J Comp Physiol A (2017) 203:973–982
DOI 10.1007/s00359-017-1205-5

ORIGINAL PAPER

Short-term peripheral sensitization by brief exposure to pheromone components in *Spodoptera littoralis*

S. López[1] · A. Guerrero[1] · M. J. Bleda[2] · C. Quero[1]

Received: 27 February 2017 / Revised: 31 July 2017 / Accepted: 8 August 2017 / Published online: 29 August 2017

Abstract In insects, the olfactory system displays a high degree of plasticity. In *Spodoptera littoralis*, pre-exposure of males to the sex pheromone has been shown to increase the sensitivity of the olfactory sensory neurons at peripheral level. In this study, we have investigated this sensitization effect by recording the electroantennographic responses of male antennae to the major sex pheromone component (*Z*,*E*)-9,11-tetradecadienyl acetate and to the minor components (*Z*,*E*)-9,12-tetradecadienyl acetate and (*Z*)-9-tetradecenyl acetate. Responses to the conjugated diene acetate at 1 and 10 µg and to the unconjugated ester at 10 µg at three different times (11, 22 and 33 min) after pre-exposure ($T = 0$ min) were significantly higher than those at $T = 0$, whereas no increase of sensitivity to the pheromone was elicited by any dose of the minor monoene acetate. In addition, pre-exposed antennae to sub-threshold amounts (0.1, 1 and 10 ng) of the major pheromone component also induced an increased response to the chemical at different times (5 and 15 min) after exposure. Our results revealed that pre-exposed isolated antennae display a short-term higher sensitivity at the peripheral level when compared to naive antennae. In addition, we provide evidence of a peripheral sensitization mediated not only by the major pheromone component, but also by the minor unconjugated diene acetate, and the induction of this sensitivity appears to be dependent on the pre-exposure dose and the time span between pre-exposure and subsequent recordings. Possible implications of the sensitization effect displayed by the minor component for a more effective discrimination of the pheromone bouquets of other closely related species are highlighted.

Keywords Sensitization · *Spodoptera littoralis* · Pheromone · Electroantennography

✉ C. Quero
cqlqob@cid.csic.es

[1] Department of Biological Chemistry and Molecular Modelling, IQAC (CSIC), Barcelona, Spain

[2] Institute of Advanced Chemistry of Catalonia (IQAC, CSIC), Barcelona, Spain

Introduction

Moths rely mainly on their olfactory system to perceive chemical cues, which provide the necessary information to locate resources, e.g., for finding potential mates, food or suitable oviposition sites. In this regard, the ability of males to find females is mediated by species-specific pheromone blends emitted by conspecific females, which activate pheromone-sensitive male olfactory sensory neurons (OSNs) with a high degree of sensitivity (Hansson 1995). In this process, a series of behavioral responses are induced in both sexes to facilitate a successful mating. It is well known, however, that odorant-evoked behavioral responses can be affected by pre-exposure to female-produced sex pheromone or synthetic attractants reducing male attraction to the pheromone, as observed, f.i., in the cabbage looper *Trichoplusia ni* (Kuenen and Baker 1981), the Oriental fruit moth *Grapholita molesta* (Figueredo and Baker 1992), the tobacco budworm moth *Heliothis virescens* (Daly and Figueredo 2000), or the oblique banded leaf roller *Choristoneura rosaceana* (Stelinski et al. 2003a). Changes in odorant-induced behavioral output can be induced by exposure to different odors in two directions: habituation and sensitization. Habituation is the process in which the behavioral response to a repeated or long presentation of a specific stimulus is reduced (Duerr and Quinn 1982; Rankin et al. 2009). Sensitization, in contrast, involves a gradual increase in the response to a

