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Chem. Senses 34: 763–774, 2009
doi:10.1093/chemse/bjp060
Advance Access publication September 21, 2009

Plant Terpenes Affect Intensity and Temporal Parameters of Pheromone Detection in a Moth

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Abstract

In moths, the components of the female pheromone blend are detected in the male antennae by pheromone olfactory receptor neurons (Ph-ORNs) expressing narrowly tuned olfactory receptors. Responses to sex pheromones have generally been thought to be independent from the odorant background. However, interactions between pheromone components and plant volatiles have been reported at behavioral and detection levels. To document the mechanisms of such interactions, we analyzed Ph-ORN responses of *Spodoptera littoralis* to the main pheromone component, Z9E11-14:Ac, in the presence of 4 monoterpenes. To mimic natural contexts in which plant odors and pheromone emanate from different sources, the 2 stimuli were presented with different temporal patterns and from independent sources. Linalool reversibly reduced the firing response to Z9E11-14:Ac and produced an off effect. Geraniol and geranyl and linalyl acetates reduced the responses to Z9E11-14:Ac with a longer time course. Pulses of linalool over prolonged pheromone stimulation resulted in a discontinuous firing activity. Pulses of pheromone were better separated over a background of linalool, compared with odorless air. The data confirm that plant compounds may modulate the intensity and the temporal coding by Ph-ORNs of pheromone information. This modulation might positively affect mate location at high pheromone density especially nearby a pheromone source.

Key words: odorant interactions, olfaction, olfactory receptor neurons, plant volatile compounds

Introduction

Moth sex pheromones constitute a remarkably efficient communication system serving mate recognition and mate location. A high specificity of the detection is essential to the recognition of the proper mate. The components of the female-emitted pheromone blend are detected in the male antennae by specialized olfactory receptor neurons (Ph-ORNs). Each Ph-ORN expresses an olfactory receptor (OR) type narrowly tuned to one component of the pheromone blend (e.g., Jacquin-Joly and Lucas 2005). The perception of the specific pheromone blend triggers upwind navigation and in-flight orientation by the male. It involves that the sensory system is able to monitor the fast changes in the pheromone path in the atmosphere.

Under natural conditions, however, pheromones are emitted among complex mixtures of other volatile compounds, including those released by surrounding vegetation. There are now growing evidences that some of the volatile organic compounds within this rich odorant environment, whose concentrations overcome that of pheromone, affect its perception. However, the mechanisms are still not clear and probably not unique. Many aspects of moth behavior are modulated by plant volatile compounds (Landolt and Phillips 1997; Reddy and Guerrero 2004). Enhancement of attraction of male moths to the sex pheromone by adding plant volatile compounds to the lure has been reported in several moth species, *Plutella xylostella* (Reddy and Guerrero 2000), *Spodoptera exigua* (Deng et al. 2004), *Cydia pomonella* (Light et al. 1993; Yang et al. 2004), and *Helicoverpa zea* (Light et al. 1993), for instance, and suggests that it might be an universal phenomenon.

Much effort has been devoted to investigate the potential use of pheromones for the control of insect pest species. The success of these alternative methods relies not only on the high attractivity and specificity of synthetic attractants but

also on their capacity to keep the same level of attractivity in various environments. Thus, it is of paramount importance to determine whether the presence of an odorant background alters the perception of the pheromone and particularly the capacity of the Ph-ORNs to properly code the quality, intensity, and temporal parameters of the pheromone signal.

The capacity of some volatile organic compounds to interfere with pheromone detection by Ph-ORNs has been acknowledged for a long time. Schneider et al. (1964) showed that a stimulation with a high dose of geraniol suppressed the response of the Ph-ORNs to the natural pheromone in *Antheraea pernyi*. Geraniol, linalool, and other terpenes reduced receptor potential and decreased firing response of Ph-ORNs to the pheromone in the tortricid moth, *Adoxophyes orana* (Den Otter et al. 1978). Similar inhibition of Ph-ORNs by geraniol was also observed in ermine moths (Van der Pers et al. 1980). Linalool inhibited the after response of the Ph-ORNs to (*Z,E*)-4,6-hexadecadiene, a pheromone analog, in *Bombyx mori* (Kaissling et al. 1989). Altogether, these data collected repeatedly from various species suggest that general odorants interact with pheromone primary detection as a noise that decreases the detection of the specific signal.

However, contrasting with a general inhibitory effect, Ph-ORNs were found to respond to high doses of plant volatiles in *Agrotis segetum* (Hansson et al. 1989). Linalool and a green leaf volatile, (*Z*)-3-hexenol, were found to increase the responses of Ph-ORNs to (*Z*)-11-hexadecenal, the main pheromone component, when presented as a blend to the antennae of male *H. zea*, a noctuid moth (Ochieng et al. 2002). This led the authors to postulate that synergy at the Ph-ORN level could significantly contribute to the enhanced male behavioral response observed in several species (Landolt and Phillips 1997).

This discrepancy of effects of the same odorants between species led us to reinvestigate the effects of linalool, geraniol, linalyl acetate, and geranyl acetate on pheromone detection in a different species, the Egyptian cotton leafworm moth, *Spodoptera littoralis*. We recorded the firing activity from Ph-ORNs tuned to (*Z*,*E*)-9,11-tetradecadienyl acetate (Z9E11-14:Ac), the main component of the pheromone blend produced by female *S. littoralis*. Z9E11-14:Ac elicits successive steps of the male sex behavior, from wing fanning to oriented flight to the source in a wind tunnel (Haines 1983; Quero et al. 1996). Linalool is present in the odor of undamaged leaves of cotton (Röse et al. 1996; Jönsson and Anderson 1999) and maize (Gouinguené et al. 2003), 2 major host plants of *S. littoralis*. Its release is increased in damaged plants (Gouinguené et al. 2003). The activity of linalool was compared with that of geraniol, a structural isomer, and of their acetate derivatives, geranyl and linalyl acetates. The 3 compounds are components of the volatile emissions of several plant species. Furthermore, geranyl acetate has been found as a minor constituent of the emissions of maize (Gouinguené et al. 2003; Degen et al. 2004). To mimic a natural odor context in which volatiles from plants and from conspecifics emanate from temporally and spatially distinct points, sources for both compounds were kept separate, and the 2 stimuli were presented with different temporal patterns. Attention was paid to calibrate the stimulus concentration in air and to use ratio of pheromone–plant compound ratio consistent with natural levels. To assess statistically the effects of plant compounds, we measured response parameters related to intensity and temporal characteristics of the firing response.

