknown. Generally, the fraction of pheromone sensitive receptor cells on the antenna of the male moth is between 75% and 25% of all odor receptor cells (Schneider, 1999) and this translates into lower EAG responses to sex pheromone in females than in males (Holdcraft et al., 2016). Males have to navigate along very diluted and jagged pheromone plumes for tens or hundreds of meters to locate and mate with calling females before other males do and boosting the number of sensory neurons is one way to increase their sensitivity (Cardé, 2021). Perhaps females need fewer pheromone sensory neurons than males because they do not use the pheromone for the same purpose, but there are still other features of the male pheromone detection system that are not found in females. For instance, males have neurons specifically tuned to the minor pheromone compounds, which are essential to discriminate similar pheromone blend ratios. Furthermore, in some species males have neurons tuned to

behavioral anatagonists which prevent cross-attraction between species producing similar pheromone blends (Baker, 2008). Although *S. littoralis* females have an ORN type which sensitivity, as per dose-response curves, is equivalent to that of the major sex-pheromone male receptor cell (Ljungberg et al., 1993), there is no evidence of ORNs in females responding specifically to the minor compounds *Z*9-14:Ac and *E*11-14:Ac (Saveer et al., 2014). The spatial arrangement of the major and minor as well as the pheromone antagonist cells within and between sensilla follows distinct patterns on the male antennae. This variable distribution may help them cope with the considerable variation in pheromone concentration that they experience when flying towards calling females from great distances to the point of emission without losing the ability to detect the relative proportion of pheromone compounds in the blend (Baker et al., 2012). Because females are barely attracted to calling females (Holdcraft et al., 2016) they may not need the same pheromone ORN arrangement as males. However, the electrophysiological evidence that we have so far indicates that females probably are inefficient at discriminating their own sex pheromone blend from similar ones, and they seem to be relatively insensitive to natural doses of their own sex pheromone too. So, how can we explain the substantial evidence that exposure to sex pheromone affects female behavior (Holdcraft et al., 2016)? We provide some ideas that may shed light on this apparent contradiction.

Firstly, we believe that to unambiguously stablish pheromone autodetection it should be demonstrated that females can behaviorally discriminate their own sex pheromone blend from similar ones. Otherwise they may just be detecting pheromone compounds irrespective of their taxonomic origin. This approach is standard in sex pheromone identification where males are presented with different blend ratios and often prefer the one produced by conspecific females (Allison & Cardé, 2016a), but as far as we know this procedure has not been employed in pheromone autodetection tests (Holdcraft et al., 2016). Some moth species appear to rely only on one pheromone compound, because females either lack minor pheromone compounds or these do not appear to increase male responses. Although in these species having just one pheromone receptor cell type would be sufficient for pheromone detection by males or females, the vast majority of moth species use two or more pheromone compounds in the blend (Allison & Cardé, 2016b). Secondly, many olfactory autodetection tests apply unnaturally high pheromone doses for prolonged periods of time, and this practice may result in altered odor perception. This is due to the fact that olfactory receptor neurons are promiscuous and respond to several odorants, but they do so with different sensitivities. While at natural concentration ranges a given odorant stimulates only a limited spectrum of receptor

neuron types, at abnormally high concentrations additional receptor types that would not typically respond to that odorant at natural doses may also be excited, and at even higher concentrations the most sensitive receptors may become adapted (Si et al., 2019; Del Mármol et al., 2021).

The dose-response curves of *Z*8-12:Ac and *E*8-12:Ac ORNs of male *G. molesta* show high specificity to the middle doses but lower discrimination at the highest doses. The displacement of the dose-response curve along the concentration axis reflects the affinity of the odorant for the odorant receptor, and the height of the curve (the number of spikes per second or firing rate) indicates the maximum number of olfactory receptors activated (Rospars, 2013). Furthermore, at high stimulus concentrations small impurities present in synthetic compounds could cause false positives, especially when they are more volatile than the target compound (Schorkopf et al., 2019). Thus, although necessary to stablish the ED50 from dose-response curves to determine receptor sensitivity and specificity in electrophysiological tests (Rospars et al., 2008; Rospars, 2013; Kaissling, 2019; Si et al., 2019), unnaturally high stimulus concentrations may convey a distorted odor representation to the brain and induce behaviors that may or may not be adaptive, leading to flawed conclusions regarding the function of that stimulus. For instance, the behavioral tests that demonstrated pheromone autodetection in *G. molesta* employed relatively high sex pheromone doses (10 to 100 µg loaded on rubber septum) for prolonged periods of time (up to 10 h) (Stelinski et al., 2006, 2014; Kuhns et al., 2012). In these studies both EAG and mating were reduced 24 h after exposure, so it is likely that females experienced acute sensory adaptation or behavioral habituation due to the strong and lengthy stimulation (Kuhns et al., 2012). Our SSR study shows that some female neurons respond to sex pheromone, but they are not particularly sensitive, and they also respond to the other stimuli, so their natural ligand is probably not the pheromone. The exposure of females to unnaturally high pheromone concentrations in the autodetection experiments may have stimulated, or even adapted, unspecific neurons, and falsely signalled the detection of non-pheromone odors which could have triggered behavioral responses that the sex pheromone at natural concentrations would never have had.

Calling females are undeniably a more natural source of pheromone stimulus than synthetic blends, and indeed when used to test autodetection the calling period of target females is affected (Harari et al., 2011; Sadek et al., 2012; Rehermann et al., 2016). This change in behaviour is very reasonable evidence of pheromone autodetection yet, as with synthetic stimuli, if conspecific females are not compared with heterospecific females that produce similar blends, then there is no definitive demonstration that females can discriminate their

own sex pheromone from similar ones. Certainly, comparing the effect of pheromone blends from different species may be easier done with synthetic blends than with live females, as it would allow precise manipulation of blend composition and dose.

Testing other compounds in addition to the sex pheromone on an adequate number of cells in both males and females was decisive in classifying cell types by HCA in our study. Analyzing males and females simultaneously is relevant considering the notorious methodological variation across olfactory setups (Gorur-Shandilya et al., 2019), but also because it serves to compare the known pheromone responses of males with the hypothetical response of females. The clear separation of cells by stimulus type in both sexes indicates that the absence of sex-pheromone specific cells in females was not due to an inadequate sample size or deficient test stimuli. In male *G. molesta* 29% of the sensilla trichodea do not respond to pheromone (Ammagarahalli & Gemeno, 2015), which is similar to the 38% that we report here. Of these, only two cells responded to the trinary plant blend. The presence of male cells that respond specifically to plant volatiles is believed to increase their possibilities of finding females, since these odorants can have a synergic effect on male response to pheromone (Varela et al., 2011a; Barros-Parada et al., 2018; Kong et al., 2020). In females the unspecific cells were dominant (90%) and only 6% belonged in the plant category. This is not surprising, since the plant blend compounds were selected for the strong responses that they elicited in sensilla trichodea of male OFM (Ammagarahalli & Gemeno, 2015). Testing further compounds relevant to the female biology will probably increase the number of cells responding to host odorants, as indicated by receptor molecular studies (Chen et al., 2020). Females also have a higher number of auricilic sensilla than males (Ammagarahalli & Gemeno, 2015), and recordings from these sensilla would probably increase the number of ORNs that respond to plant odorants (Ansebo et al., 2005; Ruschioni et al., 2015).

My SSR experiment further revealed that females of *G. molesta* have 3% of their sensilla specifically tuned to ethyl *trans*-cinnamate, a plant volatile that males release during courtship and serves as an intersexual signal and probably as an intersexual signal too (Baker et al., 1981b; Nishida et al., 1982, 1985; Löfstedt et al., 1990; Conner & Iyengar, 2016). Studies on *H. virescens* revealed that 33% out of 184 female ORNs tested responded to hairpencil compounds and to an interspecific behavioral antagonist (Hillier et al., 2006). Furthermore, 19% out of 202 cells in males were of the same type (Hillier & Vickers, 2007; Hillier et al., 2007). Contrastingly, we found no male cells responding to ethyl *trans*-cinnamate, but male *G. molesta* produce additional hairpencil compounds (Baker et al., 1981b; Nishida et al., 1982, 1985), that were not tested and that they could sense with specific ORNs. Similarly, the

percentage of plant-responding cells may increase by sampling additional ecologically relevant plant stimuli. Furthermore, several cells with relatively specific responses to hairpencil and plant odors were classified as unspecific by the HCA due to their relatively weak response, but this picture may change with larger ORN samples and more refined statistical analyses.

As predicted, my study demonstrates that the organization of the olfactory sensory system of male and female OFM appears to be shaped by the ecological function of each sex. Females and males seem to be relatively unable to detect their own pheromones but have cells that respond to ecologically relevant plant stimuli and for the pheromone of the other sex. Although some female cells that respond to female sex pheromone have been described for other species, our results show that their physiological and biological relevance have in general been overrated. However, it has been reported that female behaviour can be altered under mating disruption conditions where the environmental level of pheromone probably surpasses the natural concentration released by females (Harari et al., 2011). Studies on moths regarding female response towards different pheromone blends at various concentrations in the field are necessary to determine the actual effect autodetection could have when mating disruption is implemented.

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Table S 3.1. Number (mean ± SEM) and proportion (per cent) of different sensillum types on scaled and scale-free areas of the flagellum of *G. molesta* females. Counts from scaled and scale-free areas were from different antennae/insect.

