Received: 30 November 2020 | Revised: 16 November 2021 | Accepted: 13 January 2022

DOI: 10.1111/phen.12382

ORIGINAL ARTICLE

Physiological Entomology: How Insects Work - Linking Genotype to Phenotype

Royal Entomological Society

Laboratory investigation of pheromone pre-exposure in *Lobesia botrana* males indicates minor role of desensitization in the field

Aitor Gavara | Vicente Navarro-Llopis | Jaime Primo | Sandra Vacas

Centro de Ecología Química Agrícola, Instituto Agroforestal del Mediterráneo, Universitat Politècnica de València, Valencia, Spain

Correspondence
Aitor Gavara, Vicente Navarro-Llopis, and Sandra Vacas, Centro de Ecología Química Agrícola, Instituto Agroforestal del Mediterráneo, Universitat Politècnica de València, Edificio 6C, Camino de Vera s/n, 46022 Valencia, Spain.
Email: aigavi@etsiamn.upv.es, sanvagon@ceqa.upv.es, and vinallo@ceqa.upv.es

Funding information
Conselleria de Agricultura, Medio Ambiente, Cambio Climtico y Desarrollo Rural (Generalitat Valenciana), Grant/Award Number: S8456000

Abstract

The role of desensitization in mating disruption (MD) of European Grapevine Moth (EGVM), *Lobesia botrana* (Dennis and Schiffermüller) (Lepidoptera: Tortricidae), is studied by exposing males to different airborne pheromone concentrations for various time intervals prior to evaluate their behavioural response. By using different types of dispensers, male moths are exposed to three decreasing pheromone concentrations (up to the minimum concentration able to desensitize them) for 1, 3, and 24 h. The airborne pheromone concentration present in all treatments is accurately quantified using gas chromatography–mass spectrometry equipment to compare these data with actual airborne pheromone concentrations under field conditions. The effect of pheromone exposure on *L. botrana* males is studied in wind tunnel and electroantennogram assays, immediately and 24 h after the exposure period. Results show that the reduction of response to the pheromone depended on the dose and the duration of the treatment, but the lowest dose tested did not produce desensitization. These results support that the most important mechanisms of MD acting in fields treated against EGVM are the competitive ones and that the non-competitive ones only may become relevant very close to pheromone dispensers. Our results support the hypothesis of cross-adaptation to other chemical stimuli in the pre-exposed moths to their pheromone although more studies are needed to draw stronger conclusions.

KEYWORDS
European grapevine moth, habituation, Lepidoptera, mating disruption, peripheral adaptation, Tortricidae

INTRODUCTION

The European Grapevine Moth (EGVM), *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae), is a key pest widespread among wine producing areas with a particular impact in southern Europe. The damage associated with this pest is produced after larvae feeding on the grape and following increases in susceptibility to fungal infections, such as *Botrytis cinerea* (Persoon: Fries) (Sclerotiniaceae) (Ioriatti et al., 2011). This pest has been traditionally managed with pesticides, but given the rising awareness about the detrimental and toxic effects on human health and the environment, the use of more environmentally friendly control strategies, such as mating disruption (MD), is gaining importance (Ioriatti et al., 2009; Witzgall et al., 2010). This pest control technique relies on the release of synthetic sex pheromones to delay, reduce or prevent mating and, therefore, reduce the pest population on the crop (Witzgall et al., 2010). MD has demonstrated effectiveness against important lepidopteran pests so nowadays it is widely used with successful results (Witzgall et al., 2010). MD was used against EGVM over 249,000 ha of vineyards in Europe in 2017 (Lucchi &

Benelli, 2018) by releasing the main component of its sex pheromone, (*E*,*Z*)-7,9-dodecadienyl acetate (Lucchi et al., 2018; Pasquier & Charmillot, 2005) to the crop environment to prevent mating of the target species (Ioriatti et al., 2011).

MD treatment has typically consisted of hand-applied passive dispensers (small plastic containers filled or impregnated with the pheromone) deployed in the field at high densities of more than 200 dispensers ha^{-1} (Lucchi et al., 2018). However, another type of dispensers, the programmable automatic aerosol devices, have demonstrated similar efficacy at very low densities, 2–5 units ha^{-1} (Miller & Gut, 2015). Hand-applied dispensers release the pheromone passively through their walls at relatively constant rates. In contrast, aerosol devices release the pheromone via programmed discharges making this system independent of external conditions (Benelli et al., 2019; Evenden, 2016).

The mechanisms involved in MD against pests are classified as competitive or non-competitive. In the case of the competitive mechanisms, males are able to respond to the females' signal because they are not impaired physiologically. In this case, disruption is the result of competition between females and dispensers (Evenden, 2016; Miller & Gut, 2015). Non-competitive mechanisms result from an effect on signal composition or physiological impairments to the female or male and include among others: (1) desensitization, which includes sensory adaptation of the peripheral level and habituation of the response in the central nervous system; and (2) camouflage, when a female's plume becomes imperceptible because of its combination with the released plume (Evenden, 2016, Miller & Gut, 2015). Under field conditions, although there is one main mechanism, pest populations are being affected simultaneously by different MD mechanisms (Evenden, 2016, Miller & Gut, 2015). Therefore, greater understanding of the mechanisms governing MD under field conditions should guide development of pheromone dispensers with improved efficacy and reduced cost (Gordon et al., 2005). Moreover, understanding the mechanisms and their relative importance in depth could lead to a greater standardization of MD product comparisons (Judd et al., 2005).

Sensory adaptation has not been demonstrated in all insect orders. In the case of Lepidoptera, it is described as a disrupting mechanism in many studies under laboratory conditions (D'Errico et al., 2013; Judd et al., 2005; Stelinski, Gut, et al., 2003; Stelinski, Gut, & Miller, 2005; Stelinski, Miller, et al., 2003; Suckling et al., 2018). In these studies, the electrophysiological and behavioural male responses decreased after prolonged pheromone exposures, but an increase of both parameters was demonstrated in other cases after pulsed or brief pheromone pre-exposures (Mori & Evenden, 2014; Quero et al., 2014; Stelinski, Gut, Epstein, et al., 2005). However, its role is not clear in MD-treated fields due to the high pheromone concentrations needed to disrupt males. Although competitive attraction by false-trail following seems to be the main mechanism acting in fields treated with passive dispensers (Miller et al., 2006), there are studies highlighting that this mechanism could be ineffective without others acting in conjunction (Cardé et al., 1998; Sanders, 1996).