Materials and methods

Insects

Spodoptera littoralis were reared in the laboratory on an artificial diet at 22 °C, at 60–70% relative humidity, and under a 16:8 h light:dark (LD) photoperiod till emergence. Sexes were separated at pupal stage and maintained in different climatic chambers under an inverted LD regime. Male adults were provided with a 10% sucrose solution. One- to 3-day-old males were used for electrophysiological studies.

Electrophysiology

Male moths were anesthetized with CO_2 and restrained in a Styrofoam holder. A chlorinated silver wire was inserted into the abdomen to serve as reference electrode. One antenna was fixed with small strips of adhesive tape on the surface of the holder. Single sensillum recordings were obtained from trichoid hairs using tip recording (Kaissling 1974). The tips of a few olfactory hairs were cut off using sharpened forceps. These sensilla were sampled among the long trichoid hairs that have been shown to house one ORN tuned to Z9E11-14:Ac (Ljungberg et al. 1993; Quero et al. 1996). The recording electrode filled with sensillum saline (10^{-3} M Ca^{++} solution after Pezier et al. [2007] modified from Kaissling and Thorson [1980]) was slipped over the end of the cut trichoid hair.

Both electrodes were connected to a preamplifier NL 102 (Digitimer). The signal was amplified (×1000) and filtered (0.2–10 kHz). It was digitized at 10 kHz and 12 bits with a Data Translation DT3001 board (Data Translation). Spike firing was analyzed using Awave software (Marion-Poll 1995) to detect and sort spikes and calculate the time of occurrence of individual spikes. Receptor potentials were recorded according to the same procedures except for the filters set from DC to 10 kHz.

Experiments were started less than 1 min after connecting the recording electrode to a sensillum, and the recording session lasted less than 10 min for one sensillum. Experiments were repeated on at least 5 different animals, with a maximum of 5 sensilla for the same insect. Recording sessions from 2 sensilla of a same insect were separated by 5 min.

Stimulus chemicals

(*Z*,*E*)-9,11-tetradecadienyl acetate (“pher,” >97% purity checked by gas chromatography [GC], CAS 50767-79-8)

was synthesized in the laboratory (courtesy of Martine Lettere). Dilutions were prepared in hexane (>98% purity, CAS 110-54-3) from Carlo-Erba.

Linalool (*lin*, racemic, 97% purity, CAS 78-70-6), geraniol (*ger*, 96% purity, CAS 106-24-1), geranyl acetate (mixture of isomers, >97% purity, CAS 16409-44-2) and Linalyl acetate (97% purity, CAS 115-95-7) were purchased from Sigma. White mineral oil from Sigma (CAS 8042-47-5) was used to prepare volume-to-volume dilutions of the 4 terpenes.

Olfactory stimulation

Olfactory stimuli were delivered with a programmable olfactometer that used distinct sources for pheromone and plant compounds.

Airflows

Air coming from the building supply was charcoal filtered and humidified. To create 8 equal flows, the main flow of pure air was first divided by 2 in a Y connector (model 1/8″ P514, Upchurch Scientific). The resulting flows were divided into 4 equal flows by a 5-port manifold (model P-115, Upchurch Scientific). Each of the 8 flows was connected to a miniature electrovalve (model LHDA1233115H, The Lee Company). The output of each valve was connected to a stimulus source through a polytetrafluorethylene (PTFE) tubing (1.32 mm inner diameter [i.d.], 15 cm length).

Stimulus sources

Odorant solutions were contained in 4-ml glass vials, closed by septum corks. The inlet and outlet of the sources were made of 2 hypodermic needles (18-g size) inserted through each septum and connected to PTFE tubing. For terpenes, the vial contained 1 ml of mineral oil mixed with the appropriate volume of compound to achieve dilutions of 1%, 0.1%, or 0.01% v/v. For pher, a volume of hexanic solution was dropped off onto a piece of PTFE tube (1.32-mm i.d., 15 mm length) to ensure a final deposit of 0.1, 1, or 10 μg. The tube was inserted into the input needle of the vial after evaporation of the solvent.

Stimulation pencil

An 8-channel stimulation pencil was made by sealing 8 PTFE tubes (1.32-mm i.d., 35 cm length) with epoxy glue into a 10-cm stainless steel cylinder (7-mm i.d.). Each PTFE tube ended exactly at the opening of the metal cylinder and was connected from its other side to a stimulus source. A low-binding plastic pipette cone (volume 1 ml), with its tip cut to make an opening of 3- to 4-mm diameter, was inserted at the output of the pencil body. Thus, each odorized channel was kept separated and odorants mixed only in the plastic cone. The stimulation pencil was mounted on a micromanipulator, and the outlet of the cone was positioned 6 ± 2 mm from the insect antenna. The position of the stimulator pencil with respect to the antenna was kept constant throughout an experiment.

Stimulus sequences

Programming of the electrovalves was performed using an 8-channel Valve Bank (AutoMate Scientific). The antenna was permanently bathed by pure air at 440 ml/min delivered by 2 identical channels that mixed in the stimulation cone. During stimulation periods, pure air was replaced by either odorized air (pheromone or plant odor) or air passing over pure mineral oil (blank). The channels with plant odor and pheromone were simultaneously activated to apply binary mixtures. A triggering signal was used to synchronize the acquisition of the electrophysiological signal with the stimulation program.

Every sensillum was challenged with a series of 4 stimulations applied in an alternate order from one sensillum to another. The series included an odorless stimulation (blank), a combination of pher plus a terpene applied according to various time schedules, pher plus blank, and a terpene plus blank. Sensilla were let under pure air during 1 min between stimulations. Odorant or blank was applied either as a short (0.5 s unless indicated) single presentation designated as "puff," a prolonged (2.5 s) stimulation ("background"), or pulsed stimuli ("pulses" of 0.1 s). Pher was evaluated at 1-μg load. and monoterpenes, particularly lin, were used as 1% v/v dilutions, unless indicated. The different experiments are described hereafter.

Experiment 1

Puffs of lin and pher were delivered single, simultaneously or one after the other.

Experiment 2

A puff of one compound was delivered in the middle of a background of the other one.