Sensillum type	Scaled area (n=8)		Scale-free area (n=5)		Total	
	number	%	number	%	number	%
Trichodea	466.61 ± 23.32	31.31 ± 00.83	1215.00 ± 51.56	76.03 ± 00.71	1681.61	54.44
Auricillica	768.90 ± 37.36	51.53 ± 01.24	80.40 ± 04.39	05.05 ± 00.31	849.3	27.49
Coeloconica	157.35 ± 09.36	10.61 ± 00.59	73.80 ± 6.61	04.66 ± 00.49	231.15	7.78
Basiconica	11.65 ± 06.13	00.72 ± 00.36	101.70 ± 20.20	06.22 ± 00.94	113.35	3.66
Chaetica	86.22 ± 01.86	05.83 ± 00.21	95.40± 03.31	06.03 ± 00.42	181.62	5.87
Styloconica	0	0	40 ± 2.04	02.00 ± 00.15	31.8	1.02
Total	1490.72 ± 78.03		1598.10 ± 88.27		3088.82	

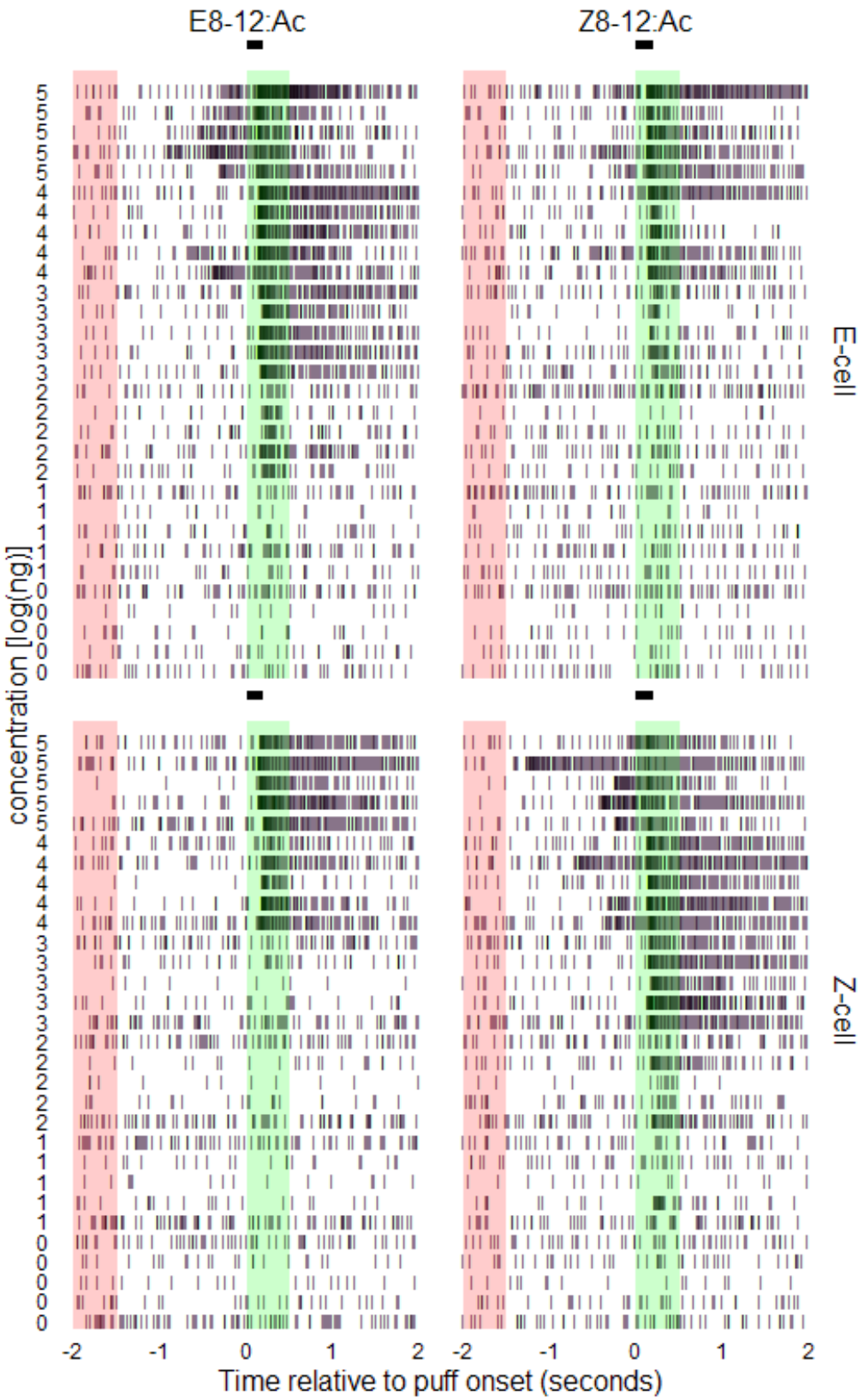

Figure S 3.1. Firing activity of 5 *Z*8-12:Ac (top) and 5 *E*8-12:Ac (bottom) pheromone receptor neurons of male *G. molesta* stimulated with several doses (left axis) of the two sex pheromone compounds (right axis) to create the dose response curves shown in **Figure 3.2**. The pink shadowed area indicates the time considered as pre-puff basal spiking activity and the green area indicates the time of post-stimulation response used in the analysis. Horizontal line above indicates the duration of the stimulation (200 ms).

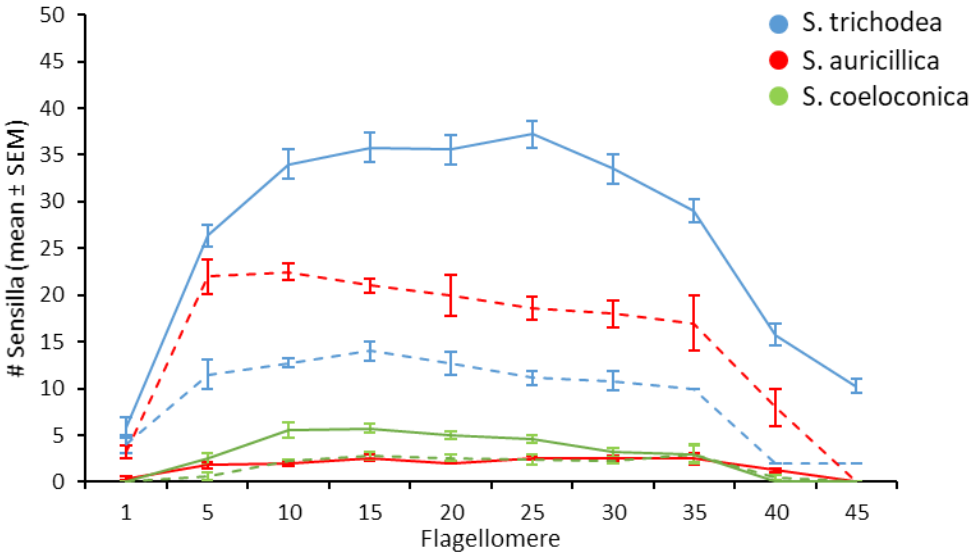

Figure S 3.2. Abundance of three major sensillum types in scaled (dotted lines) and scale-free (solid lines) areas along the flagellum of *G. molesta* females.