In particular, for the EGVM, competitive disruption was demonstrated to be the main factor for an effective MD. The competition between females and dispensers increases with the dispensers' pheromone dose, whereas camouflage was described to be one mechanism that possibly acts near the dispensers (Schmitz et al., 1995). With regard to the non-competitive mechanisms, Schmitz et al. (1997) excluded their effect under field conditions, despite their demonstration at very high doses in laboratory assays. Nevertheless, Trimble and Marshall (2010) pointed out the impossibility to examine the relationship between these processes and the airborne pheromone concentration due to the lack of an accurate control of the emission sources as seen in Schmitz et al. (1997), in which pheromone amounts were not quantified with absolute data.

Previous laboratory assays, aimed at elucidating the role of non-competitive mechanisms in treated fields, have been performed by exposing males with presumed high airborne pheromone concentrations but, in many cases, exposure duration was no longer than 60 min and it did not provide any precise data on exposure concentrations (Judd et al., 2005; Stelinski, Gut, et al., 2003; Stelinski, Gut, & Miller, 2005; Stelinski, Miller, et al., 2003). However, it is still unknown whether longer duration of conditioning, as moths experience in MD-treated fields, could increase the disrupting effect at different airborne pheromone concentrations.

For this purpose, in this study, the response of EGVM males was assessed after treatments combining different airborne pheromone concentrations and exposure duration by means of electroantennography (EAG) and wind tunnel assays conducted immediately and 24 h after treatment.

METHODS

Insects

L. botrana individuals were obtained from a 2-year-old colony, annually restocked with field-collected individuals, reared at our facilities at Universitat Politècnica de València (Valencia, Spain), fed on semi-synthetic diet—based on grape juice, crushed dried vine leaves, brewer's yeast, wheat germ, and corn flour in a 6:2:1:1:1 ratio—in a climatic chamber. Individuals were kept under 18:6 (L:D) photo-period at 22°C and 70% RH. Assays were made with 3–4 days old adult males, kept separated from females in different chambers. Due to the lack of accentuated sexual dimorphism in adult stage, sexing and selection of males were made in the pupal stage.

Moths pheromone exposure

Male moths were kept in 1.3-L glass chambers, in which the different pheromone airborne concentrations were supplied (Figure S1). To reach such concentrations, the glass chamber with males was joined upstream to a smaller glass chamber in which different passive dispensers were introduced. An air compressor (Jun-air Intl. A/S,

Norresundby, Denmark) coupled with an AZ 2020 air purifier system (Claind Srl, Lenno, Italy) was connected to the dispenser pre-chamber to provide a 20 ml min^{-1} ultrapurified airflow. Downstream the conditioning glass chamber, a Pasteur pipette loaded with 400 mg Porapak-Q cartridge was connected to collect and quantify the pheromone emitted at known intervals.

The different doses were provided by three different types of passive dispensers: Lobetec, commercial dispensers employed for MD in vineyards (Lobetec, SEDQ SL, Barcelona, Spain); Septum, commercial rubber septa monitoring dispensers Grapemone (OpenNatur, Lleida, Spain); and Vial, experimental dispenser, which consisted of 4-ml low-density polyethylene tubes (5 and 1.4 cm in diameter; Kartell, Fisher Scientific, Madrid, Spain) loaded with 50 mg cotton impregnated with 30 mg pheromone (ca. 95% purity). These experimental dispensers were prepared to obtain an intermediate emission level between MD and monitoring dispensers. Once prepared, these dispensers were aged for 24 h in a laboratory fume hood to stabilize their emission.

The different treatments tested resulted from combining two parameters, airborne pheromone quantity—determined by the different dispensers—and duration of exposure. Groups of 10 male moths were exposed to each of the three doses for 1, 3, and 24 h, which resulted in nine treatments. Each exposure treatment was replicated using 20 and 10 males for wind tunnel and EAG assays, respectively.

Before each treatment, the experimental assembly was provided with the same airflows and dispensers for 24 h to saturate the bonding sites of the glass chamber, ensuring a balance exchange of pheromone molecules. In this way, when the males were introduced and the different exposure times were performed, all the pheromone released reached the Porapak filter. This procedure was confirmed in preliminary tests for every type of dispenser, obtaining constant amounts of airborne pheromone into the glass chamber.

In the present study, the response of males to pheromone was studied by wind tunnel assays and EAG tests, immediately and 24 h after exposure to different pheromone treatments according to Miller and Gut (2015). During those 24 h before the biological tests, male-exposed groups were kept in mesh rearing cages (Bugdorm, Entomopraxis, Barcelona, Spain) to allow ventilation, in the same chamber conditions as those described for the stock colonies and isolated from females.

Airborne pheromone quantification for exposure treatments

Chemicals and reagents

All solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany). (*E*,*Z*/*E*)-7,9-dodecadien-1-yl acetate (ca. 95%) was supplied by Ecología y Protección Agrícola S.L. (Carlet, Valencia, Spain). A straight chain fluorinated hydrocarbon ester—heptyl 4,4,5,5,6,6,7,7,8,8,9,9,9-tridecafluorononanoate (TFN)—was selected as an internal standard for airborne pheromone quantification to improve both sensitivity and selectivity for the spectrometric method optimization (Gavara et al., 2020).

Quantification of the airborne pheromone in the conditioning chamber

Given that hourly collections of pheromone on Porapak filters for some dispenser treatments were below detection limits, the airborne pheromone quantifications were performed after 24-h exposure treatments for each type of dispenser in triplicate.

After the treatment, the Porapak filters were eluted with 20% Et_2O/Hexane (12 ml) to recover the pheromone collected during the 24-h period. Each solution was concentrated up to ca. 1.5 ml using a rotary evaporator set at 30°C, and finally to 0.5 ml in a 2-ml gas chromatography (GC) screw-cap vial (Fisher Scientific SL, Madrid, Spain) blown down with a gentle stream of nitrogen. Ten microliters of an internal standard solution of TFN were added to the solution for the final pheromone quantification by GC coupled with mass spectrometry. The samples were analysed using a TSQ 8000 Evo (Thermo Fisher Scientific, Waltham, Massachusetts) triple quadrupole MS/MS instrument operating in selected reaction monitoring mode using electron ionization (EI+), coupled with a Thermo Scientific™ TRACE™ 1300 GC. All injections were made onto a ZB-5 (30 m × 0.25 mm × 0.25 mm) fused silica capillary column (Phenomenex Inc., Torrance, CA). The oven was held at 60°C for 1 min then raised at 10°C min^{-1} up to 110°C, maintained for 5 min, raised at 3°C min^{-1} until 150°C and finally raised at 35°C min^{-1} up to 300°C held for 5 min. The carrier gas was helium at 1.5 ml min^{-1}.