In Experiment 2.1, the antenna was stimulated with a puff of pher either in lin or blank background, a blank puff in lin background, or a blank puff in blank background.

Experiment 2.2 was symmetrical to Experiment 2.1 with a puff of lin in a background of pher.

We performed similarly additional series with lower doses of pher (0.01, 0.05, and 0.1 μg) combined with lin 1% and 2 dilutions (0.01% and 0.1%) of lin associated with 1 μg pher.

Experiment 3

Geraniol (ger), geranyl acetate, or linalyl acetate at 1% v/v dilution was evaluated in the place of lin in independent series as in Experiment 2.

Experiment 3.1: A puff of pher in a background of each terpene as in Experiment 2.1.

Experiment 3.2: A puff of each terpene in a background of pher as in Experiment 2.2. An additional series was performed with a puff of geraniol of 1 s.

Experiment 3.3: A puff of pher delivered 0.1 or 0.5 s after a puff of ger.

Experiment 4

A background was applied alone during 0.5 s and then mixed with the other odor pulsed (pulses of 0.1 s delivered at 5 pulses/s) during 1.9 s. Six independent experiments were realized using lin, ger 1%, and ger 0.1% and the standard combination of presentations.

Experiment 4.1: Pulsed pher in continuous terpene.

Experiment 4.2: Continuous pher in pulsed terpene.

Calibration and maintenance

The sources of terpenes were renewed weekly. Pher loading was changed daily. A new cone was used every day. Before testing a new compound, the vials were replaced, and the stimulation pencil was decontaminated by passing a flow of charcoal-filtered air for 1 h through the pencil heated at 160 °C. Contaminated air was evacuated from the electrophysiological setup by an exhaust fan.

The airflows in the 8 channels were regularly checked with an electronic flowmeter (Humonics ADM 1000, J & W Sc) and set at 220 ± 10 ml/min. We checked that a single-valve opening produced a square stimulus by recording the signal with a hot-wire anemometer whose probe was placed at 10 mm from the cone output. The various stimulus sequences did not modify the flow, except a transient pressure change at opening and closing of the valve (signal change of ca. 10% of the total flow). The hot-wire anemometer indicated that the air reached the probe 10–15 ms after the opening of the valve. The opening of the valve was used as the time marker for the stimulus onset.

We used solid-phase microextraction (SPME) to quantify the concentrations of odorants released during stimulation. Fibers (Supelco) coated with 100-μm polydimethylsiloxane were conditioned for at least 10 min at 270 °C. To sample the odors from the stimulus source, the fiber was introduced in the cone opening. The sampling time was 5–15 min. The samples were analyzed immediately after collection by GC on a Delsi Nermag DN 200 equipped with an apolar column (RTX-5SilMS, 30 m × 0.25 mm i.d. × 0.25 mm degrees of freedom, Restek). The injector temperature was 270 °C. The oven was programmed from 60–120 °C at 20 °C/min and then to 320 °C at 30 °C/min. Helium was used as the carrier gas. The flame ionization detector was set at 300 °C. Five samples were analyzed for each concentration and compound. Concentrations of linalool and geraniol in the airflow were deduced from the absolute amounts recovered on the SPME fiber using the method reported by Bartelt and Zilkowski (1999, 2000) for nonequilibrium quantification of airborne volatiles from an air stream.

Data analyses

For Experiments 1–3, peristimulus time histograms were built using 100 ms as a time bin and used to determine maximum firing. To compare the responses, we measured the number of spikes within periods (1–3) defined with respect to application of the stimuli. The 3 periods are period 1—the time of the puff (whatever its occurrence and duration), period 2—the time interval that just followed the puff (odorless in Experiment 1 and with background in Experiments 2 and 3), and period 3—the 0.5-s period following the end of background application. Values were normalized to numbers of spikes emitted per second. Values from a same sensillum were dependent and the numbers of spikes per second between series (means ± standard errors of the mean) were compared using Wilcoxon signed-rank unilateral tests for paired data.

Shorter bins (20 ms) were used to analyze the responses to pulsed stimulation in experimental series 4. The numbers of spikes per bins were used to test correlation between firing and the frequency of the pulsed stimulus. We used the Lomb-Scargle periodogram according to the procedure adopted by Barrozo and Kaissling (2002) to analyze the capacity of Ph-ORNs to generate a periodic response. Calculations were performed using the program developed in R language and available at http://research.stowers-institute.org/efg/2005/LombScargle/ (Glynn et al. 2006).

Results

Pheromone and terpene aerial concentrations delivered by the stimulator

The mean aerial concentrations of linalool at the output of the stimulator were 0.02, 0.7, and 3.0 ppm (i.e., 0.12, 4.0, and 20 ng/ml) for the 0.01%, 0.1%, and 1% v/v sources, respectively. The 1% v/v geraniol source delivered a 0.8-ppm concentration in air (5 ng/ml). The concentrations of pheromone released by the sources of 1 and 10 μg were estimated to 1.9 and 3.7 ppb (19 and 38 ng/ml), respectively.

Responses to single puffs of linalool, pheromone, and their blend

Experiment 1

Responses to a puff of pheromone were phasic (Figure 1a). The firing rate reached a maximum within 300 ms after the onset of the puff and rapidly declined. Linalool 1% applied alone clearly reduced the basic firing activity of Ph-ORNs (1.5 ± 0.7 vs. 4.9 ± 1.2 spikes/s in blank, Figure 2a). Firing response to pheromone + linalool was reduced by 41% compared with pheromone alone. The response latency was not modified (Figures 1b and 2a).

The response to the blend showed a clear increase of firing after the end of the stimulation (Figures 1b and 2a). This

off-rebound was never observed after stimulation by pheromone alone. The level of firing activity was not significantly different from control after linalool alone ($P = 0.46$). A puff of linalool presented 0.1 s after the end of the puff of pheromone was not followed by a rebound (Figure 2b).