stimulus after a previous exposure without any learned association (Bernays and Chapman 1994; Grubb and Thompson 2004). While olfactory habituation is mainly related to changes at the antennal lobe level (Das et al. 2011), sensitization has also been suggested to affect outer dendritic segments in peripheral OSNs (Mukunda et al. 2016), albeit the neural basis still remains unclear. In Lepidoptera, as cited above, pre-exposure to sex pheromone induces habituation and, therefore, a decrease in the response to the natural attractant (Bartell and Lawrence 1973; Figueredo and Baker 1992; Daly and Figueredo 2000). Regarding sensitization, in insects there are scarce reports that often refer to a cross-effect mediated by plant volatiles which modulates the subsequent response to pheromone compounds. For instance, a constant exposure to plant volatiles in *C. rosaceana* and *Argyrotaenia velutinana* induced an increase in the response to plant volatiles and to the major pheromone component (*Z*)-11-tetradecenyl acetate (Stelinski et al. 2003b). In this case, octopamine was suggested to play a key role in the sensitization process, since octopamine-injected individuals with no pre-exposure responded similarly than pre-exposed insects. Also, in *Rynchophorus palmarum* a first stimulation of acetoin reversibly sensitized the response of OSNs to the aggregation pheromone (4*S*)-2-methyl-(5*E*)-hepten-4-ol (Saïd et al. 2005). To our knowledge, only on the Egyptian cotton leafworm *Spodoptera littoralis* and *Agrotis ipsilon,* a sex pheromone-mediated sensitization (behaviorally, in the latter) has been reported (Anderson et al. 2003, 2007; Guerrieri et al. 2012; Quero et al. 2014; Abrieux et al. 2016). *S. littoralis* is a polyphagous pest of more than 80 agricultural crops, among others cotton, maize, rice, alfalfa, soybean and vegetables. The sex pheromone depends on the origin of the strain and is composed of up to eleven 14-carbon acetates, being (*Z,E*)-9,11-tetradecadienyl acetate (*Z9,E*11-14:OAc) always the major component (Muñoz et al. 2008; Saveer et al. 2014). In this insect, modulation of the pheromone perception was firstly reported by Anderson et al. (2003, 2007), who showed a long-term sensitization when males were pre-exposed to the female sex pheromone. Thus, pre-exposed males responded more significantly than naive males in behavioral assays conducted 27 h after exposure (Anderson et al. 2003). In addition, recordings from antennal lobe neurons 24 h post-exposure revealed an increase in the sensitivity of interneurons and a lower response threshold in pre-exposed males (Anderson et al. 2007). However, no changes of the peripheral receptors were detected in electroantennographic recordings indicating that experience of the pheromone may elicit changes in the central nervous system (Anderson et al. 2007). Later, Guerrieri et al. (2012) reported an increase of the peripheral sensitivity to the main pheromone component on pre-exposed males to the natural attractant. In addition, one gene encoding pheromone binding protein-3 (PBP3) showed a small but significant upregulation upon pre-exposure, and one glomerulus responsive for processing the major pheromone component was significantly enlarged in pre-exposed males relative to naive insects (Guerrieri et al. 2012).

Recent studies from our group have provided evidence of a short-term sensitization after a brief exposure to the major pheromone component on antennae of *S. littoralis* in electroantennographic recordings (Quero et al. 2014). One of the major questions arising from this report is whether the increased response to the pheromone is solely restricted to the major component alone or to other biologically active compounds as well, such as other pheromone components, plant volatiles, etc. To address this point, we have pre-exposed excised antennae of *S. littoralis* to the major pheromone compound and to the minor components (*Z,E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12-14:OAc) and (*Z*)-9-tetradecenyl acetate (*Z*9-14:OAc) at different times after exposure, and measured the EAG responses to subsequent stimuli of the specific compound. The two minor compounds have been selected based on the olfactory receptors tuned to both compounds, i.e., SlitOR6 has been found highly specific to the unconjugated diene acetate (Montagné et al. 2012), and SlitOR13 appeared also to be tuned to the same component and to the monoene acetate as well although in a less sensitive manner (de Fouchier et al. 2015). In addition, we wonder whether pre-stimuli with very low doses of the major compound, similar to the mean amount present in pheromone glands (Martínez and Camps 1988), would also induce sensitization. This would disclose the threshold of the sensitization effect.

Materials and methods

Insects

Spodoptera littoralis specimens were obtained from a laboratory colony regularly maintained at the Institute of Advanced Chemistry of Catalonia (Barcelona, Spain). Larvae were reared at 25 ± 2 °C and 65 ± 10% RH with a reversed 16:8 h L:D photoperiod on an artificial diet slightly modified from the previously reported (Poitout and Bues 1974). Newly emerged males were isolated individually in cubic plastic containers (14 × 17 × 9 cm) until use and provided with a 10% sucrose solution ad libitum. Virgin males of 1–4 days old were used in all the assays.

Chemicals

*Z*9,*E*11-14:OAc (>95% purity by GC analysis), *Z*9,*E*12-14:OAc (94.5%), and *Z*9-14:OAc (95%) were purchased from Bedoukian Research, Inc. (Danbury, CT, USA).

n-Hexane (SupraSolv®) was obtained from Merck (Darmstadt, Germany).