For each target compound—TFN (the internal standard) and EGVM pheromone, (*E*,*Z*)-7,9-dodecadien-1-yl acetate—the MS/MS method was optimized in tests carried out previously (Gavara et al., 2020) by selecting the precursor ion and the product ions that provided the highest selective and sensitive determinations: 393-373 and 164-79, precursor–product ions (m/z) employed to obtain the chromatographic areas for TFN and (*E*,*Z*)-7,9-dodecadien-1-yl acetate, respectively.

The amount of pheromone and the corresponding chromatographic areas were connected by fitting a linear regression model, $y = a + bx$, where y is the ratio between pheromone and TFN areas and x is the amount of pheromone. Data obtained were divided by the total air volume that went through the conditioning chamber during the treatment to obtain the airborne pheromone concentrations as mg or μg per m^3.

Wind tunnel tests

To investigate physiological impairments in males generated by previous pheromone exposure, their ability of plume odour detection was evaluated in wind tunnel tests. A plexiglass wind tunnel (63 × 63 × 215 cm, Analytical Research Systems, Gainesville, Florida) with charcoal filters in its terminal section and a centrifugal fan in the

opposite part allowed the aspiration and filtration of the air flow set at 2 m s^{-1}. The wind tunnel was housed in a climatic room, kept at 22°C, 70% RH, and lit at 10 lux with a lamp.

A Delta trap (Biagro SL, Valencia, Spain) with a sticky base and baited with a monitoring pheromone dispenser (Grapemone lure; OpenNatur, Lleida, Spain) was placed at the end of the flight section as a pheromone stimulus. A 30-cm plexiglass stand, as the take-off area to release males, was placed at the opposite end, 120 cm downwind the trap.

Males were individually placed in glass tubes (7 cm length and 3 cm diameter), with both ends capped with muslin pieces, for 20 min in the same room of the wind tunnel for their acclimatization. Then, still confined in the tube, males were transferred to the tunnel stand and maintained for 2 min of acclimatization before assaying activity. After this time, the muslin was removed and male behaviour responses to the pheromone stimulus were observed and recorded. The pheromone stimulus remained inside the tunnel from the beginning of the acclimatization to the end of the experiment. Males not showing any response in the next minute were considered inactive. However, being there any type of behaviour after their release, the males were considered as active and were observed for two additional minutes. Moth behaviour was scored for wing fanning, take-off, zigzag flight and reaching the source, all behavioural steps involved in moth mating behaviour. Reaching the source included landing on the delta trap surface (landing on the trap but not entering into it) or sticking on the base (getting into the trap and landing on the sticky base). Twenty different males were tested for each of the treatments on different days and from different generations. Although in an ideal experimental design, replications of all treatments should be done in the same days to avoid the possible effects of environmental parameters such as the atmospheric pressure (Mori & Evenden, 2014; Wins-Purdy et al., 2008), this was not possible to perform in our study due to the high number of treatments and the short period in which EGVM males are active (2 h after the scotophase).

EAG assays

EAG assays were carried out to determine if there was any change of response of male moths exposed to pheromone treatments. Males were immobilized into test tubes in an ice bath to excise their heads, keeping the antennae attached to extend their lifespan. Among two and five terminal segments of the antenna were removed with a scalpel (Schmitz et al., 1997). Silver wire electrodes were used, previously impregnated with salt-free hypoallergenic electrode gel (Spectra 360, Parker Laboratories, Inc., Fairfield, New Jersey), to increase the electrical contact. The head was set on the reference electrode and the distal part of the antenna was set on the recording electrode.

One side of a glass elbow-sized tube (L-tube) was placed at less than 2 cm distance from the connected antenna. The other side was connected to a Tygon hose (Saint-Gobain SA, Courbevoie, France) through which a wet and carbon-filtered airflow (50 ml min^{-1}) passed, delivered by a Syntech CS-55 stimulus controller (Ockenfels Syntech GmbH, Kirchzarten, Germany). A pore-sized opening in the elbow part of the L-tube was used to introduce the tip of a Pasteur pipette previously charged with a 1-cm^2 piece of filter paper (Whatman No. 1; Scharlab, Barcelona, Spain) impregnated with each stimulus. The EAG responses were recorded with a Syntech IDAC 2 acquisition controller and the GC-EAD 32 (v. 4.3) software was employed for data recording and acquisition (Ockenfels Syntech GmbH, Kirchzarten, Germany).

Every antenna was stimulated as follows: (1) clean air (clean piece of paper inside the pipette, to account for the flow distortion caused by the paper), ensuring the correct adjustment of the antenna and the absence of mechanical responses (ca. 0 mV); (2) 1-hexanol (98%), by impregnation of the filter paper piece with 5 μl of a 0.001 mg ml^{-1} solution in *n*-hexane, as a control exposure to make sure that the antenna was alive and properly connected; and (3) (*E*,*Z*)-7,9-dodecadien-1-yl acetate by impregnation of the filter paper piece with 5 μl of a 0.001 mg ml^{-1} solution in *n*-hexane. The solvent control (5 μl of *n*-hexane) was also performed to verify the absence of response to the solvent (ca. 0 mV in all cases). The stimuli were triggered with an air push through the pipette (50 ml min^{-1}, 0.5 s), in which the sample was volatilized reaching the antennae preparation. Every stimulus was done with a different Pasteur pipette and a different piece of filter paper to avoid contamination and mixed responses. Every stimulus was triggered 20 s after loading the corresponding filter paper. The time between the different stimuli was about 60 s. Each male moth was tested only once, so only one antenna was tested. Data registered were the maximum potential triggered by the stimuli (mV). Ten antennae replicates were performed for every treatment with different males from different generations.

Data analysis

Statistical analyses were performed using SPSS v. 16 (Armonk, New York) software. For the study of each behavioural activity in the wind tunnel tests—activation, wing fanning, take-off, zigzag flight and reach the source—analyses were done using a χ^2 test to find statistical differences ($p < 0.05$) among the different treatments immediately and 24 h after the treatments.

The analysis of variance (ANOVA), followed by post hoc Fisher's least significant difference (LSD) test at $p < 0.05$, was used to study the differences observed among the EAG results. All the ANOVA analyses were applied to these data without transformation, as they fulfilled the homoscedasticity requirements and the residuals fitted normal distributions (the standardized skewness and kurtosis values ranged from −2 to +2 in a normal probability plot).

RESULTS

Airborne pheromone concentrations in the different exposure treatments

The mean airborne pheromone concentrations provided by each type of dispenser were determined as: Lobetec, 1.95 ± 0.24 mg m^{-3}; Vial; 7.44 ± 1.83 μg m^{-3}; and Septum, 1.97 ± 0.44 μg m^{-3}.