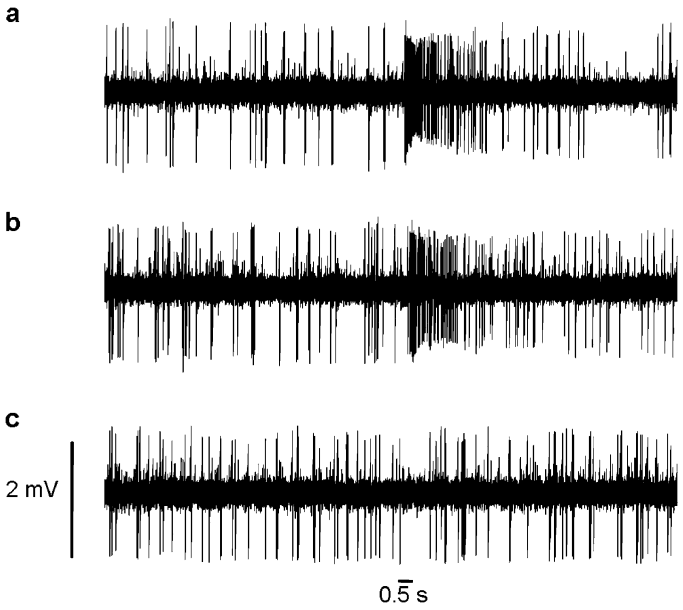

Figure 1 Samples of extracellular recordings from a Ph-ORN showing responses to single puffs of 0.5 s of **(a)** 1 μg of pher (Z9E11-14: Ac), **(b)** a blend of 1 μg of pher plus linalool (1%), and **(c)** linalool 1% alone. Stimulus bar = 0.5 s. Vertical bar = 2 mV.

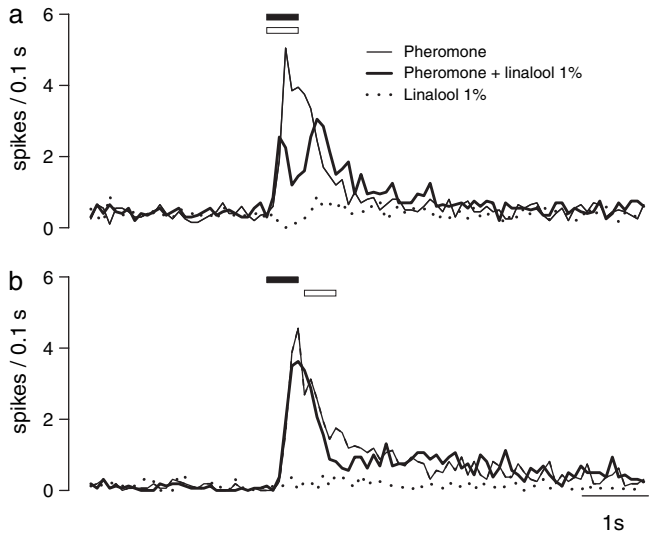

Figure 2 Experiment 1. Linalool reduced the response to pheromone and elicited an off-response when presented together with pheromone **(a)**. Presented 0.1 s after the end of the pheromone puff, linalool reduced the postfiring, which did not rebound at the end of linalool presentation **(b)**. Mean peristimulus time histograms of 20 (a) and 16 (b) replicates. Time bin = 0.1 s. Black and white bars show pheromone and linalool or mineral oil (blank) presentations, respectively.

To determine the origin of the inhibitory effect of linalool, we analyzed the amplitude of the sensillar potential. The latter was lower with the blend of pheromone + linalool than with only pheromone (–3.5 vs. –6.2 mV, $n = 20$, $P = 0.011$). Linalool elicited a small depolarization that was not significantly different from that observed with pure mineral oil. No rebound was observed.

Responses to a puff of one odor in a background of the other one

Experiment 2.1: a puff of pheromone in linalool background

The background of linalool reduced the response of Ph-ORNs to pheromone (Figure 3a). The maximum (Figure 3a) and the average firing rates during the puff of 1 μg pheromone (period 1, Figure 4b) were reduced by the half in presence of linalool 1%, as compared with neutral background. Similarly, linalool background reduced the postpheromone firing compared with neutral background (period 2, Figure 4b). But there was an increase in firing just after linalool background was turned off (period 3, Figure 4b). Firing rates during period 3, in the absence of pheromone puff, did not significantly differ with and without linalool (blank) (7.1 ± 1.7 vs. 6.5 ± 1.4 spikes/s). Responses to pheromone puff (period 1) were still reduced by 43% at linalool 0.1% background.

Experiment 2.2: a puff of linalool in pheromone background

A single puff of linalool 1% temporarily inhibited the response to 1-μg pheromone (Figure 3b). This effect was

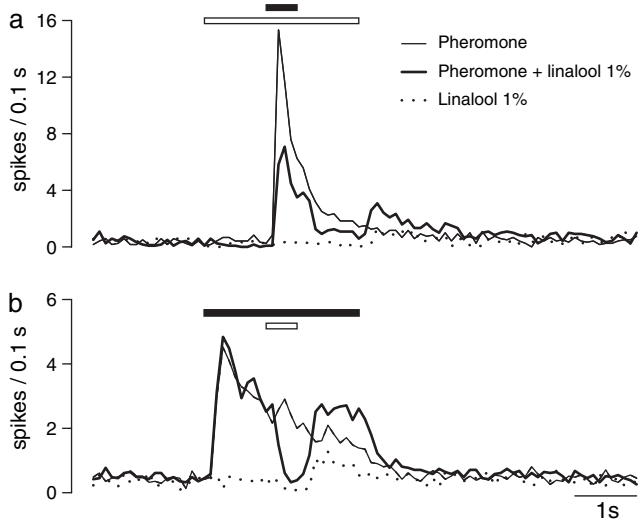

Figure 3 Experiments 2.1 and 2.2. A background of linalool reduced the response to a puff of pheromone, which was followed by a rebound **(a)**. Response to a sustained stimulation with pheromone was temporally inhibited by a puff of linalool and followed by a rebound **(b)**. Mean peristimulus time histograms of 20 (a) and 31 (b) recordings. Time bin = 0.1 s. Black and white bars indicate pheromone and linalool or mineral oil (blank) presentation, respectively.