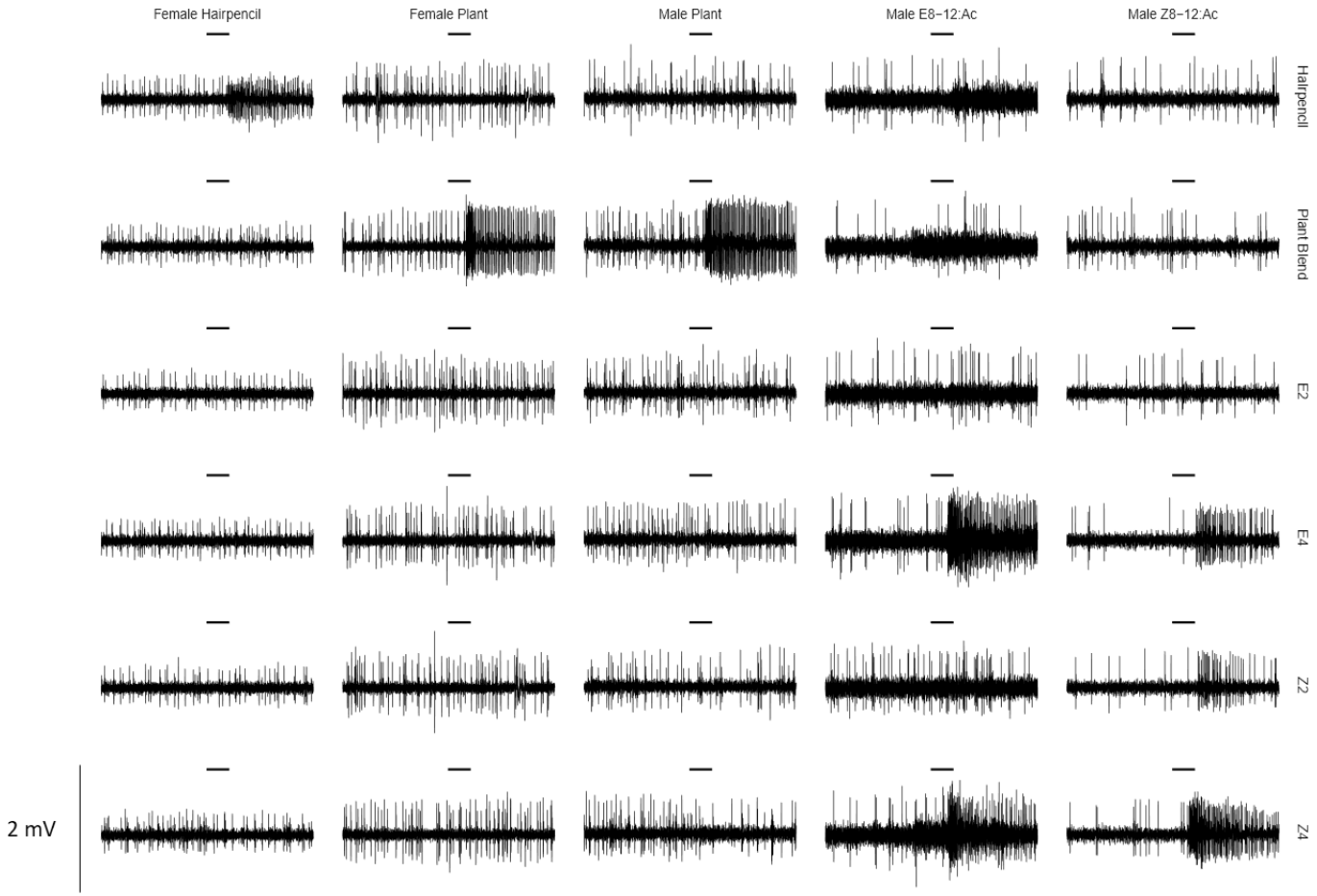

Figure S 3.3. Representative traces of the specific ORN types (top) shown in **Figure 3.3**. Each cell is stimulated with the 6 test stimuli. The horizontal line above records indicate the duration of stimulation (200 ms).

CHAPTER IV. EAG RESPONSES OF ADULT *LOBESIA BOTRANA* MALES AND FEMALES COLLECTED FROM *VITIS VINIFERA* AND *DAPHNE GNIDIUM* TO LARVAL HOST-PLANT VOLATILES AND SEX PHEROMONE

Abstract

We analysed electroantennogram (EAG) responses of male and female adults of the European grapevine moth *Lobesia botrana* (Denis et Schiffermüller) (Lepidoptera: Tortricidae) collected as larvae from grapevine (*Vitis vinifera* L.) and flax-leaved daphne (*Daphne gnidium* L.). The host-plant odorants tested were either *V. vinifera*-specific [1-octen-3-ol, (*E*)-β-farnesene, (E)-4,8-dimethyl-1,3,7-nonatriene], *D. gnidium*-specific (2-ethyl-hexan-1-ol, benzothiazole, linalool-oxide, ethyl benzanoate), or were shared by both host-plants (linalool, methyl salicylate). Sex pheromone compounds were also tested. Male response to the major pheromone component (*E*7,*Z*9-12:Ac) was higher than to any other stimuli, whereas response to the minor pheromone components (*E*7,*Z*9-12:OH and *Z*9-12:Ac) was not different from the response to the plant odorants. Female response to pheromone was lower or not different from that to plant odorants. Methyl salicylate elicited higher response in females and (*E*)-β-farnesene elicited higher responses than several other plant odorants in both sexes. Non-significant interaction between host-plant odorant and sex indicated absence of sex specialization for host-plant volatile detection. Lack of significant interaction between plant volatile and larval host-plant suggested no specialization for plant-volatile detection between *V. vinifera* and *D. gnidium* individuals.

Keywords: electroantennogram, host plant, volatiles, Tortricidae, sex

Introduction

The European grapevine moth, *Lobesia botrana* (Denis et Schiffermüller) (Lepidoptera: Tortricidae), is one of the most important pest species affecting vine (*Vitis vinifera* L.) and is responsible for severe economic losses in the vineyards of the Palaearctic region (Ioriatti et al., 2011). *L. botrana* has colonized areas of central Africa, western Asia and more recently the Americas (Chile, Argentina and the USA), making it a global grapevine pest (Torres-Vila, 2000; Ioriatti et al., 2011). Conventional control relays heavily on pesticide use, but the practice of alternative environmentally-friendly methods has increased in the last decades. The use of semiochemicals - mainly sex pheromones and plant volatiles - in pest control is a promising tool (Norin, 2007). Mating disruption (MD) is a very effective way to control insect pests, however it is rarely effective under high population densities and needs to be complemented with insecticide treatments in an Integrated Pest Management framework (Ioriatti et al., 2011). The success of MD in the control of *L. botrana* has stimulated research on the response of males to its sex pheromone (Arn et al., 1988; Torres-Vila et al., 1997; El-Sayed et al., 1999; Sans et al., 2016). However, there is growing evidence that females detect and change their calling behaviour in response to conspecific pheromone (i.e. pheromone 'autodetection') (Holdcraft et al., 2016), and that this may play a role in MD (El-Sayed and Suckling, 2005; Harari et al., 2015). El-Sayed and Suckling (El-Sayed and Suckling, 2005) determined that exposure to sex pheromone did not alter the behaviour of *L. botrana* females, but Harari et al. (2015) observed a reduction in calling behaviour and mating success of pheromone-exposed females. A comparative electrophysiological study with sex pheromone in both sexes could help explain some of these inconsistencies.

Sex bias in olfactory studies is not limited to sex pheromone and comprises plant-volatile stimuli too. Plant volatiles attract insects to their feeding, mating and oviposition sites, and help them avoid non-hosts (Krieger and Breer, 1999; Bruce and Pickett, 2011). Many studies on host selection in insects with non-dispersive larval stages have mainly centred on females because they are the ones that lay the eggs (Tasin et al., 2005, 2010; Agosta, 2006; Tasin, Bäckman, Bengtsson, Varela, et al., 2006), while males have received less attention. Although *L. botrana* develops mainly on *V. vinifera*, it remains polyphagous and may perform better on other wild or cultivated hosts that contribute with a higher nutritional value, decrease natural enemy pressure or enhance larval installation and survival (Thiéry and Moreau, 2005; Torres-Vila et al., 2012). Among these alternative hosts, the flax-leaved daphne, *Daphne gnidium* L., is especially important as this thymelaeaceous, evergreen and sclerophylous shrub is considered the putative wild host of *L. botrana* (Maher and Thiéry, 2006; Torres-Vila and Rodríguez-

Molina, 2013 and references therein). Tasin et al. (2005, 2010) investigated antennal and behavioural responses of *L. botrana* females to *V. vinifera*'s volatiles and compared them to the responses elicited by *D. gnidium* chemical cues. They reported that *L. botrana* females respond both to common and specific compounds of each host and to their mixture. Still, females were more attracted to *D. gnidium*'s than to *V. vinifera*'s complete blend (Tasin et al., 2010). Some studies have explored male *L. botrana* responses to plant volatiles (Masante-Roca et al., 2007; von Arx et al., 2011, 2012), but few have compared male and female EAG responses (Vitagliano et al., 2005), and none, that we know, has compared individuals collected from different host plants.

In the present study we compare electroantennogram (EAG) responses of males and females of *L. botrana* collected as larvae from *V. vinifera* and *D. gnidium* to plant volatiles from each host. We hypothesize that the response to these compounds may somehow reflect a preference for *L. botrana*'s more suited host, shedding light on the occurrence of an evolutionary host shift whereby the new moth-host association is either linked to adaptive changes or to more recent host-selection processes. In addition, we explore sex differences in the response to pheromone and plant stimuli.

Materials and methods

Moth strains and tested individuals

Larvae were collected from *V. vinifera* and *D. gnidium* in several locations in Extremadura, Spain, in 2014 (**Table 4.1**). These locations, and their agroecological contexts, were considered adequate to prevent or minimize gene flow between populations. For instance, the distance between Jaraicejo daphne stands (TJ population) and the nearest vineyards was about 12 km, and daphne plants were unknown in the Guareña vineyards (VG1 and VG2 populations). Insects were collected during May in the larval stage (mostly 4th–5th instar larvae) on grapevine inflorescences or daphne shoots. More than 50 larvae were collected per population in either commercial vineyards (>10 ha) or daphne stands (100 plants scattered over >1 ha). Larval development was completed in the laboratory at 25 ± 1ºC and 60 ± 10 % RH, under a long-day photoperiod (16L:8D) on a semi-synthetic diet (Stockel et al., 1989) to discard parasitized or diseased larvae and to produce similar-sized adults irrespective of population origin and host plant. Adults obtained from each strain were used as parents to produce virgin F1 adults for EAG tests as follows. Pairs of 2-3-day-old adults were caged in 22-ml clear plastic containers with water ad libitum, and green grapevine berries (vine strains) or daphne leaves (daphne strains) added into each container to promote egg laying. Containers and plant

material with the eggs attached were transferred before hatching to rearing containers provided with the same semi-synthetic diet, and pupae were delivered to the University of Lleida and then sexed and kept in separated environmental chambers under a 18L:6D photoregime at 23 ± 1°C. Adults were fed 10% sucrose solution and the EAG tests were performed 2-4 days after emergence.