Electroantennographic recordings

The electroantennogram apparatus was commercially available from Syntech (Hilversum, The Netherlands) and the methodology used was based on standardized protocols (Acín et al. 2010). Briefly, one antenna from a non-anesthetized male was excised, and mounted on an electrode holder (Syntech, Kirchzarten, Germany). The basal tip of the antenna was placed on the reference electrode, and the distal tip, from which the last 2–3 antennomeres had been previously cut, on the recording electrode. A drop of conductive gel Spectra 360 (Parker Lab. Inc., Hellendoorn, The Netherlands) was added to each electrode to facilitate adhesion of the antenna. A flow of humidified pure air (ca. 750 mL/min) was continuously directed over the preparation through the main branch of a glass tube (7 cm long × 5 mm diameter) to clean the environment of the antennae and prevent desiccation. The holder containing the antenna was placed 1.0 cm below the main branch of the air-delivery tube. Test stimulations were carried out by giving puffs of air (ca. 200 mL/min) for 200 ms through a Pasteur pipette with the aid of a CS-01 stimulus controller (Syntech). The pipette contained a piece of round filter paper (Whatman No.1) (2.5 cm diameter), which contained either the solvent (hexane, 10 µL) alone as control or the corresponding amount of the pheromone component that had been dissolved in hexane at the required concentration, so that 10 µL of the solution provided the required dose for the experiment. The solvent was allowed to evaporate before the tests. Two consecutive stimulations of each testing dose and compound were applied at 60-s intervals over the antennae. Control puffs were applied before and after each pair of stimuli to determine the baseline depolarization of the antennae. The output signals were amplified (10×), filtered (DC to 1 kHz) with an IDAC-2 interface (Syntech), further amplified (10×), digitized on a PC and analyzed with the EAG Pro Version 2.0 (2005) (Syntech). The whole EAG preparation was enclosed in a Faraday cage (70 × 65 × 60 cm) connected to the ground to prevent extraneous electric signals. The net electroantennographic responses were calculated by subtracting the mean response to control before and after each stimulus from the mean response to the pheromone component.

Experiments

All the assays were carried out during the insects' scotophase (10:00 am–18:00 pm). First, EAG dose–response profiles for the major pheromone component (10–10^5 ng) were obtained for the excised right and left antenna of the insects, to discard any laterality in the olfactory response, as could occur f.i. in social bees (Anfora et al. 2010). Recordings for both antennae were obtained from 8 males. In pheromone pre-exposure trials, two different set of assays were defined. In the first set (Experiment I), one randomly selected antenna of each male was excised and initially stimulated with a puff over 1 or 10 µg of either *Z*9,*E*11-14:OAc, *Z*9,*E*12-14:OAc or *Z*9-14:OAc, and subsequent responses to puffs over 1 or 10 µg of the same compound were recorded at three different times (11, 22 and 33 min). Ten antennae were tested for each compound and dose. In the second set (Experiment II), one antenna of each male was labeled as "treated" and the other one as "naive" (control). After being excised, treated antennae were placed on the EAG holder and pre-exposed to a puff of different doses (0.1, 1 and 10 ng) of *Z*9,*E*11-14:OAc, and then subjected to puffs over 1 µg of this compound at 5 or 15 min after the pre-exposure. The net EAG responses were compared to those of the naive antennae, which were stimulated only with puffs over 1 µg of the major component at 5 or 15 min after being excised. Ten antennae were tested for each pre-exposure dose and time.

Statistical analysis

Prior to the analysis, data were checked for normality and outliers. When needed, a log-transformation was applied to normalize the data. Mean depolarization values to the doses of the major pheromone component were compared using Kruskal–Wallis and Wilcoxon matched-pairs signed-rank non-parametric tests. For Experiment I, in which each insect had a profile with four sequential and correlated responses over time, a multilevel model (linear random-intercept regression model, LRIRM) was fitted using restricted maximum likelihood (REML) estimation method taking into account the variability within insect and between insect. Time, doses and their first order interaction were the covariates of the model at cluster (i.e., insect) or level-2. For each LRIRM, residual diagnostics, goodness of fit of the model, estimation of standard deviations at insect (level-2, $\sqrt{\sigma_u^2}$) and observation levels (level-1, $\sqrt{\sigma_e^2}$), conditional intraclass correlation coefficient (ρ) and determination coefficient (R^2) (Snijders and Boske 2012) were also performed. As the adjustment of the model predicted log-depolarization mean values and their 95% confidence intervals, the exponential function was computed on these values to express them on the original scale. Percent increases derived from the predicted values and their 95% confidence intervals were also calculated. All the analyses were conducted using the statistical software Stata 12.0 (StataCorp 2011) and tests were two-sided for a significance level $\alpha = 0.05$.