Wind tunnel assays

Data obtained from the wind tunnel tests were expressed as the percentage of male moths performing the different actions registered. Eighty percent of the control moths were scored for activation, wing fanning, and take-off. Zigzag flight was observed for 50% of the control moths, whereas only 40% finally reached the source (Figure 1).

Immediately after exposure

The different treatments only had moderate effects on male activation (Figure 1). The exposure treatments with Septum showed the highest proportion of active moths without differences against control treatment ($p > 0.05$); however, this value decreased significantly to less than 50% when moths were exposed to Lobetec dispensers for 3 and 24 h ($\chi^2 = 3.956$, $p = 0.047$ and $\chi^2 = 5.227$, $p = 0.022$ respectively).

Changes in wing fanning activity were similar to those recorded for the activation. Moths activity was reduced after exposure to Lobetec dispensers, to 40%, 20%, and 25% when exposed for 1, 3, and 24 h, compared with non-treated moths ($\chi^2 = 6.667$, $p = 0.01$; $\chi^2 = 14.400$, $p < 0.001$ and $\chi^2 = 12.130$, $p < 0.001$). The exposure to Septum and Vial did not significantly alter wing fanning behaviour as compared with the control moths ($p > 0.05$).

Regarding take-off, moths started to behave significantly different to control immediately after being exposed to Vial dispensers for 3 and 24 h, reducing their response by 50% ($\chi^2 = 5.227$, $p = 0.020$ and $\chi^2 = 3.956$, $p = 0.047$). The most significant differences were obtained by exposing males to Lobetec, regardless of the duration of

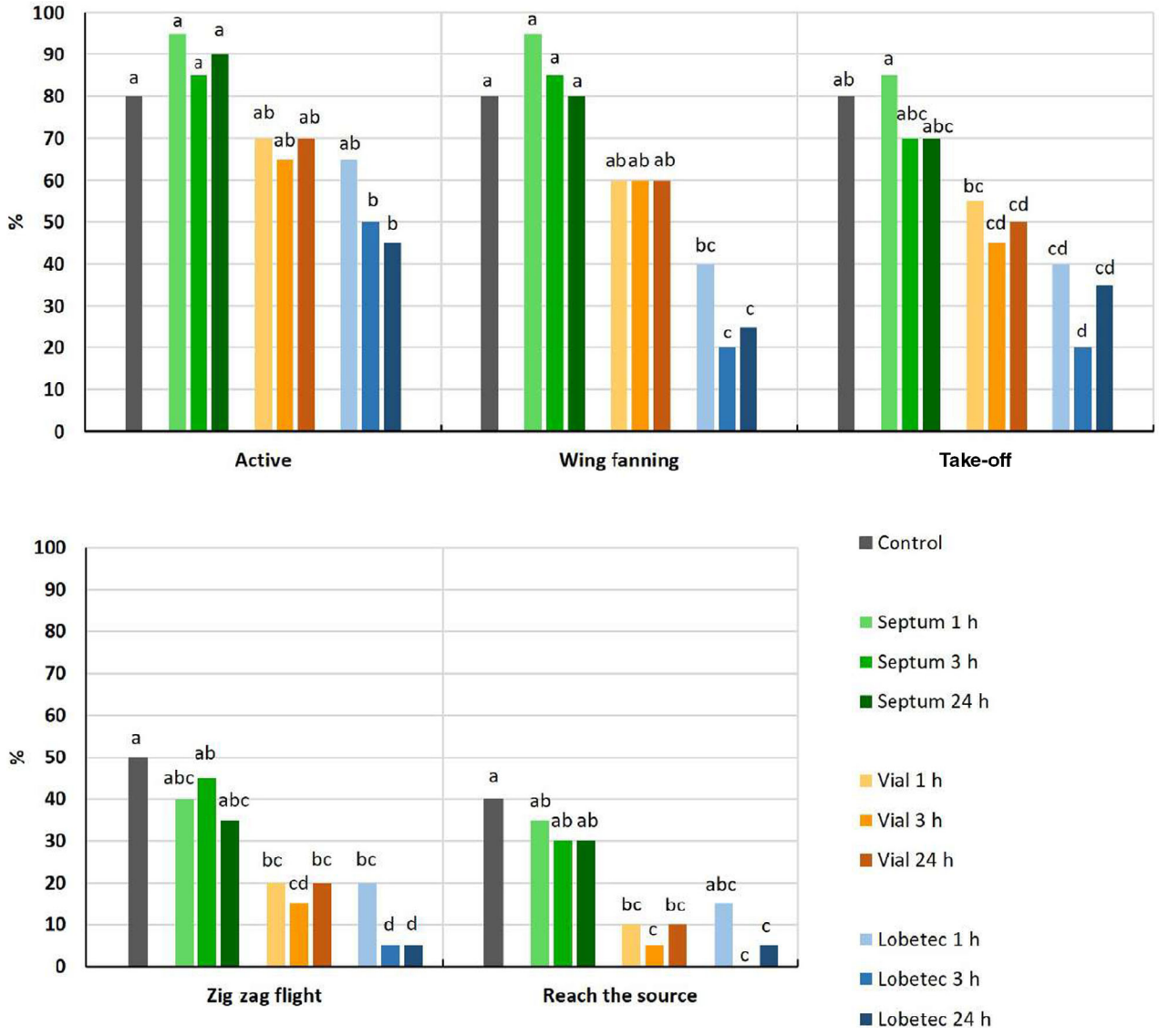

FIGURE 1 Percentage of male moths that showed activation, wing fanning, take-off zigzag flight and reached the source immediately after being exposed to the different treatments. For each parameter, bars labelled with different letters show significant differences among treatments (χ^2 test, $p < 0.05$)

Physiological Entomology: How Insects Work - Linking Genotype to Phenotype

exposure ($\chi^2 = 6.667$, $p = 0.01$; $\chi^2 = 14.400$, $p < 0.001$ and $\chi^2 = 8.286$, $p = 0.004$ for 1, 3, and 24 h respectively), reducing their response up to even 80% of the control males' response. Septum did not affect male take-off ($p > 0.05$).

In the case of moths that performed orientation flight and the typical zigzag flight of many lepidopterans (Figure 1), the results were similar to those showed for take-off without statistical differences in Septum against control ($p > 0.05$). However, significant differences were observed when males were exposed to Vial in front of the naïve ones, reducing their response from 50% to ca. 20%, regardless of the exposure duration ($\chi^2 = 3.956$, $p = 0.047$; $\chi^2 = 5.584$, $p = 0.018$ and $\chi^2 = 3.956$, $p = 0.047$, respectively, for 1, 3, and 24 h stimulus duration). When exposed to Lobetec for longer duration (3 and 24 h), the percentage of moths performing this behaviour significantly decreased to the lowest values, less than 10% ($\chi^2 = 10.157$, $p = 0.001$ for both treatments).