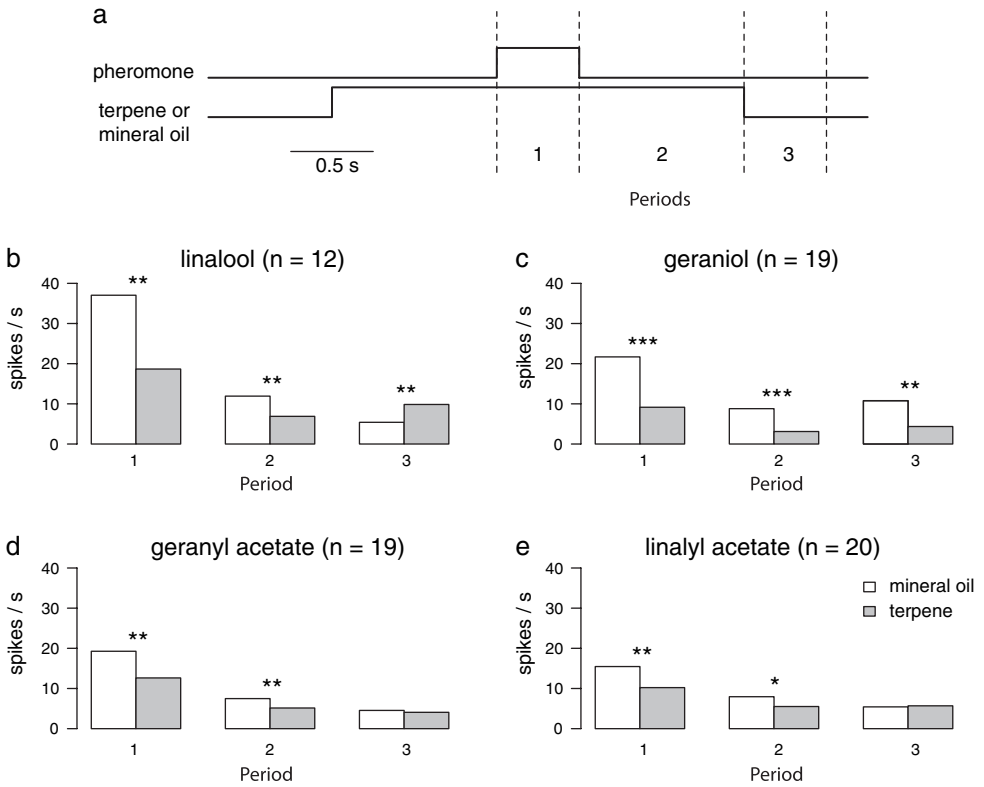

Figure 4 Experiment 3.1. As for linalool **(b)**, a background of geraniol **(c)**, geranyl acetate **(d)**, or linalyl acetate **(e)** reduced the mean firing rates of the Ph-ORNs during the pheromone puff (period 1) and after it (period 2), compared with a blank background (mineral oil, white bars). The firing rate was increased after the background was turned off (period 3) for linalool (b) but not for geraniol (c). Mean firing rates were calculated from the number of spikes emitted during period 1 (puff of pheromone), period 2 (the 1 s after the puff in presence of background), and period 3 (0.5 s after the turning off of terpenes; see **a**). Stared differences are significant at 5% *, 1%**, or 1% *** (Wilcoxon signed-rank test for paired values). Responses to the different terpenes were recorded from different insects; *n*: number of replicates.

reversible. In a pheromone background, about twice as less spikes were emitted by Ph-ORNs during the time of the linalool puff than during control puffs (period 1: 11 vs. 24 spikes/s; Figure 5b). Ph-ORN firing dramatically re-increased after the puff of linalool in pheromone background, overcoming firing level without linalool (Figure 3b). Firing during this period was 31% greater in linalool than in control background (period 2, Figure 5b). Firing rate was also higher during period 3 after linalool puff, compared with a neutral puff (Figure 5b). The on- and off effects of the linalool puff were very fast. The first occurred between 120 and 150 ms after the onset of linalool, whereas the second was observed within 200 ms after the end of the puff (Figure 3b). The 0.01% and 0.1% doses of linalool did not modify significantly the firing during puff (period 1) or after it (periods 2 and 3).

In a diluted background of pheromone (0.1 μg), firing was reduced by 64% during the linalool puff and increased by 17% and 31% during periods 2 and 3, respectively. Lower doses of pheromone (0.01 and 0.05 μg) did not elicit firing increase (<3 spikes/s during the puff), and no effect of linalool could be evidenced.

Linalool specificity: compared effects with geraniol and geranyl and linalyl acetates

Experiment 3.1: a puff of pheromone in terpene background

As with linalool (Figure 4b), firing during the puff of pheromone (period 1) and the following second (period 2) were lower with any of the 3 other terpenes than with mineral oil as a background (Figure 4c–e). With geraniol, Ph-ORN firing was lower than in blank during the 0.5 s that followed background off (period 3, Figure 4c). With linalool in turn, firing was stronger than in blank background during period 3 (Figure 4b). No differences with blank were observed at the same period with geranyl and linalyl acetates (Figure 4d–e).

Experiment 3.2: a puff of terpene in pheromone background

Puffs of geraniol or geranyl or linalyl acetates at 1% dilution did not modify the firing rate compared with blank puffs (Figure 5c–e). However, lengthening stimulation by geraniol to 1 s resulted in a reduction of the firing rate during the puff compared with oil control (26 vs. 35 spikes/s).