Table 4.1. Moth larval host-plants and field collection sites

Code	Host plant	Collection date	Municipality	Site	WGS84 coordinates
VA	*V. vinifera*	May/14/2014	Arroyo de San Serván	El Calvario	38.853554, -6.442819
VG1	*V. vinifera*	May/22/2014	Guareña	Sartenillas	38.887461, -6.148765
VG2	*V. vinifera*	May/22/2014	Guareña	Pozo Calero	38.895705, -6.137196
TA	*D. gnidium*	May/14/2014	Arroyo de San Serván	Dehesa Grajera	38.860016, -6.436377
TJ	*D. gnidium*	May/20/2014	Jaraicejo	La Sarna	39.668019, -5.794076
TM	*D. gnidium*	May/20/2014	Madroñera	Dehesa de la Solana	39.442581, -5.791424

Test stimuli

We selected a set of behaviorally active volatile compounds from *V. vinifera* and *D. gnidium* (Tasin et al., 2005, 2010) (**Table 4.2**). The components of the sex pheromone of *L. botrana* (Sans et al., 2016) [(7E,9Z)-dodeca-7,9-dienyl acetate (E7,Z9-12:Ac), (7E,9Z)-dodeca-7,9-dienol (E7,Z9-12:OH), and (Z)-9-dodecenyl acetate (Z9-12:Ac)], with an isomeric purity > 93% (Pherobank, Wageningen, The Netherlands) were also tested. Chemicals were dissolved in GC-grade n-hexane (Sigma-Aldrich, Spain) and loaded in 10 µl aliquots of 10 ng/µl of pheromone compounds or 10 µg/µl of plant compounds onto n-hexane pre-cleaned filter paper pieces (0.5 x 1 cm, Whatman #1, Sigma-Aldrich, Spain). Filter papers formulated with solvent (n-hexane) alone were a control for solvent and puffing effects. The filter paper pieces were inserted into Pasteur pipettes five minutes after stimulus load, and the stimulus pipettes were stored in glass test tubes sealed with PTFE-coated screw caps until used. New stimuli cartridges were prepared each day, and a given stimulus cartridge was used for no more than 12 stimulations. Test tubes for keeping stimulus pipettes were rinsed with acetone and heated at 250ºC overnight before being reused.

Table 4.2. Plant stimuli for EAG tests

Compound	Abbr.	Host	CAS number	Product number (Sigma Aldrich)	Lot number	Purity[a] (≥ %)
1-Octen-3-ol	1OL	*V. vinifera*	3391-86-4	O5284	PR 03904AQ	98
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	DMN	*V. vinifera*	19945-61-0	[d]		
(*E*)-β-farnesene	FAR	*V. vinifera*	18794-84-8	73492		90
2-Ethyl-1-hexanol	2EH	*D. gnidium*	104-76-7	04050	BCBJ9176V	99
Benzothiazole	BEN	*D. gnidium*	95-16-9	W325600	STBC5100V	96
Ethyl benzanoate	EBZ	*D. gnidium*	93-89-0	W242209	STBC8296V	99
Linalool oxide[b]	LOX	*D. gnidium*	60047-17-8	62141	BCBM5843V	97
Linalool[c]	LOL	Both	78-70-6	L2602	STBC9155V	97
Methyl salicylate	MSL	Both	119-36-8	[d]		

[a] As indicated by manufacturer
[b] Mixture of isomers
[c] Racemic
[d] Present from Ashraf El-Sayed, New Zealand

Electroantennograms

Moths were immobilized with CO2 for 10 s and were restrained in a handcrafted poly (methyl methacrylate) insect holder. To reduce antennal movement, the head was gently squeezed with forceps - which did not affect EAG responses -, and the terminal antennal segments were excised to facilitate electric contact. Electrodes were assembled by inserting gold filaments within drawn-glass capillary tubes containing physiological saline solution (0.2M KCl). The ground electrode was inserted in the head through the mouthparts and the recording electrode contacted the tip of one antenna. The signal from the recording electrode was pre-amplified (10 X gain, Universal single ended probe, Syntech, Germany), high-pass filtered at 0.1 Hz, and digitalized (IDAC-4, Syntech, Germany). Air flows were generated by two diaphragm aquarium pumps connected to a 3-way solenoid valve (CS-55, Syntech, Germany). A 0.5 l/min flow of charcoal-filtered and humidified air blew continuously over the insect preparation through a 5-mm internal-diameter stainless steel tube placed 15-20 mm from the preparation. The stimulus pipette tip was inserted through a hole on the wall of the continuous flow tube, 110 mm from the exhaust end. A 0.5 s-long 0.2 l/min charcoal-filtered (but not-humidified) air puff was passed through the pipette to stimulate the preparation. The flow of continuous humid air decreased by 0.2 l/min during stimulation. The air around the preparation was constantly exhausted to minimize contamination. One antenna per insect was stimulated with

all test compounds presented in randomized order, with a time interval of at least 60 s between puffs.

Statistical analyses

The response to n-hexane was subtracted from the response to the test compounds in the same antenna as a control for antennal responses to physical and solvent stimulation. In the rare cases where the subtraction resulted in a negative value, a zero was used. Data were transformed [log (x + 0.1)] when needed to improve model fit. Data were analysed with linear models [lm()] using R software (R Core Team, 2019). Models testing the effects of sex, odorant stimulus, larval host-plant and their interactions were compared with ANOVAs, preferring the simplest model that was not significantly different from the next complex one. Six individuals of each sex and host-plant were analysed. Pair-wise comparisons among treatment means were performed with estimated marginal means (library *emmeans* in R, Lenth et al. 2018). Raw data, R codes and selected R outputs are available online (https://repositori.udl.cat/handle/10459.1/65163).

Results

All compounds elicited measurable EAG traces that varied between -0.004 and -3.71 mV after *n*-hexane subtraction **(Fig. 4.1**, **Fig. S4.1**). Sex, but not host plant, had a significant effect on the response to *n*-hexane, which elicited an average response of -0.17 ± 0.05 mV and -0.62 ± 0.09 mV in females and males, respectively. The model that best explained EAG responses with a dataset containing all the odorant stimuli (pheromone and plant) included as factors (a) larval host-plant, (b) odorant, (c) sex, and (d) odorant*sex interaction (**Table 4.3A**). Thus, a substantial part of the variance in the model (i.e. relatively high sum of squares) was associated to the interaction between sex and odorant, which resulted from the much higher male than female response to pheromone compounds **(Fig. 4.1**). The next important factor contributing to the model was the odorant, whereas the contributions of sex and larval host-plant, although significant, were inferior, as indicated by their relatively smaller sums of squares (**Table 4.3A**). Due to the small host-plant effect in **Fig. 4.1** the individuals from the two host plants are mixed, but in **Fig. S4.2** they are shown separately.

As the odorant*sex interaction was significant, we performed pairwise comparisons among odorants within each sex, and between sexes within each odorant **(Fig. 4.1**, **Table S4.1**). In males, the strongest response was to the major pheromone compound (*E*7,*Z*9-12:Ac), followed by the minor pheromone acetate (*Z*9-12:Ac), which in turn was not different from (*E*)-β-farnesene. In addition, all plant compounds (except 1-octen-3-ol and linalool) and the minor