Forty percent of the control moths finally reached the pheromone source (Delta trap) and Septum exposure did not statistically differ from control treatment ($p > 0.05$). This value started to significantly decrease immediately after exposure to Vial ($\chi^2 = 4.800$, $p = 0.028$; $\chi^2 = 7.025$, $p = 0.008$ and $\chi^2 = 4.800$, $p = 0.028$, respectively, for 1, 3, and 24 h stimulus duration), whereas the lowest values were observed after exposure to Lobetec during 3 and 24 h, 0% and 5% of the tested moths, respectively ($\chi^2 = 10.000$, $p = 0.002$ and $p = 0.028$; $\chi^2 = 7.025$, respectively).

Twenty-four hours after the exposure

When treated-moths were kept under a free-pheromone environment for 24 h, their behaviour responses in the wind tunnel experiments were the same as those displayed by the naïve moths for all the parameters studied ($p > 0.05$ in all cases).

EAG assays

Immediately after the exposure

The treatment had significant effects ($F = 19.133$, df = 9.90, $p < 0.001$) on the EAG signals triggered by the main component of the pheromone (Figure 2a). The exposure to the Septum dispensers did not significantly affect male EAG responses compared with those displayed by control moths, regardless of the time of exposure ($p > 0.05$ in all three cases), although the 1 h treatment differed significantly from the 24 h exposure ($p < 0.01$). When moths were exposed to Vial dispensers for 1 h, antennal response was not significantly altered compared with control moths ($p > 0.05$); however, the treatments with Vial dispensers during 3 and 24 h reduced significantly the response ($p < 0.01$), showing values lower than 3.53 mV (reductions of above 29%). The maximum reduction (83% reduction compared

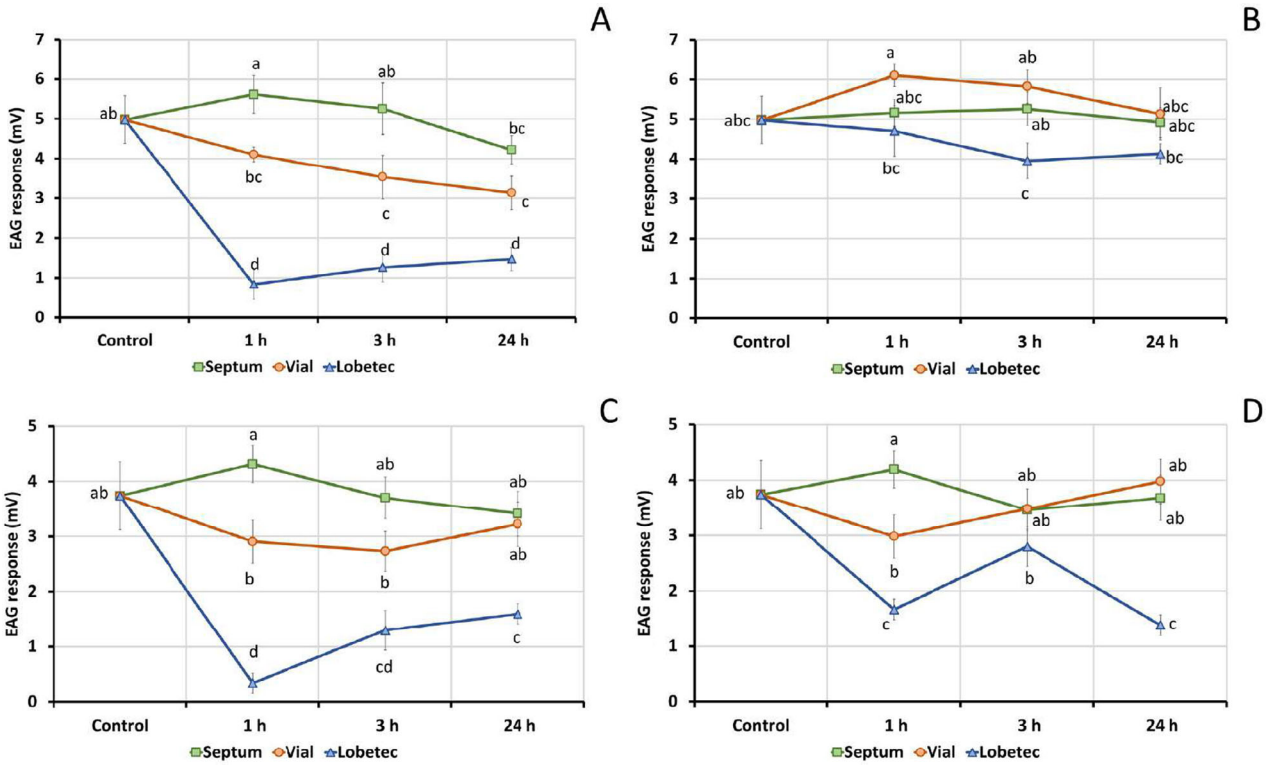

FIGURE 2 EAG response (mV) triggered by the main component of the pheromone in the antenna of male moths ($n = 10$) (a) immediately and (b) 24 h after their exposure to the different treatments: Septum, Vial and Lobetec dispensers for 1, 3, and 24 h; and by 1-hexanol (c) immediately and (d) 24 h after their exposure to the same treatments. Different letters indicate significant differences among treatments (ANOVA LSD test, $p < 0.05$). ANOVA, analysis of variance; EAG, electroantennography; LSD, least significant difference

with the control, with only 0.84 mV) was reached by the treatments with Lobetec, regardless of exposure duration ($p > 0.05$).

Similar results were obtained in the case of the responses triggered by 1-hexanol ($F = 10.003$, df = 9.90, $p < 0.001$) (Figure 2c). Olfactory detection was not significantly affected when male moths were exposed to treatments with Septum or Vial dispensers, when compared with the control ($p > 0.05$). However, highly significant reductions were observed with treatments with Lobetec dispensers ($p < 0.001$); reaching EAG signal reductions higher than 67%, up to 0.34 mV.

Twenty-four hours after the exposure

When male moths were kept for 24 h under a free-pheromone environment, EAG responses recovered to control levels. For the pheromone stimulus (Figure 2b), the responses were completely recovered, not significantly differing from those of control moths ($F = 1.770$, df = 9.90, $p > 0.05$). Nevertheless, males exposed to Lobetec displayed the lowest registered values, which significantly differed in some cases from those of the treatments with Septum or Vial.