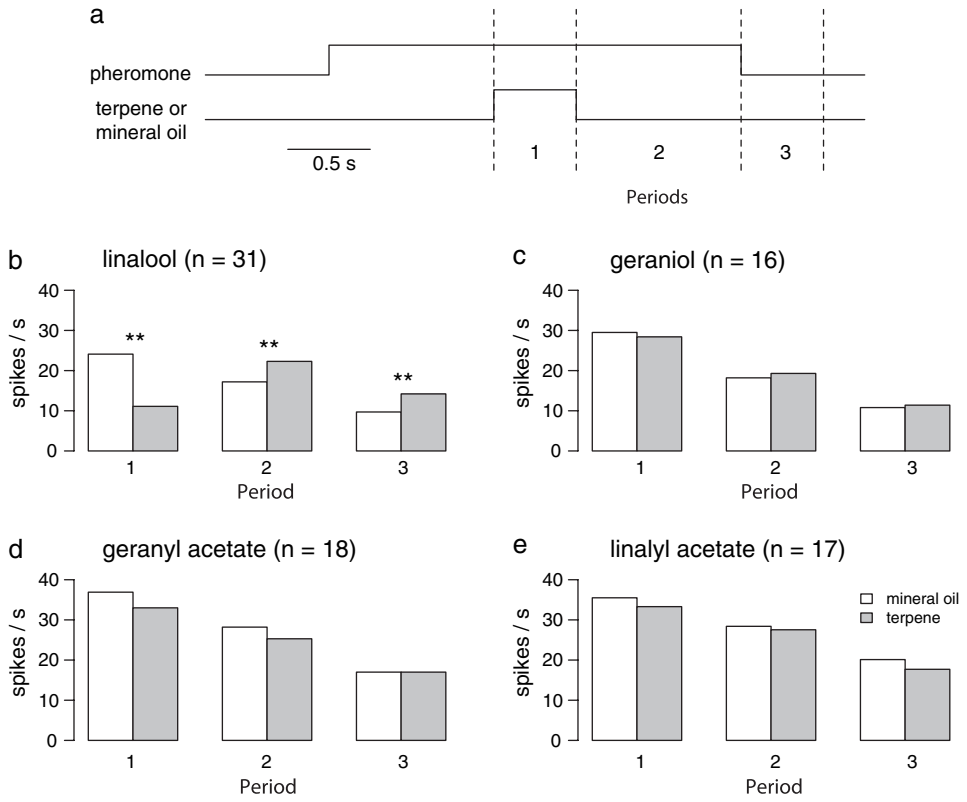

Figure 5 Experiment 3.2. Contrary to linalool **(b)**, a 0.5-s puff of geraniol **(c)**, geranyl acetate **(d)**, or linalyl acetate **(e)** did not reduce the mean firing rates of the Ph-ORNs in response to a pheromone background (period 1), compared with a control puff (mineral oil, white bars). A rebound of the firing rate was observed after the terpene puff was turned off (period 2) for linalool only (b). Mean firing rates were calculated from the number of spikes emitted during period 1 (puff of terpene), period 2 (the 1 s after the puff in presence of background), and period 3 (0.5 s after the turning off of pheromone; see **a)**. Stared differences are significant at 1%** or 1%*** (Wilcoxon signed-rank test for paired values). *n*: number of replicates.

Experiment 3.3: a puff of geraniol before pheromone

Ph-ORNs tended to fire fewer spikes during the pheromone puff delivered 0.1 s after geraniol than after blank (35 vs. 49 spikes/s, $P = 0.06$). Inhibition was significant with a 0.5-s interval between geraniol and pheromone (55 vs. 44 spikes/s) applications.

Responses to pulsed stimuli in a background

Experiment 4.1: pulsed pheromone in uninterrupted terpene background

In both blank and linalool backgrounds, the Ph-ORNs globally fired in synchrony with the pulsed stimulus (Figure 6a). The response amplitude was far greater to the first puff than to the following ones and then slowly decreased in blank background. In turn, with linalool, the 10 pheromone puffs triggered 10 peaks of firing with the same amplitude, which was globally lower than in control. The periodogram analyses showed that Ph-ORNs fired in rhythm with a significant period of 227 ms and a normalized power (NP) value of 10 ($P < 0.05$) when they were challenged with pheromone pulsed at a periodicity of 200 ms without linalool background. In turn, the period of the firing was much closer (201 ms) to the period of pheromone stimulation, and the rhythmicity was more marked (NP = 17, $P < 10^{-4}$) with linalool background.

In contrast to linalool, only a weak response to the first pheromone puff was observed in geraniol 1% background applied uninterrupted. No significant period was found by the Lomb-Scargle analysis. There was also a clear increase of firing after geraniol had been turned off (Figure 6b). In a 1% geraniol background, the responses to pheromone were not modified relative to control (Figure 6c).

Experiment 4.2: prolonged pheromone stimulation in pulsed linalool background

The Ph-ORNs showed a phasic–tonic response to pheromone (Figure 6d). Firing kept lower during pheromone application in linalool background compared with terpene-free background. More interestingly, its amplitude clearly oscillated with linalool puffs. As in Experiment 2.1, a clear off-response was observed when linalool was turned off. The periodogram analysis confirmed that the Ph-ORNs challenged with linalool pulsed at a periodicity of 200 ms in pheromone background fired in rhythm with linalool pulses with a calculated period of 203 ms and a NP value of 14 ($P < 0.001$). Inhibition due to linalool puffs created a discontinuity

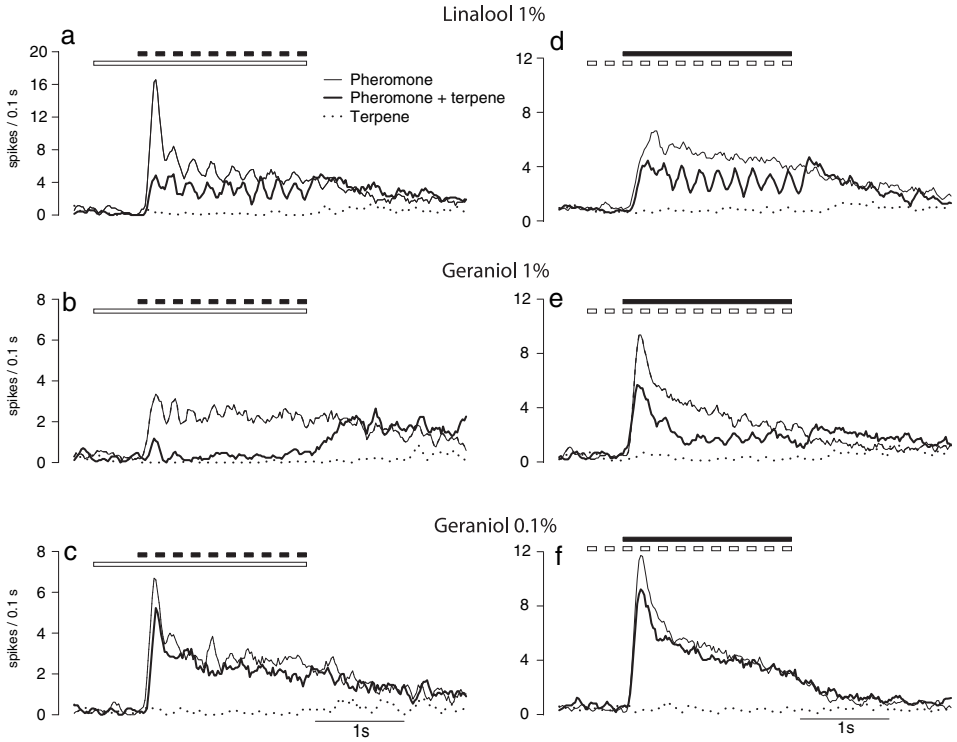

Figure 6 Experiment 4. Firing responses to pulsed stimulations in an odorant background. Linalool **(a)**, but not geraniol **(b**, **c)** backgrounds improved the time resolution of 10 pheromone pulses at 5/s (left column). Correspondingly, 0.1-s linalool pulses **(d)**, but not geraniol **(e**, **f)**, created a periodicity in a pheromone-sustained stimulation. Pulses of pure air in a terpene background (a–c) or pulses of a terpene in a pure air background (d, e) were applied as control (dotted lines). Mean peristimulus time histograms of 16 recordings (a), 12 recordings (b, c), or 17 recordings (d–f). Time bins = 20 ms. Black and white bars show pheromone and a terpene or mineral oil control presentations, respectively.

that mimicked a pulsed pheromone stimulation although the ORNs were uninterruptedly stimulated by pheromone here.