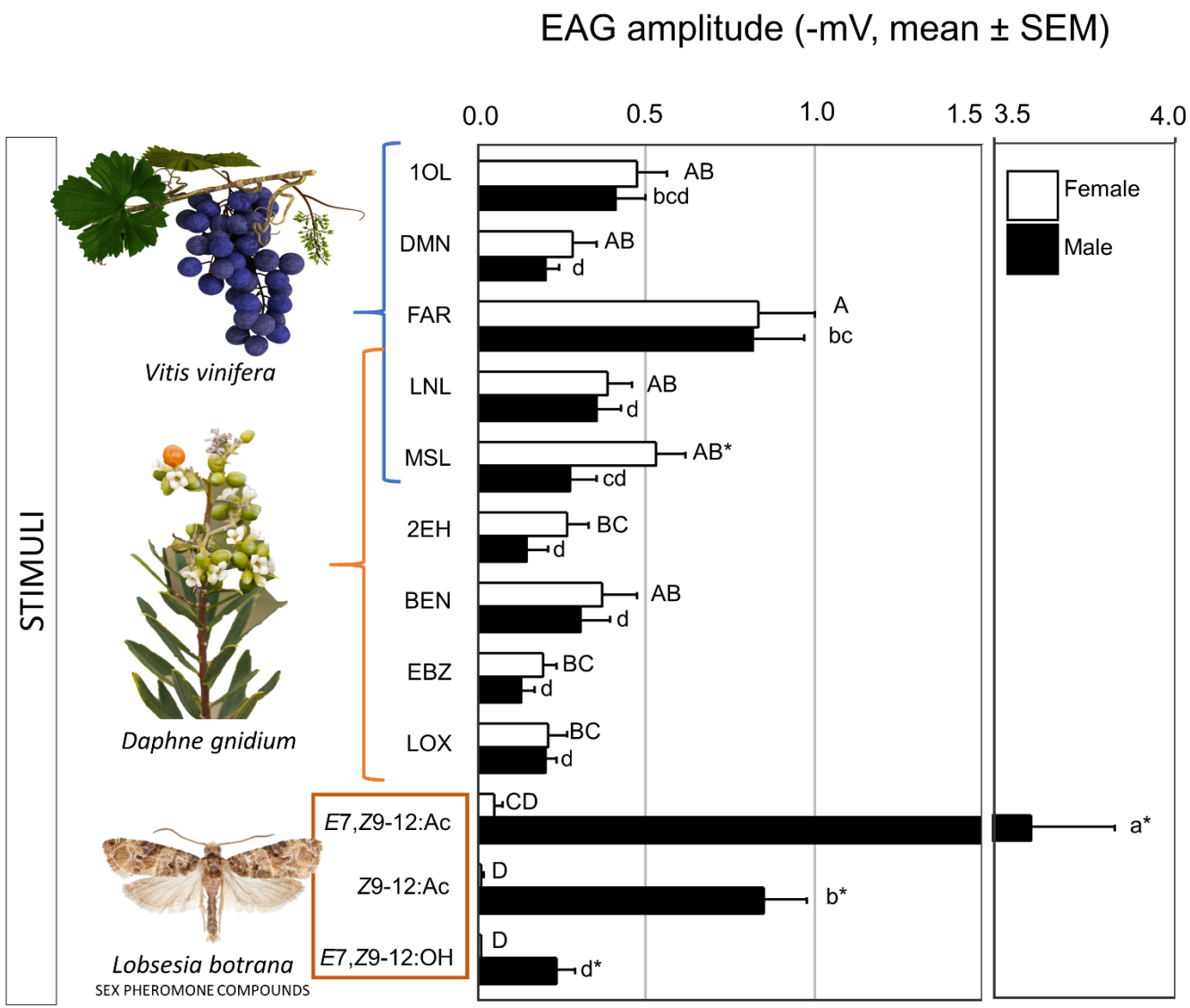

Figure 4.1. Electroantennogram responses of *L. botrana* to host plant odorants and individual pheromone compounds. Individuals from the two host plants (*V. vinifera* and *D. gnidium*) are mixed. (capital letters for females, lowercase for males), and (*) indicates significant difference between sexes (Tuckey´s pairwise test after ANOVA [Table 3A], $P < 0.05$). Plant compounds: 1OL, 1-octen-3-ol; 2EH, 2-ethyhexan-1-ol; BEN, benzothiazole; DMN, (*E*)-4,8-dimethyl-1,3,7-nonatriene; EBZ, ethyl benzanoate; FAR, (*E*)-β-farnesene; LNL, linalool; LOX, linalool oxide; MSL, methyl salicylate.

pheromone alcohol (*E*7,*Z*9-12:OH) produced lower responses than (*E*)-β-farnesene (**Fig. 4.1**, **Table S4.1**). In females, the response to (*E*)-β-farnesene was larger than to any pheromone compound and about 2 to 4-times higher than to the other plant stimuli. The response to the major pheromone compound in females was not different than the response to the plant compounds 2-ethyl-1-hexanol, ethyl benzanoate and linalool oxide. In further pairwise comparisons, sex was compared within each odorant and, except for the expected higher response of males to the sex pheromone stimuli, the only difference between sexes was a higher female response to methyl salicylate **(Fig. 4.1**, **Table S4.1**). Regarding the significant effect of the larval host-plant, we found that EAG amplitude was larger in the individuals from *V. vinifera* than in those from *D. gnidium* (**Table S4.1, Fig. S4.2**).Considering the different biological nature of sex pheromones and plant odorants, and the large odorant * sex interaction, the original dataset was split in two smaller subsets, one containing only pheromone stimuli (*pheromone dataset*) and another containing only plant stimuli (*plant dataset*), and each subset was analysed independently following the same ANOVA procedure as with the complete dataset. The model that best explained the *plant dataset* included the factors (a) larval host-plant, (b) plant odorant and (c) sex, without any significant interaction among them (**Table 4.3B**). Here, as with the entire dataset, the largest effect was due to plant odorant, followed by larval host-plant and sex. With the *plant dataset*, the response to (*E*)-β-

Table 4.3. ANOVA results using different data sets. (A) All the odorant stimuli (pheromone and plant compounds), (B) plant compounds only, (C) pheromone compounds only.

A. Pheromone and plant stimuli					
Source of Variation	**Df**	**Sum Sq.**	**Mean Sq.**	**F**	**Pr(>F)**
Larval Host-Plant	1	5.2	5.20	15.1	0.00013
Odorant	11	53.1	4.83	14.1	< 2e-16
Sex	1	7.0	7.03	20.4	9.6e-06
Odorant * Sex	11	83.5	7.59	22.1	< 2e-16
Residuals	239	82.1	0.34		
B. Plant stimuli					
Source of Variation	**Df**	**Sum Sq.**	**Mean Sq.**	**F**	**Pr(>F)**
Larval Host-Plant	1	5.3	5.33	13.78	0.00027
Plant Odorant	8	26.7	3.34	8.64	5.3e-10
Sex	1	3.1	3.15	8.13	0.00483
Residuals	187	72.3	0.39		
C. Pheromone stimuli					
Source of Variation	**Df**	**Sum Sq.**	**Mean Sq.**	**F**	**Pr(>F)**
Pheromone Compound	2	26.3	13.2	66.3	6.3e-16
Sex	1	70.1	70.1	353.6	< 2e-16
Pheromone Compound * Sex	2	15.1	7.5	38.0	2.1e-11
Residuals	60	11.9	0.2		

farnesene was higher than the response to any other compound, while the lowest responses were to 2-ethyl-1-hexanol and ethyl benzanoate (**Table S4.2**). EAG amplitude was larger in females than in males and, here again, *V. vinifera* individuals produced larger EAG responses than *D. gnidium* individuals (**Table S4.2**). In the analysis of the *pheromone dataset*, the preferred model included the factors (a) pheromone compound, (b) sex and (c) their interaction (**Table 4.3C**). The interaction was significant because females responded similarly to the three pheromone compounds whereas males responded differently to each one of them (**Table S4.3**).

Discussion

EAG responses to the three pheromone compounds were significantly larger in males than in females as previously reported for the major pheromone compound (Vitagliano et al., 2005). This is common to most moth species and it is probably related to the presence of a larger number of sex-pheromone olfactory receptor neurons (ORNs) in males than in females (De Bruyne and Baker, 2008). In males the EAG response ratios of *Z*9-12:Ac and *E*7,*Z*9-12:OH, relative to *E*7,*Z*9-12:Ac, were 0.25 ± 0.05 and 0.06 ± 0.02 (mean ± SEM), respectively. These ratios correspond roughly with the proportion of the three pheromone components in the female blend [1:0.2:0.01, reviewed in (Sans et al., 2016)]. Possibly, the proportion of ORNs tuned to each of the three pheromone compounds is relatively similar to the proportion of these compounds in the blend, as occurs in other moth species (Ammagarahalli and Gemeno, 2014) and references therein. A single-cell electrophysiology study shows that 50% of the *L. botrana* male ORNs respond to *E*7,*Z*9-12:Ac (De Cristofaro et al., 2008), which supports this hypothesis.

Female moth response to conspecific sex pheromone remains relatively unexplored (Holdcraft et al., 2016). In *L. botrana* there is behavioural evidence for autodetection of the major pheromone compound (Harari et al., 2015). In our study, none of the pheromone compounds elicited significantly higher responses than *n*-hexane in females (data not shown, $P > 0.05$). De Cristofaro et al. (2008) found that 7% of the *L. botrana* female ORNs respond to *E*7,*Z*9-12:Ac. Whether these cells are specific to the major compound or respond to other pheromone compounds, or whether there are ORNs specifically tuned to each pheromone compound in females, needs to be determined.

Analysis of the *plant dataset* revealed larger female than male EAG amplitudes, that is, regardless of the plant stimulus or host-plant population the EAG amplitude was larger in females than in males. Although the effect of sex was significant, the plant-odorant*sex

interaction was not, which means that regardless of larger EAG female responses, the relative response to the panel of plant odorants was not different between sexes. Larger female than male overall EAG amplitude to plant volatiles could result from females having more plant-sensitive ORNs than males. In several moth species the effect of sex on EAG responses to plant compounds depends largely on the compounds tested (Suckling et al., 1996; Shu et al., 1997; Ansebo et al., 2004; Vitagliano et al., 2005), while in other cases the responses are consistently larger in one sex than in the other, as in *Hyles lineata* F. (Lepidoptera: Sphingidae) (Raguso, 1996) or *Agrotis segetum* Denis et Schiffermüller (Hansson et al., 1989). In general, reported sex difference in EAG amplitude to plant compounds is relatively small, i.e. less than 1-fold, as we found in *L. botrana*, which suggests that plant detection is important to both sexes, even though males do not need to locate the host plant to lay eggs.

Methyl salicylate produced significantly larger EAG responses in females than in males (1.89 times). Higher female than male EAG responses to this compound have also been reported in another tortricid moth, *Cydia strobilella* L. (Ahman et al., 1988) (1.46 times higher), and in a sphingid moth (Raguso, 1996) (2.63 times higher). In *Cydia pomonella* L. the response to methyl salicylate also appears to be larger in females than in males (Ansebo et al., 2004). Methyl salicylate is a common plant compound involved in stress signalling (Shulaev et al., 1997), and it attracts natural enemies (James and Price, 2004). This odorant discourages female *Mamestra brassicae* L. oviposition (Ulland et al., 2007). Both, male and female *M. brassicae* show similar EAG responses to methyl salicylate (Rojas, 1999), and both have an olfactory receptor neuron type that is very specific and sensitive to this compound (Ulland et al., 2007). An odorant receptor protein (EpOR1) highly sensitive to methyl salicylate has been identified in the tortricid moth *Epiphyas postvittana* Walker and has a similar level of expression in both sexes (Jordan et al., 2009). Methyl salicylate is also one of the most active compounds eliciting locomotor behaviour of *L. botrana* larvae in a servosphere (Becher and Guerin, 2009). Thus, this compound is probably important for *L. botrana*, and further behavioural studies concerning it are warranted.