For the 1-hexanol stimulus (Figure 2d), some treatments still had a significant effect on the EAG responses ($F = 5.652$, df = 9.90, $p < 0.05$). Although the males exposed to all the treatments done with Septum and Vial dispensers responded similarly to control moths ($p > 0.05$), those treated with Lobetec during 1 and 24 h kept lower values ($p < 0.05$)—1.67 and 1.38 mV, respectively, representing 55.35% and 63.11% of signal reduction compared with control.

DISCUSSION

In our study, the response of EGVM males to pheromone is assessed after treatments combining different airborne pheromone concentrations and exposure duration by means of EAG and wind tunnel assays. We found that males can be disrupted non-competitively by high airborne pheromone concentrations, suggesting that peripheral adaptation is the mechanism that desensitizes and prevents males to reach the pheromone source.

Miller and Gut (2015) pointed out the importance of studying which mechanisms could act to reach MD when developing new pheromone dispensers. Depending on the acting mechanisms, different dispenser densities and emission rates can be preferable. For instance, for pests that can be controlled via competitive mechanisms, the lower pheromone quantity capable of attracting males would be sufficient but if desensitization benefits MD efficacy, the lower amount which maximizes this effect would be the lower rate of pheromone required.

Our wind tunnel assays show that the exposure of males to certain pheromone concentrations significantly reduced the percentage of individuals who responded to standard conditions. The higher pheromone concentration statistically reduces the activity of males in all the parameters studied whilst the behaviours performed by males under the lower concentration did not differ from those performed by the control moths. Under the vial pheromone concentration different results are obtained following the sequence of the studied behaviours. Although the first steps studied (activation and wing fanning) do not differ from control moths, the results show that the last steps of the behavioural sequence (from take-off to reach the source) are significantly affected compared with control moths. In this way, whilst there are studies in which only activation and wing fanning have been used to detect signs of habituation in male behaviour (Bartell & Lawrence, 1973; Suckling et al., 2018), our results highlight the importance of studying the time-sequenced behaviours: taking flight in the stimulus direction, the zigzag flight and the arrival to the source. In fact, the most important effects have been observed in the final phases of the oriented behaviour, as reported in similar studies (Judd et al., 2005; Trimble, 2012).

The exposure to the highest pheromone dose (1.95 mg m^{-3} with Lobetec) produced the lowest EAG responses, incapacitating male moths to orient themselves and reach the pheromone source. By contrast, the lower doses tested performed differently. Although the treatment with 7.44 μg m^{-3} (Vial) is able to immediately prevent males from orienting their flight and reaching the source, the concentration of 1.97 μg m^{-3} (Septum) is not enough to produce this effect. This suggests that the amount of airborne pheromone capable to disrupt non-competitively EGVM males would be between this range of concentrations. These results agree with the lower EAG responses triggered by the pheromone when males are exposed to concentrations of 1.95 mg m^{-3} (regardless the period of exposure) or 7.44 μg m^{-3} (for 3 or more hours), suggesting that peripheral adaptation is the mechanism acting, in this case, immediately after male pre-exposure.

Schmitz et al. (1997) studied non-competitive mechanisms of EGVM through two different experiments: by recapturing males previously exposed to commercial MD dispensers after 24 h, and measuring the male antennal response against different concentrations impregnating several rubber septa with different flow levels. Results of recapture experiments demonstrate lower captures of pheromone-exposed males during 3 and 8 h. The results of the EAG show significant signal reductions using the higher pheromone flows and a very quick recovery, reaching 70% of the pre-treatment amplitude response after 5 min. To explain the apparent contradiction of both experiments the authors concluded that different mechanisms were acting in both experiments. The smaller response would be produced by peripheral adaptation in the EAG assays, whilst habituation of the central nervous system would participate in the release-recapture field assay. Despite this logical statement, no EAG assays are done to corroborate habituation and reject long lasting peripheral adaptation in the field as suggested later by Miller and Gut (2015). Moreover, the different dispensers used in both experiments could lead to very different levels of pheromone concentration and thus, results could not be comparable.

The results of this study support desensitization as a non-competitive MD mechanism that can be triggered in EGVM with high amounts of pheromone and support that long-lasting peripheral

adaptation could be the acting mechanism in the moth releasing experiment—as demonstrated for several tortricid species, for example, *Choristoneura. rosaceana* (Harris) (Stelinski, Gut, et al., 2003; Stelinski, Miller, et al., 2003), *Pandemis pyrusana* Kearfott (Stelinski, Gut, & Miller, 2005) and *C. pomonella* (Judd et al., 2005; Stelinski, Gut, & Miller, 2005)—due to the lack of capacity to find the source in the wind tunnel was accompanied by lower EAG amplitudes. Despite the lack of statistical differences among the different treatments in our EAG assays, when responses were evaluated 24 h after the treatment, the lower signals showed by the pre-exposed moths to the highest concentration may suggest a gradual recovery. However, further assays are needed to ensure that pre-exposed moths do not present habituation in periods shorter than 24 h in the pheromone-free atmosphere to prove our hypothesis and reject firmly the past results.

In other studies, pheromone dose has been reported as a dependent parameter increasing peripheral adaptation (Judd et al., 2005; Schmitz et al., 1997; Stelinski, Gut, et al., 2003; Stelinski, Gut, & Miller, 2005), whilst the effect of exposure time has been demonstrated in other studies (Judd et al., 2005; Wins-Purdy et al., 2008). In experiments exposing *Cydia pomonella* (L.) males to its pheromone (Judd et al., 2005), they found that pre-exposure duration seemed to have more effect on reducing the responsiveness than the dose. In our results, this dependency of time can also be seen since the effects of the treatments with Septum and Vial on EAG responses are higher as the time of exposure increased. The plateau of peripheral adaptation is reached with the 7.44 μg m^{-3} treatment between 1 and 3 h exposure periods. Nevertheless, the strongest effects (80% of EAG signal inhibition) are obtained after 1 h with the highest dose, 1.95 mg m^{-3}. Stelinski, Miller, et al. (2003) determined a plateau after 15 min of treatment with 36 ng pheromone ml^{-1} (ca. 36 mg m^{-3}), reducing about 60% the EAG signal for the tortricid *C. rosaceana*. They hypothesized that not all the receptors adapt completely because of the biological disadvantage for males to become completely anosmic to its pheromone, something that needs to be proved.