Results

Dose–response curves and laterality

EAG responses from right and left antennae were compared to determine possible differences in sensitivity between them. A similar response profile was observed for both antennae (Kruskal–Wallis test for all doses: $p = 0.788$), with an expected increase of the response in a dose-dependent manner (Fig. 1). No significant differences were apparent between the response of both antennae at any of the doses tested (Wilcoxon matched-pairs signed-rank test: for 10 ng, $p = 0.116$; 100 ng, $p = 0.176$; 1 µg, $p = 0.116$; 10 µg, $p = 0.484$; and 100 µg, $p = 0.779$). Based on the absence of laterality, the antennae to be tested in experiments I and II were randomly selected for each individual.

Experiment I: effect of pre-exposure to different pheromone components

A pre-stimulus of the antenna with 1 or 10 µg of *Z*9,*E*11-14:OAc elicited a significant increase of the response to subsequent stimuli of this chemical at different times (Fig. 2a). Thus, dose of 1 µg induced a significant increase of the response of 41.6, 63.3 and 78.2% at 11, 22 and 33 min, respectively, compared to the response obtained with the first stimulus (control, $T = 0$ min) (Table 1). At the dose of 10 µg, the increase of the response was slightly lower but statistically significant (27.5, 49.3 and 44.4% for 11, 22 and 33 min, respectively) vs control (Fig. 2a; Table 1). With regard to the minor component *Z*9,*E*12-14:OAc, the increase in sensitivity was only observed at the highest dose (10 µg), eliciting an increase of 49.5, 51.1

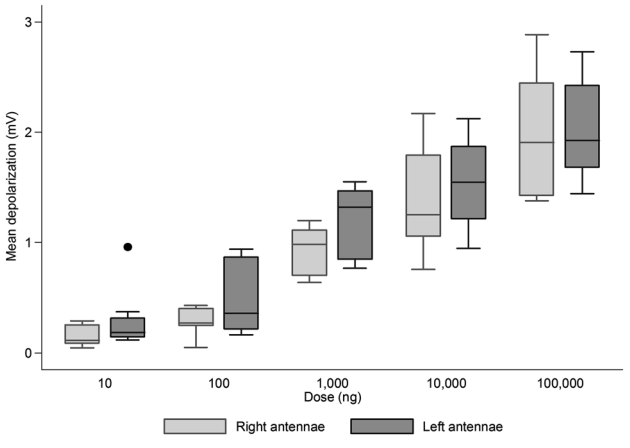

Fig. 1 Dose-response profile of right and left antennae of virgin *S. littoralis* males in response to five doses (10–100,000 ng) of *Z*9,*E*11-14:OAc. No significant differences were found between the response of both antennae at any of the doses tested (Wilcoxon matched-pairs signed-rank test at $\alpha = 0.05$). *Line* inside the *box* represents the median depolarization of the corresponding data set; *black dot* outlier