(*E*)-β-farnesene elicited the strongest response in both sexes, despite having the lowest vapor pressures of all the plant compounds tested. (*E*)-β-farnesene is one of the key volatiles that conform the most attractive *V. vinifera* volatile blend to *L. botrana* mated females, but has not been detected in volatiles collections from *D. gnidium* (Tasin et al., 2010). Relatively specific ORNs to this compound have been described in sensilla trichodea or auricillica of males and females of the tortricid moths *C. pomonella* (Ansebo et al., 2005) and *Grapholita molesta* Busk (Ammagarahalli and Gemeno, 2015). Vitagliano et al. (2005) reported larger EAG responses to

(*E*)-β-farnesene in *L. botrana* males than in females. The difference with our study could be due to the use of different puff stimuli for the standardization of EAG responses (*Z*-3-hexenol in their study, *n*-hexane in ours).

Although global EAG amplitude to plant compounds was larger in the individuals collected from *V. vinifera* than in those from *D. gnidium*, relative sensitivity to plant compounds did not differ between the two groups, as indicated by the lack of a significant host*odorant interaction, which suggests that odour blends containing mixtures of these *compounds* should be perceived similarly by the two host-plant insect groups. Behavioural responses to plant odours require brain integration of antennal input from each individual odorant in the odour blend (Haverkamp et al., 2018). Therefore, lack of statistical differences in odour discrimination at the antennal level does not imply that insects from each host would show similar preference for the two host-specific odour blends. Differences in plant preference could still occur if there are not differences at the EAG level.

The set of plant odorants tested in our study (4 *Vitis*-specific, 3 *Daphne*-specific and 2 common to both hosts) was chosen based on behavioural activity, host specificity and commercial availability. Other plant odorants like (*E*)-β-caryophyllene, which is one of the essential components in the *V. vinifera* blend (Tasin, Bäckman, Bengtsson, Ioriatti, et al., 2006), or the volatiles of tansy (*Tanacetum vulgare* L.), which flowers attract *L. botrana* adults, although it is not a suitable larval host (Gabel et al., 1992), were not included in our study. It is possible that EAG differences among the two host groups may show with other plant compounds. Yet, our study provides a first assessment on the effect of larval host-plant on *L. botrana* olfaction, and the lack of host-odour specificity suggests that if an evolutionary host-shift from *D. gnidium* to *V. vinifera* has taken place in the past (Torres-Vila and Rodríguez-Molina, 2013) it has not been accompanied by a significant modification of the moth sensory system. Reproductive isolation, if any, has not substantially modified sex pheromone detection either.

Conclusion

Our study reveals no antennal discrimination by male and female *L. botrana* adults collected as larvae from *D. gnidium* or *V. vinifera* to a set of volatiles released by both hosts or specific to each one of them. This finding suggests that males and females have a similar peripheral odour detection system to this set of plant volatiles and argues against host-plant volatile-detection specificity between individuals collected in one host or the other. Reproductive isolation, if any, has not substantially modified sex pheromone detection either. Two plant volatiles, methyl salicylate and (*E*)-β-farnesene, have shown significant antennal responses and could

have a relevant role in behaviour. Small, but significant pheromone response in females grant future SSR studies to accept or discard the pheromone autodetection hypothesis, which could be relevant in MD control strategies.

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Table S 4.1. Pairwise significance tests for the factors A) larval host-plant, B) odorant by sex, and C) sex by odorant in the ANOVA performed with the complete dataset (Table 4.3A, main text). Different letters in each section indicate significant differences among groups (Tukey´s test, P < 0.05)

A. Larval host-plant						
host	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
Vitis	0.400	0.0204	239	0.361	0.442	a
Daphne	0.302	0.0154	239	0.273	0.334	b
B. Odorant by sex						
sex = female						
odor	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
far	0.781	0.1447	239	0.5418	1.125	a
msl	0.571	0.1058	239	0.3961	0.822	ab
1ol	0.499	0.0926	239	0.3466	0.720	ab
lnl	0.426	0.0789	239	0.2953	0.613	ab
ben	0.380	0.0705	239	0.2637	0.548	ab
dmn	0.330	0.0611	239	0.2288	0.475	ab
2eh	0.301	0.0559	239	0.2092	0.434	bc
lox	0.273	0.0507	239	0.1898	0.394	bc
ebz	0.272	0.0505	239	0.1889	0.392	bc
major	0.138	0.0256	239	0.0957	0.199	cd
minac	0.110	0.0205	239	0.0766	0.159	d
minoh	0.107	0.0198	239	0.0740	0.154	d
sex = male						
odor	**emmean**	**SE**	**df**	**lower. CL**	**upper. CL**	**group**
major	3.629	0.6141	239	25.999	5.065	a
minac	0.832	0.1408	239	0.5960	1.161	b
far	0.807	0.1366	239	0.5784	1.127	bc
1ol	0.392	0.0664	239	0.2809	0.547	bcd
lnl	0.367	0.0621	239	0.2631	0.512	cd
ben	0.323	0.0547	239	0.2317	0.451	d
msl	0.289	0.0490	239	0.2074	0.404	d
lox	0.268	0.0454	239	0.1921	0.374	d
minoh	0.268	0.0453	239	0.1917	0.373	d
dmn	0.261	0.0441	239	0.1867	0.364	d
ebz	0.195	0.0330	239	0.1398	0.272	d
2eh	0.184	0.0311	239	0.1318	0.257	d
C. Sex by odorant						
odor = 1ol						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.499	0.0926	239	0.3466	0.720	a
m	0.392	0.0664	239	0.2809	0.547	a
odor = 2eh						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.301	0.0559	239	0.2092	0.434	a
m	0.184	0.0311	239	0.1318	0.257	a
odor = ben						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.380	0.0705	239	0.2637	0.548	a
m	0.323	0.0547	239	0.2317	0.451	a
odor = dmn						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.330	0.0611	239	0.2288	0.475	a
m	0.261	0.0441	239	0.1867	0.364	a
odor = ebz						

sex	response	SE	df	lower.CL	upper.CL	.group
f	0.272	0.0505	239	0.1889	0.392	a
m	0.195	0.0330	239	0.1398	0.272	a
odor = far						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.807	0.1366	239	0.5784	1.127	a
m	0.781	0.1447	239	0.5418	1.125	a
odor = lnl						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.426	0.0789	239	0.2953	0.613	a
m	0.367	0.0621	239	0.2631	0.512	a
odor = lox						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.273	0.0507	239	0.1898	0.394	a
m	0.268	0.0454	239	0.1921	0.374	a
odor = major						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	3.629	0.6141	239	25.999	5.065	a
m	0.138	0.0256	239	0.0957	0.199	b
odor = minac						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.832	0.1408	239	0.5960	1.161	a
m	0.110	0.0205	239	0.0766	0.159	b
odor = minoh						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.268	0.0453	239	0.1917	0.373	a
m	0.107	0.0198	239	0.0740	0.154	b
odor = msl						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.571	0.1058	239	0.3961	0.822	a
m	0.289	0.0490	239	0.2074	0.404	b

Table S 4.2. Pairwise significance tests for the factors A) larval host-plant, B) plant odorant, and C) sex in the ANOVA performed with the plant dataset (Table 3B, main text). Different letters in each section indicate significant differences among groups (Tukey´s test, P < 0.05)

A. Larval host-plant						
host	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
vitis	0.416	0.0261	187	0.368	0.471	a
daphne	0.300	0.0188	187	0.265	0.339	b
B. Plant odorant						
odor	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
far	0.804	0.1067	187	0.619	1.045	a
1ol	0.443	0.0587	187	0.341	0.575	b
msl	0.399	0.0529	187	0.307	0.518	bc
lnl	0.397	0.0527	187	0.306	0.516	bc
ben	0.352	0.0467	187	0.271	0.457	bc
dmn	0.293	0.0389	187	0.226	0.381	bc
lox	0.274	0.0363	187	0.211	0.355	bc
2eh	0.233	0.0309	187	0.179	0.303	c
ebz	0.230	0.0305	187	0.177	0.298	c
C. Sex						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
f	0.401	0.0263	187	0.352	0.456	a
m	0.311	0.0186	187	0.276	0.350	b

Table S 4.3. Pairwise significance tests for the pheromone compound*sex interaction [A) Pheromone compound by sex, B) sex compound by pheromone] in the ANOVA performed with the pheromone dataset (Table 3C, main text). Different letters in each section indicate significant differences among groups (Tukey´s test, P < 0.05)

A. Pheromone compound by sex						
sex = female						
odor	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
major	0.138	0.0194	60	0.1040	0.183	a
minac	0.110	0.0155	60	0.0833	0.146	a
minoh	0.107	0.0150	60	0.0805	0.141	a
sex = male						
odor	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
major	3.629	0.4665	60	28.059	4.693	a
minac	0.832	0.1069	60	0.6432	1.076	b
minoh	0.268	0.0344	60	0.2069	0.346	c
B. Sex compound by pheromone						
Major pheromone compound (*E*7, *Z*9-12:Ac)						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
m	3.629	0.4665	60	28.059	4.693	a
f	0.138	0.0194	60	0.1040	0.183	b
Minor pheromone compound *Z*9-12:Ac						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
m	0.832	0.1069	60	0.6432	1.076	a
f	0.110	0.0155	60	0.0833	0.146	b
Minor pheromone compound (*E*7, *Z*9-12:OH)						
sex	**response**	**SE**	**df**	**lower.CL**	**upper.CL**	**.group**
m	0.268	0.0344	60	0.2069	0.346	a
f	0.107	0.0150	60	0.0805	0.141	b