We also found a reduction of the 1-hexanol EAG amplitudes in a similar extent as the pheromone results. This effect is also observed by D'Errico et al. (2013) and Stelinski, Miller, et al. (2003) when exposing male moths to their pheromone, inducing sensory adaptation for the pheromone components and the control stimuli used. Both authors attributed this effect to the fatigue produced due to the very high amounts of stimuli employed. These results motivate further detailed studies on these interactions since this effect has a potential utility for developing new dispensers, for example, in the use of MD blends that targets multiple species but could contain antagonists (Evenden et al., 1999; Porcel et al., 2015; Suckling et al., 2016, 2018).

The pheromone concentrations tested here and in the aforementioned studies are still high. In our case, 1.97 ± 0.44 μg m^{-3} is not able to desensitize EGVM males. Recent studies based on volatile collections and chemical analysis have allowed the determination of the absolute airborne pheromone concentrations present in fields treated with MD (Gavara et al., 2020). Accordingly, the average maximum concentration detected in vineyards treated with passive dispensers was of about 40 ng m^{-3}, which is, by far, lower than the concentration needed to trigger the non-competitive mechanisms in laboratory conditions (at least 7 μg m^{-3}). Thus, the average airborne concentration found in treated fields is insufficient to disrupt moths non-competitively.

However, these mechanisms still could be acting near the dispensers. Cardé et al. (1998) suggested habituation of male pink bollworm moths after close contact with Isomate "rope" dispensers using field flight tunnels. These data could indicate that, in treated fields, males could experience this instantaneous and short-duration peripheral adaptation. Regarding this phenomena, Miller and Gut (2015) discussed about the behavioural differences between two different species, *Grapholita molesta* (Busck) and *C. pomonella*. *Grapholita molesta* would need substantially less pheromone to be disrupted since the peripheral adaptation they suffer diminishes the orientation behaviour near the dispensers; meanwhile, *C. pomonella* is able to get closer to the dispensers without experimenting desensitization. Accordingly, microencapsulated and female-equivalent dispensers are effective against *G. molesta* but not against *C. pomonella*. Our wind tunnel results obtained for the highest concentration (commercial dispenser) suggest that *L. botrana* could be experiencing the same phenomenon as *G. molesta* near the dispensers, although it should be proved for shorter exposition times. This would support the dominance of competitive disruption of *L. botrana*, mechanisms that could also be promoted in the field by the already demonstrated canopy-pheromone interaction (Karg et al., 1994), especially relevant when employing MD aerosol devices (Gavara et al., 2020). Leaves have been proven to adsorb part of the pheromone emitted by the dispensers in treated fields; thus, due to the higher amounts of pheromone emitted by the aerosol devices, the leaves near them could release high amounts of pheromone in a short period of time. These results support the results of Welter et al. (2005) and McGhee (2014) in which fewer moths are trapped in front of an aerosol device.

In conclusion, EGVM males can be disrupted non-competitively by high airborne pheromone concentrations, suggesting that peripheral adaptation is the mechanism that desensitize and prevent males to reach the pheromone source. Due to the high amounts of pheromone needed to trigger this mechanism, it seems clear that it only acts at short distances from the dispenser in field conditions, becoming especially relevant for the aerosol devices.

ACKNOWLEDGMENTS

This work was supported by *Conselleria de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural* (*Generalitat Valenciana*) under grant agreement S8456000. The authors would like to thank Ana Castellar for language editing.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS

Aitor Gavara, Sandra Vacas, Vicente Navarro-Llopis and Jaime Primo designed the experiments. Aitor Gavara, Sandra Vacas and Vicente Navarro-Llopis performed the experiments and analysed the results.

All authors contributed to the writing of the manuscript and have read and agreed to the published version of the manuscript

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Aitor Gavara https://orcid.org/0000-0003-1607-9131

REFERENCES

Bartell R.J., Lawrence L. A. (1973) Reduction in responsiveness of males of Epiphyas postvittana (Lepidoptera) to sex pheromone following previous brief pheromonal exposure. *Journal of Insect Physiology*, 19, (4), 845–855.

Benelli, G., Lucchi, A., Thomson, D. & Ioriatti, C. (2019) Sex pheromone aerosol devices for mating disruption: challenges for a brighter future. *Insects*, 10, 308.

Carde Ring T., Staten Robert T., Mafra-Neto Agenor (1998) Behaviour of pink bollworm males near high-dose, point sources of pheromone in field wind tunnels: insights into mechanisms of mating disruption. *Entomologia Experimentalis et Applicata*, 89, (1), 35–46.

D'Errico, G., Faraone, N., Rotundo, G., De Cristofaro, A. & Trimble, R.M. (2013) Sensory adaptation of antennae and sex pheromone-mediated flight behavior in male oriental fruit moths (Leptidoptera: Tortricidae) after prolonged exposure to single and tertiary blends of synthetic sex pheromone. *Environmental Entomology*, 42, 548–557.

Evenden, M. (2016) Mating disruption of moth pests in integrated pest management: a mechanistic approach. In: *Pheromone communication in moths*, Vol. 24. California: University of California Press, pp. 365–394.

Evenden M.L., Judd G.J.R., Borden J.H. (1999) Pheromone-mediated mating disruption ofChoristoneura rosaceana: is the most attractive blend really the most effective?. *Entomologia Experimentalis et Applicata*, 90, (1), 37–47.

Gavara, A., Vacas, S., Navarro, I., Primo, J. & Navarro-Llopis, V. (2020) Airborne pheromone quantification in treated vineyards with different mating disruption dispensers against *Lobesia botrana*. *Insects*, 11, 289.

Gordon, D., Zahavi, T., Anshelevich, L., Harel, M., Ovadia, S., Dunkelblum, E. et al. (2005) Mating disruption of *Lobesia botrana* (Lepidoptera: Tortricidae): effect of pheromone formulations and concentrations. *Journal of Economic Entomology*, 98, 135–142.

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

Ioriatti, C., Bagnoli, B., Lucchi, A. & Veronelli, V. (2009) Vine moths control by mating disruption in Italy: results and future prospects. *Redia*, 87, 117–128.

Judd Gary J.R., Gardiner Mark G.T., DeLury Naomi C., Karg Gerhard (2005) Reduced antennal sensitivity, behavioural response, and attraction of male codling moths, Cydia pomonella, to their pheromone (E,E)-8,10-dodecadien-1-ol following various pre-exposure regimes. *Entomologia Experimentalis et Applicata*, 114, (1), 65–78.

Karg G., Suckling D. M., Bradley S. J. (1994) Absorption and release of pheromone ofEpiphyas postvittana (Lepidoptera: Tortricidae) by apple leaves. *Journal of Chemical Ecology*, 20, (8), 1825–1841.