As with linalool, firing to pheromone was dramatically decreased when geraniol 1% was pulsed in background and an off-response was observed when geraniol was turned off (Figure 6e). In contrast to pulsed linalool, firing did not oscillate. Some peaks of firing activity were observed at the time of pheromone puffs but a Lomb-Scargle analysis did not point a significant period. In the same conditions, geraniol 1% did not modify the ORN responses to the pheromone as compared with blank background (Figure 6f).

Discussion

Linalool and the other 3 monoterpenes antagonized pheromone in *S. littoralis*

The main 2 effects of linalool were a reversible reduction of the firing to the main pheromone component, Z9E11-14:Ac, followed by a poststimulus rebound. The inhibition was observed during linalool application, and the activity recovered rapidly when linalool was turned off. This result is consistent with the observations by Pophof and van der Goes van Naters (2002) in *B. mori*. To explain inhibition, we exclude an inhibitory transduction pathway because linalool did not elicit a hyperpolarization of the sensillum potential. More probably, this reduced response in presence of linalool is of the same nature as mixture suppression, a common mode of interaction between odorants, resulting in a lower measured response to blends compared with the level of response expected from the amplitudes of the responses to individual compounds (e.g., in insects: De Jong and Visser 1988; Getz and Akers 1997). We postulate that linalool prevented pheromone molecules to reach, or bind, to one of the olfactory proteins involved in reception: a pheromone-binding protein or the OR. However, other mechanisms of inhibition cannot be excluded depending on the amount of linalool. Pophof and Van der Goes van Naters (2002) showed that high doses of linalool (50 µl at source) inhibited the firing activity elicited in Ph-ORNs by the G protein activator, NaF, and the diacylglycerol analog, 1,2 dioctanoyl glycerol. They concluded that linalool interfered with the transduction cascade at steps downstream the binding of pheromone on the ORs. Furthermore, because the amplitude of elementary receptor potentials was slightly but significantly reduced in presence of linalool, they proposed that linalool affected ion channels directly.

Such a transductional effect cannot explain the poststimulus rebound. We observed that not only the firing rate increased at the end of linalool application but also it was even

greater compared with the stimulation with pheromone only. To explain the off-response to linalool, Pophof and Van der Goes van Naters (2002) proposed a combined excitatory and inhibitory effect for linalool, with a shorter time course for inhibition, a phenomena proposed by Stange and Kaissling (1995) to explain the complex effects of volatile anesthetics. An inhibition of the response to a specific ligand, followed by a rebound was also observed with iodobenzene for the benzoic acid cells of female *B. mori* by De Brito Sanchez and Kaissling (2005). The authors proposed that iodobenzene had a bimodal activity, the general inhibitory effect being followed by a slower excitatory effect. But, in contrast to Kaissling et al. (1989), we never observed excitatory responses to linalool from the hundreds of Ph-ORNs we recorded, at the linalool concentrations we applied. In contrast to iodobenzene, which hyperpolarized the Ph-ORNs of *B. mori* (De Brito Sanchez and Kaissling 2005), *S. littoralis* Ph-ORNs were not hyperpolarized by linalool. Thus, iodobenzene and linalool probably trigger different mechanisms of inhibition. Furthermore, the off effect of linalool was not observed when linalool was applied after pheromone (Figure 2b) although a small depression of basic activity followed with a small rebound could be observed with linalool alone (Figure 2a). In *S. littoralis*, we presume that the burst of firing reflects a postresponse of the Ph-ORNs to pheromone still present in the sensillum lymph or on the antennal cuticle at the end of stimulus application rather than a specific excitatory activity of linalool on the Ph-ORNs. This postresponse could be due either to pheromone accumulation or to partial desadaptation. With the first hypothesis, linalool could reduce the diffusion of pheromone molecules and make pheromone accumulate sufficiently to elicit a final overstimulation after linalool had disappeared. According to the desadaptation hypothesis, linalool could reduce the adaptability of the Ph-ORNs to pheromone. Less adapted neurons would then be more sensitive to the remaining pheromone when the linalool is turned off. ORNs are very susceptible to adaptation (Kaissling et al. 1987), and sensory adaptation is known to occur with the 2 modes of presentation of Z9E11-14:Ac we used (Zufall and Leinders-Zufall 2000). Repetitive stimulation (10 pulses at 5 pulses/s) causes short-term adaptation, whereas the 2.5-s stimulation may be assimilated to adaptation to maintained stimuli, according to the categories defined by Zufall and Leinders-Zufall (2000). An inhibition of response due to an effect of linalool on the ion channels (proposed by Pophof and Van der Goes van Naters [2002]) can also result in lower adaptation so that our desadaptation hypothesis for the rebound is also consistent with the hypothesis of a transductional mode of action for linalool proposed by the latter authors.

Antagonism of the 4 terpenes for Ph-ORNs was fully reversible, and the firing rate recovered very quickly after the compounds had been turned off. This fast recovery was clearly evidenced in the repeated linalool-pulses experiments. The time to recover to normal firing activity after linalool exposure was even shorter than the return to a normal activity after a pheromone stimulus. Although terpenes could be cleared off the sensillum by a simple desorption, the dynamic of the off period, which is similar to that of pheromone pleads for an active process, like the enzymatic catabolism of pheromones. As stressed by Vogt (2005), the insect olfactory tissues are constantly submitted to potentially toxic chemicals from the environment. Vogt (2005) argued that these xenobiotic compounds have driven the enrichment of the olfactory tissues of biotransformation enzymes that can degrade volatile molecules. Besides specific enzymes for the catabolism of pheromones, the sensilla might contain enzymes able to metabolize natural environmental odors (Rybczynski et al. 1990).