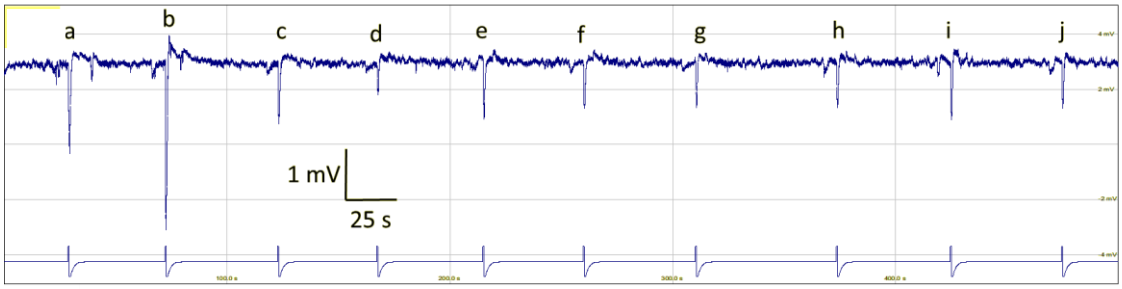

Figure S 4.1. EAG trace of a *Lobesia botrana* male collected from *Vitis vinifera* (VG2 population, EAG file 130601407). Stimuli: a) (*E*)-β-farnesene, b) major sex pheromone compound (*E*7,*Z*9-12:Ac), c) 1-octen-3-ol, d) puff and solvent control (*n*-hexane), e) methyl salicylate, f) linalool, g) minor pheromone compound (*E*7,*Z*9-12:OH), h) benzothiazole, i) 2-ethylhexan-1-ol, j) (*E*)-4,8-dimethyl-1,3,7-nonatriene. Top trace, EAG; bottom trace, puffs (0.5 s duration).

GENERAL DISCUSSION AND CONCLUSIONS

General Discussion

In a time when awareness of environmental and human health is increasing, it is more important than ever to push research forward towards environmentally safe methods to control insect pests. Hence, the focus is now shifting towards mechanisms underlying odor detection and processing with a special interest in pheromones, which have been shown to possess great potential in the improvement of insect control techniques like mating disruption (MD) (Miller and Gut, 2015). While MD appears as a prominent tool to control important insect pests in the field, it is important to remember that it is a complex phenomenon involving multiple disciplines, and thus, it can be affected by the inherent biology of the insect or variations in environmental cues (Cardé and Minks, 1995; Miller and Gut, 2015). Applying this information to develop new technologies - such as automated dispensers - will help boost their efficiency and reduce their cost, getting a step closer to sustainable agriculture (Benelli et al., 2019). My thesis contributes to increase our knowledge on the biology of two tortricid moths and will serve as a basis to explore the behavior and neuroethology of more species in the future. Furthermore, it gives specific response strategies to overcome some the challenges that can emerge from the implementation of MD in the field.

Pest monitoring: circadian rhythms and effect of environmental factors

The use of the trap served to generate a clear seasonal and daily flight curve of males towards two different baits. It permitted me to correlate the captures with environmental factors, like sunset time or temperature.

In order to extend our knowledge on the biology of insects in the field we developed an affordable and durable automated pest monitoring system presented in Chapter I, that allowed me to determine the daily and seasonal flight of the Oriental fruit moth (OFM), a polyphagous pest species that produces great economical losses in Spain and other regions (Kirk et al., 2013; CABI, 2019). I confirmed that *G. molesta* male flight activity concentrates from a few hours before dusk to shortly after dark (Rothschild and Minks, 1974; Gentry et al., 1975; Kim et al., 2011). However, insect daily activity is not fixed, and its known to be influenced by external factors (Dreisig, 1980; Beck, 2012; Groot, 2014; Saunders, 2014). My annual survey indicates that photoperiodic cues serve as a relevant factor for the determination of diel flight timing of the species, with daily rhythmicity varying throughout the year to adjust to the small shifts in sunset time, as previously outlined by the experiments of

Kim et al. (2011). This phenomenon has also been observed in other species (Batiste et al., 1973a; Lucchi et al., 2018), and for this reason dispensers programmed to adjust pheromone release to the diel flight of the male moths should incorporate the changes in sunset timing as the year advances. Doing so would help save pheromone and increase the efficacy of MD against pests. However, clean photoperiodic signals can be further modified by other factors. My study demonstrates that, as observed for this and other species, changes in temperature can alter male daily flight, with high and low temperatures advancing or delaying activity periods, respectively (Batiste et al., 1973b, 1973a; Rothschild and Minks, 1974; Cardé et al., 1996; Danks, 2003; Beck, 2012; Groot, 2014; Saunders, 2014). Temperature changes could then diminish the efficacy of MD by outspreading the activity of the individuals beyond the expected hours, stressing the importance of incorporating the possible effects of this factor while adjusting the automated pheromone dispensers.

I further hypothesized that the baits used in the field could also influence male periodicity. While septa are known to release pheromone at a continuous rate, females follow an intern circadian clock that activates their calling behavior relative to sunset and other environmental cues, just as males do (Sower et al., 1970; Baker and Cardé, 1979; McNeil, 1991; Groot, 2014). We could therefore expect that the response curve obtained with septa did not represent the periodicity of male response to live females. My results from Chapter I and Chapter II show that although male catches were higher on septa than female baited traps, *G. molesta* male diel flight towards natural (i.e., females) and synthetic pheromone sources was essentially the same. Although a similar strategy where male periodicity is not dependent from pheromone availability has been observed for other species (Batiste et al., 1973a; Cardé et al., 1974; Mitchell et al., 1974; Klun et al., 1981), in other species male flight seems to be determined by female pheromone release (Shorey, 1966; Furlong et al., 1995; Zhang et al., 1998; Stevenson and Harris, 2009). This variability in strategies indicates the need to evaluate the periodicity of each pest species individually, for different results could arrive when subjected to similar external stimuli. I believe our trap will serve as a resourceful tool to achieve this goal.

Female autodetection

Apart from the environmental cues, biological factors such as age and mating status of the individual can further alter their mating activity (McNeil, 1991; Groot, 2014), and some evidence starts to emphasize a possible effect of conspecific pheromone on female behavior (reviewed in Holdcraft et al., 2016). For females, detecting highly dense female populations could be a beneficial biological trait, so that females could disperse or aggregate to increase

their reproductive fitness (Holdcraft et al., 2016). These behaviors, when considered, could have an impact on the use of semiochemical-based management techniques, like MD. However, while male moth neuronal response to pheromone is well known, studies regarding female detection of their own sex pheromone are scarce.

An analysis comparing response to pheromone in adults of both sexes served to eliminate the variability between experiments, giving strength to our procedure. It shows that, when exposed to similar conditions, the responses in males and females are dramatically different. This is a consequence of their biological cycles, which have different needs and trigger different behaviors.

Electroantennographic as well as single sensillum recording studies on different species indicate that females of several moth species can detect and respond to conspecific pheromone, a phenomenon known as autodetection (Holdcraft et al., 2016). In Chapter III and Chapter IV, I found that female electroantennographic response of two tortricid moths was of lower magnitude than that of males. This indicated that either the female antenna was equipped with a lower proportion of pheromone-responding cells, that these cells were not as sensitive as in males, or both. Characterization of *G. molesta*'s olfactory system served to solve this question. OFM male PRNs were abundant and very sensitive and specific to pheromone, confirming previous observations by Ammagarahalli and Gemeno (2014). A hierarchical cluster analysis (HCA) could classify the PRNs in terms of their ligand as two differentiated groups of cells, depending on their sensitivity to the major (*Z*8-12:Ac) or the minor (*E*8-12:Ac) compounds, that appear in a Z:E proportion (100:8) tightly related to the pheromone blend ratio of *G. molesta* females. This ratio is species specific (Byers, 2006) and combined with the time of pheromone release (Groot et al., 2014), is pivotal to achieve reproductive isolation (Allison and Cardé, 2016), any changes translating in a decrease in attraction and mating success for males (Roelofs and Brown, 1982; Knight et al., 2015).

No analog cells to male PRNs were found in OFM females, although some of the OSNs were excited by high doses of one or both pheromone compounds. However, compared to male PRNs they were less abundant, less sensitive and lacked specificity, since they would usually respond to plant volatiles as well. Overall, these results explain the lower EAG responses observed in females, and the lack of differentiation between pheromone compounds. This lack of specific PRNs for each of the pheromone compounds would render it impossible for females to detect a change in the pheromone ratio, and thus they would not be able to distinguish their species from others that share the same compounds in different proportions (El-Sayed,

2019). Females overexposed to pheromone would then in no case show a species-specific alteration of their behavior. It is possible that the exposure of females to unnaturally high pheromone concentrations in the autodetection experiments supporting the existence of autodetection may have stimulated, or even adapted, unspecific neurons, and falsely signalled the detection of non-pheromone odors which could have triggered behavioral responses that the sex pheromone at natural concentrations would never have had. Thus, considering a concentration similar to the one found in nature, females would probably be regarded as unspecific to pheromone, and even if they could encounter concentrations as high as the ones tested in the laboratory, as could happen under MD environments, the adaptive value to females would remain unclear. Future studies trying to understand the actual scope autodetection might have in response to mating disruption should assess female response towards different pheromone blends at varying concentrations in the field.