Lucchi, A. & Benelli, G. (2018) Towards pesticide-free farming? Sharing needs and knowledge promotes Integrated Pest Management. *Environmental Science and Pollution Research*, 25, 13439–13445.

Lucchi, A., Ladurner, E., Iodice, A., Savino, F., Ricciardi, R., Cosci, F. et al. (2018) Eco-friendly pheromone dispensers—a green route to manage the European grapevine moth? *Environmental Science and Pollutution Research*, 25, 9426–9442.

McGhee, P.S. (2014) *Impact of high releasing mating disruption formulations on (male) codling moth, Cydia pomonella L., behavior*. A dissertation submitted in partial fulfillment of the requirements for the degree of Entomology-Doctor of Philosophy, Michigan: Michigan State University.

Miller, J.R. & Gut, L.J. (2015) Mating disruption for the 21st century: matching technology with mechanism. *Environmental Entomology*, 44, 427–453.

Miller J. R., Gut L. J., de Lame F. M., Stelinski L. L. (2006) Differentiation of Competitive vs. Non-competitive Mechanisms Mediating Disruption of Moth Sexual Communication by Point Sources of Sex Pheromone (Part I): Theory1. *Journal of Chemical Ecology*, 32, (10), 2089–2114.

Mori, B.A. & Evenden, M.L. (2014) Efficacy and mechanisms of communication disruption of the red clover casebearer moth (*Coleophora deauratella*) with complete and partial pheromone formulations. *Journal of Chemical Ecology*, 40, 577–589.

Pasquier, D. & Charmillot, P.J. (2005) Survey of pheromone emission from different kinds of dispensers used for mating disruption in orchards and vineyards. *IOBC-WPRS Bulletin*, 28, 335–340.

Porcel, M., Sjöberg, P., Swiergiel, W., Dinwiddie, R., Rämert, B. & Tasin, M. (2015) Mating disruption of *Spilonota ocellana* and other apple orchard tortricids using a multispecies reservoir dispenser. *Pest Management Science*, 71, 562–570.

Quero, C., Vidal, B. & Guerrero, A. (2014) EAG responses increase of *Spodoptera littoralis* antennae after a single pheromone pulse. *Natural Product Communications*, 9, 1934578X1400900810.

Sanders, C. (1996) Effects of prolonged exposure to different concentrations of synthetic pheromone on mating disruption of spruce budworm moths in a wind tunnel. *The Canadian Entomologist*, 128, 57–66.

Schmitz, V., Renou, M., Roehrich, R., Stockel, J. & Lecharpentier, P. (1997) Disruption mechanisms of pheromone communication in the European grape moth *Lobesia botrana* Den & Schiff. III. Sensory adaptation and habituation. *Journal of Chemical Ecology*, 23, 83–95.

Schmitz V., Roehrich R., Stockel J. (1995) Etude du mécanisme de la confusion sexuelle pour l'Eudémis de la vigneLobesia botranaDen. et Schiff. (Lep., Tortricidae). I. Rôles respectifs de la compétition, du camouflage de la piste odorante et de la modification du signal phéromonal. *Journal of Applied Entomology*, 119, (1-5), 131–138.

Stelinski, L.L., Gut, L.J., Epstein, D. & Miller, J.R. (2005) Attraction of four tortricid moth species to high dosage pheromone rope dispensers: observations implicating false plume following as an important factor in mating disruption. *IOBC-WPRS Bulletin*, 28, 313.

Stelinski Lukasz L., Gut Larry J., Miller James R. (2003) Concentration of air-borne pheromone required for long-lasting peripheral adaptation in the obliquebanded leafroller, Choristoneura rosaceana. *Physiological Entomology*, 28, (2), 97–107.

Stelinski L. L., Gut L. J., Miller J. R. (2005) Occurrence and Duration of Long-Lasting Peripheral Adaptation Among Males of Three Species of Economically Important Tortricid Moths. *Annals of the Entomological Society of America*, 98, (4), 580–586.

Stelinski, L.L., Miller, J.R. & Gut, L.J. (2003) Presence of long-lasting peripheral adaptation in Oblique-banded Leafroller, *Choristoneura rosaceana* and absence of such adaptation in Redbanded Leafroller, *Argyrotaenia velutinana*. *Journal of Chemical Ecology*, 29, 405–423.

Suckling D.M., El-Sayed A.M., Walker J. T. S. (2016) Regulatory Innovation, Mating Disruption and 4-PlayTM in New Zealand. *Journal of Chemical Ecology*, 42, (7), 584–589.

Suckling, D.M., Stringer, L.D., Jiménez-Pérez, A., Walter, G.H., Sullivan, N. & El-Sayed, A.M. (2018) With or without pheromone

habituation: possible differences between insect orders? *Pest Management Science*, 74, 1259–1264.

Trimble, R.M. (2012) Sexual behavior of *Grapholita molesta* and *Choristoneura rosaceana* (Lepidoptera: Tortricidae) in a flight tunnel after prolonged exposure to the aerial concentration of pheromone previously measured in orchards treated with pheromone for mating disruption. *Environmental Entomology*, 41, 1481–1493.

Trimble, R.M. & Marshall, D.B. (2010) Differences in the relationship between sensory adaptation of antennae and concentration of aerial pheromone in the Oriental Fruit Moth and Obliquebanded Leafroller (Lepidoptera: Tortricidae): implications for the role of adaptation in sex pheromone-mediated mating disruption of these species. *Environmental Entomology*, 39, 625–632.

Welter, S., Pickel, C., Millar, J., Cave, F., Van Steenwyk, R. & Dunley, J. (2005) Pheromone mating disruption offers selective management options for key pests. *California Agriculture*, 59, 16–22.

Wins-Purdy, A.H., Judd, G.J. & Evenden, M.L. (2008) Mechanisms of pheromone communication disruption in Choristoneura rosaceana exposed to microencapsulated (Z)-11-tetradecenyl acetate formulated with and without horticultural oil. *Journal of Chemical Ecology*, 34, 1096–1106.

Witzgall, P., Kirsch, P. & Cork, A. (2010) Sex pheromones and their impact on pest management. *Journal of Chemical Ecology*, 36, 80–100.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Figure S1 Assembly scheme of the conditioning setup (dispenser pre-chamber, conditioning chamber and Porapak filter) to expose male moths during the experiment at different airborne pheromone amounts. Arrows mark the flow direction.

How to cite this article: Gavara, A., Navarro-Llopis, V., Primo, J. & Vacas, S. (2022) Laboratory investigation of pheromone pre-exposure in *Lobesia botrana* males indicates minor role of desensitization in the field. *Physiological Entomology*, 47(2), 126–135. Available from: https://doi.org/10.1111/phen.12382