Effects on coding of the temporal parameters of stimulus

The firing response was differently affected according to the time pattern of presentation of the plant volatiles and the pheromone. A puff of pheromone over a background of linalool elicited a steep peak of firing, although with reduced amplitude compared with response in air. By contrast, a single puff of linalool over a pheromone background reduced the firing activity almost down to the basal level. Geraniol, a structural isomer of linalool but a primary instead of a ternary alcohol, and the 2 corresponding acetates reduced the responses to Z9E11-14:Ac with a very different time course, compared with linalool. Although this difference might reflect different mechanisms of action, it could simply be due to different kinetics of diffusion inside the sensillum lymph surrounding the Ph-ORNs. Indeed, it has been shown in vertebrates that the transport of odorants to the dendrites is partly governed by their solubility within the nasal mucosa (Kurtz et al. 2004). The water solubility of geraniol, geranyl acetate, and linalyl acetate (0.4–0.7 [Weidenhamer et al. 1993; Ajisaka et al. 2000], 0.018 [Weidenhamer et al. 1993], and 0.05 [Cal 2006] g/l, respectively at 25 °C) is much lower than that of linalool (1.45 g/l at 25 °C; http://www.inchem.org/pages/sids.html). These differences between linalool and the other terpenes might account for the different dynamics observed.

Literature on moth orientation emphasizes the importance of the temporal pattern of olfactory stimuli for proper navigation. Male moths orient better to a pheromone source when the pheromone emission is pulsed, compared with flight response to continuous emission (Willis and Baker 1984; Kramer 1986). Thus, the effects of general odorants on the perception of the temporal characteristics of the pheromone signal are probably as critical for orientation as reduction in sensitivity. Discontinuous stimulation by a plant compound such as linalool would reduce but not suppress detection of pheromone, reduce adaptation, shorten the Ph-ORN response, and finally sharpen the temporal resolution. Pulses of linalool over prolonged pheromone stimulation create a temporal structure in a continuous stimulus. These 2 effects might positively affect behavior close to

the pheromone source where both the concentration and the number of pheromone eddies increase. Such a positive effect of linalool inhibition has been described by Kramer (1992) for orientation of *B. mori* males to an unnatural pheromone analog. On the contrary, geraniol appeared to totally suppress the temporal structure of the response, and we expect this compound to impair the orientation behavior.

Specificity of effects and ecological relevance

Contrary to our results, Ochieng et al. (2002) found a synergistic activity for linalool in the moth *H. zea*. In the latter species, linalool synergistically increased the firing of the ORNs tuned to Z11-16:Ald. On the contrary, we never observed a synergistic effect of linalool in *S. littoralis*, even at the lowest doses we tested. Linalool did not alter the detection of the pheromone at these low doses. Thus, we can exclude a dose-dependent type of effect, but other parameters might explain the opposite effects of linalool reported in the 2 works. First, Z11-16:Ald and linalool were released from the same filter paper by Ochieng et al. (2002), whereas we used 2 separate sources. We eliminated this possibility because when Z9E11-14:Ac and linalool were deposited on the same filter paper placed in a single vial we also observed an antagonist effect of linalool in *S. littoralis* (Party V, unpublished data). Second, the pheromones of *H. zea* and *S. littoralis* have different functional groups (aldehyde vs. acetyl), and linalool may interact differently with their corresponding olfactory proteins. But linalyl acetate and geranyl acetate also antagonized the responses to Z9E11-14:Ac although they have no hydroxyl but an acetyl group. Therefore, the type of interaction with the pheromone cannot be explained by a simple structural parameter like the similarity functional groups of the plant odorant and the pheromone compound. Ochieng et al. (2002) proposed that the coperception of plant volatiles and pheromone improves the localization of a mate because they found a synergy between linalool and the pheromone of *H. zea*. The type of interaction with pheromone detection seems to vary according to the insect species and, possibly, the chemical structure of the pheromone. With respect to the strong necessity of maintaining specificity of sex pheromone communication, one expects that the response to blends of pheromone and some plant compounds favorable to the species improves, or at least preserves, the specificity of sexual communication. Linalool is not only emitted by the main host plants of *S. littoralis* but also ubiquitously released by a number of plant species (Knudsen et al. 2006) whose value to the moth greatly varies. Different effects of linalool with respect to moth species deserve further investigations because they could indicate specific adaptations of the olfactory systems for peculiar blends of plant odorants and pheromones.

The pheromone–plant system constitutes a valuable model for studying how the olfactory system maintains specificity of communication in a noisy environment. A point of major concern is to determine whether the effects observed in the laboratory apply to natural conditions. Emissions of volatile compounds by plants have been extensively quantified in controlled environment, but it is very difficult to extrapolate to concentrations under field conditions. At the insect scale, airflows and eddies provide scattered distribution of odors with patches of high concentration alternating with pockets of extremely diluted odorants. Pheromones are generally produced in very low amounts, and natural outdoor concentrations are difficult to estimate. Pheromone concentration in a cotton field treated for mating disruption was measured at 0.0013 ppb (Koch et al. 2002, 2009). Mean atmospheric concentrations of monoterpenes from plant origin range from tens of parts per trillion to several parts per billilon (review in Kesselmeier and Staudt 1999). But these ambient concentrations are probably much lower compared with concentrations near plants. For instance, the concentration of some carbonyl compounds over rape field can reach 18 ppb (Muller et al. 2002). Because the ratios rather than the absolute amounts appear critical to analyze interactions between chemicals, it is worth comparing the concentration ratios of pheromone to plant compounds present in laboratory versus field conditions. Above data indicate that plant volatile compounds overcome the pheromone concentration by more than 10^6 in the field. In turn, measurements in our setup indicated an aerial concentration of 3.1 ppb for the 1-μg pheromone source and 3 ppm for the most concentrated linalool source. Interactions were still observed with 0.7 ppm linalool indicating a ratio of only 1:1000 or 1:400. Thus, the relative ratios of pheromone/plant compounds that we used in our experiment fall well below the extreme range that a moth can experience under natural conditions or in an agrosystem.

Acknowledgements

We are grateful to Drs Sylvia Anton, Philippe Lucas, and Daniela Schmidt-Buesser for helpful comments on the manuscript. We thank 2 anonymous referees for constructive criticisms and references.

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Accepted August 26, 2009