Other odorants: plant volatiles and hairpencil pheromone

Although it is still unclear what evolutionary advantage autodetection could have for females, we know that males rely on pheromone cues to locate females and achieve reproduction, which would justify the need for more sensilla trichodea, more PRNs and the bigger size of the glomeruli where these cells innervate. Females, on the other hand, present a higher proportion of auricillic sensilla and, although lacking a MGC, can present instead a higher number of "ordinary" glomeruli and other sexually dimorphic glomeruli, as it is the case of *G. molesta* (Varela et al., 2009). The female olfactory system seems therefore to be better adapted to the perception of other compounds that can help them complete their life cycle.

As for other species where males conduct a complex courtship (Conner and Iyengar, 2016), I found that females of OFM have cells specialized in the perception of ethyl *trans*-cinnamate, a male hairpencil volatile. The perception and integration of information regarding male sex pheromone may serve as a last resource to avoid interspecies mating (Baker et al., 1981; Conner and Iyengar, 2016). Conversely, I found no male cells responding to ethyl *trans*-cinnamate, which contrasts with the finding of 19% cells responding to hairpencil compounds and to a behavioral antagonist in *H. virescens*, a moth that also uses courtship pheromone, where these cells are believed to serve males to avoid competition with individuals of the same species (Hillier and Vickers, 2007; Hillier et al., 2007). Nevertheless, it is possible that OFM males can still benefit from detecting conspecific males to avoid intraspecies competition, although this perception would be linked to secondary hairpencil compounds (Nishida et al., 1982, 1985).

Female brain configuration might also be representing their specialization in plant volatile detection. When exposed to a blend of *E*-β-farnesene, terpinyl acetate and methyl salicylate, I found that 6% of OFM female cells are responding to these compounds. Although scarce, many female OFM neurons still respond to these volatiles, and it is possible that a wider array of compounds would reveal a more sophisticated system.

Indeed, my EAG study on *L. botrana* starts to decode the complexity of moth response to plant volatiles. Female EAG response to plant volatiles was higher than male's, which could be an outcome from females having more plant sensitive ORNs than males. However, the results show that sex differences in EAG amplitude to plant compounds is relatively small, i.e. less than 2-fold, as found in other species (Hansson et al., 1989; Raguso, 1996), which suggests that plant detection is important to both sexes, even though males do not need to locate the host plant to lay eggs. It has been demonstrated that some plant compounds can have a synergic effect on the response of males to pheromone, believed to increase their possibilities of finding females (Varela et al., 2011).

Host preference: steppingstones and reservoirs

The EAG amplitude of individuals of *Vitis vinifera* was larger than that of *Daphne gnidium*'s. Nevertheless, both groups did not differ in their relative sensitivity to plant compounds, suggesting a similar perception by the two host-plant insect groups to odor blends containing a mixture of the tested compounds. However, each volatile or combination of volatiles produces a specific neural representation, activating a specific combination of glomeruli in the antennal lobe (Andersson et al., 2015). This structure of the neural system allows moths to detect and differentiate among plants emitting different combinations and proportions of a wide array of commonly encountered plant volatiles (Bruce et al., 2005; Haverkamp et al., 2018). Therefore, lack of statistical differences in odor discrimination at the antennal level does not imply that, when presented with the two host-specific odor blends, insects from each host would show a similar preference.

It is possible that EAG differences among the two host groups may show with other plant compounds. Yet, my study provides a first assessment on the effect of larval host-plant on *L. botrana* olfaction, and the lack of host-odor specificity suggests that if an evolutionary host-shift from *D. gnidium* to *V. vinifera* has taken place in the past (Torres-Vila and Rodríguez-Molina, 2013) it has not yet been accompanied by a significant modification of the moth sensory system. Furthermore, if reproductive isolation has happened, it has not substantially modified sex pheromone detection either. In a polyphagous species like *L. botrana,* the fact

that populations can evolve attraction to new hosts while retaining their ability to return to previous ones should raise the alarm when implementing control strategies. Even though they showed preference for their most suitable host, they could reproduce and survive in alternative ones under unfavorable conditions and use them as steppingstones to conquer new environments, expanding beyond the initially diagnosed limits. Potential new hosts should be considered while implementing MD, to assess possible new risks and reinforce control efficacy.

Laboratory vs. wild

Most of the knowledge on the biology of insects is obtained from laboratory experimentation. However, some studies seem to indicate that there exists a difference in the biology of wild and laboratory reared insects in their performance in the wild (Giebultowicz et al., 1992; Shelly and Edu, 2009). This was a prevalent question during my thesis, since most of the individuals I used in the experiments stemmed from a colony reared over 9 years in the laboratory. To evaluate the effect that developing conditions could have on male responsiveness towards pheromone, the response of wild and laboratory reared males towards septa and virgin females was registered and analyzed in the field (Chapter I). The curves obtained by wild and laboratory reared OFM males start to indicate that they respond similarly to each lure. Furthermore, both wild and laboratory male response time towards the baits corresponds to the period at which laboratory reared females have been described to call under constant conditions (Navarro-Roldán and Gemeno, 2017). Male and female *G. molesta* circadian rhythms seem then to be synchronized when exposed to the same environmental conditions, ensuring that males can find receptive females while they are responsive. Furthermore, laboratory reared female attractiveness does not differ substantially to that of wild females (Knight et al., 2015). Altogether, these results seem to corroborate that females and males reared in the laboratory for a long period are not essentially altered in their ability to produce and respond to plant volatiles and pheromone in the field. Furthermore, both sexes display similar relations to light, temperature and other environmental factors when exposed to natural conditions, ensuring that a great proportion of the population is active simultaneously. Hence, the use of laboratory reared insects should not induce an overall error in field experimentation.

Conclusions

Chapter I. Trap design

1. High time resolution traps can serve the purpose of monitoring male flight towards different baits and can be adapted to study many insect species.

Chapter II. Male flight

2. Septa baited traps capture more males than unmated female baited ones. However, male diel flight towards synthetic pheromone and calling females is not significantly different, indicating that the periodicity of response of males to sex pheromone is determined by their own rhythms rather than by pheromone availability.

3. Female calling in the laboratory and in the field seems to be similar. Furthermore, male flight in the wild coincides with female calling behavior in the laboratory.

4. Photoperiod, understood as the length of the day and its changes throughout the year and determined by time of sunset, is responsible for main shifts in daily periodicity of male and female mating behavior in the field.

5. Temperatures below or over activity thresholds restrain activity to specific periods in the day, advancing and delaying the expected periodicities.

6. Proper adjustment of automated pheromone dispensers requires to include these environmental inputs.

Chapter III: Female autodetection

7. *Grapholita molesta* female and male olfactory systems are different.

8. Males respond highly to sex pheromone compounds and are able to distinguish between the enantiomers, both at electronantennographic and single sensillum level. This allows them to identify their own species from others.

9. Females lack specific sex pheromone receptor neurons. Those that respond to pheromone compounds do it in an unspecific manner and would not allow them to distinguish the enantiomers. This is further reflected in the electroantennogram. The olfactory system of females would not allow them to discern their species from closely-related ones, thus autodetection (i.e. female detection of her own species sex pheromone) seems unlikely.

10. Females have cells specifically tuned to ethyl *trans*-cinnamate, the male hairpencil pheromone, a compound that serves as a close-range signal to prevent interspecies mating and involved in mate selection.

11. Both females and males present cells that respond to plant volatiles. The former would benefit from these cues to find suitable oviposition places, while males would increase their chances of locating females.

Chapter IV. Host plant

Plant volatiles

12. Individuals from *D. gnidium* and *V. vinifera* populations respond similarly to plant volatiles from both hostplants, highlighting the problematic of polyphagia.

13. *E*-β-farnesene elicits the highest response in both sexes.

14. Male and female responses are not significantly different to the compounds tested, except for methyl salicylate, that elicits higher response in females.

Sex pheromone

15. Higher response to all pheromone compounds in males than females. Male response to minor components is similar to that of some plant volatiles. Females seem to respond more to the main pheromone compound (*E*7,*Z*9-12:Ac) than to the minor ones.

16. There is no significant difference in the response of both populations to sex pheromone compounds, indicating that they are still able to respond to it and reproduce, so no reproductive isolation has taken place.

Laboratory vs. field observations

17. Laboratory reared individuals retain their ability to adapt to environmental cues in the field.

18. Wild and laboratory reared males show similar periodicities in the wild towards two different pheromone sources, indicating that male olfaction is not apparently altered after exposed to unnatural conditions for a long period.

19. Laboratory reared female pheromone production has not been essentially altered after being exposed to unnatural conditions for a long period.

As I have exposed in my Thesis, many factors affect the efficacy of control of agricultural pests, and it is necessary to consider them all to boost accurate practices in order to achieve a more sustainable agriculture. However, it is true that many of the products we demand from the fields end up in our garbage, while countless people still suffer from famine all over the world. Research can help increase production in a more sustainable way, but we have to understand that any true change towards a more sustainable world can only origin from our own responsible consumption

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