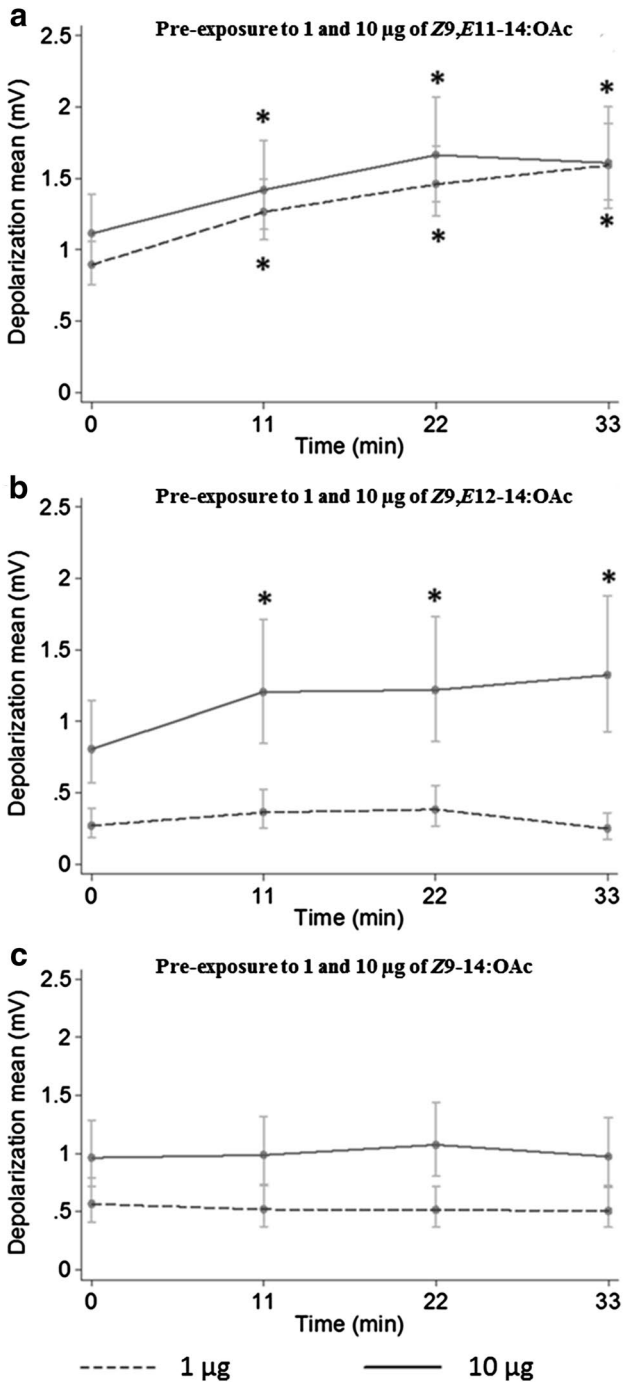

Fig. 2 Mean electroantennographic response (mV ± 95% CI) of *S. littoralis* male antennae when stimulated with different pheromone components at 11, 22 and 33 min after the first stimulus ($T = 0$ min). *Asterisks* within each dose for each compound denote statistically significant differences in the mean response at an specific time with regard to that elicited at $T = 0$ min ($p < 0.05$)

and 64.0% at 11, 22 and 33 min, respectively, vs control, similarly to the values evoked by pre-exposure to 1 µg of the major component (Fig. 2b; Table 1). The stimulus with 1 µg did not trigger any changes in the antennal response. Illustrative EAG

Table 1 Percent increase of the mean predicted response to three pheromone components of *S. littoralis* males at different times referred to that at $T = 0$ min

	Dose (µg)	Time (min)	Increase of response (%)	(95% CI)[a]
*Z*9,*E*11-14:OAc	1	11	41.6*	(23.30; 62.63)
		22	63.3*	(42.24; 87.60)
		33	78.2*	(55.19; 104.69)
	10	11	27.5*	(6.44; 52.70)
		22	49.3*	(24.69; 78.89)
		33	44.4*	(20.55; 72.95)
*Z*9,*E*12-14:OAc	1	11	32.4	(−5.98; 92.06)
		22	42.2	(−0.48; 103.30)
		33	−7.7	(−35.43; 31.90)
	10	11	49.5*	(6.38; 110.20)
		22	51.1*	(7.50; 112.42)
		33	64.0*	(16.69; 130.58)
*Z*9-14:OAc	1	11	−8.5	(−30.66; 20.81)
		22	−9.1	(−31.11; 20.02)
		33	−10.5	(−32.19; 18.13)
	10	11	2.4	(−19.72; 30.64)
		22	11.8	(−12.35; 42.63)
		33	1.1	(−21.24; 29.81)

Asterisks denote statistical significance at $\alpha = 0.05$ level

[a] 95% confidence interval

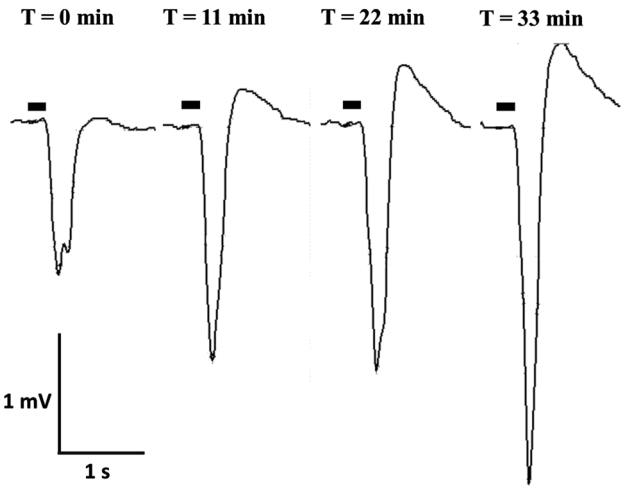

Fig. 3 Illustrative electroantennographic responses of an excised antenna to 10 µg of *Z*9,*E*12-14:OAc at $T = 0$ min (pre-exposure) and three different times after pre-exposure. *Horizontal black bar* upon each trace denotes the duration of the puffed stimulus

responses at $T = 0$, and 11, 22 and 33 min after pre-exposure are shown in Fig. 3. In contrast, the monoene minor component *Z*9-14:OAc did not induce significant changes in the response when the antennae were pre-stimulated at any of the tested doses (Fig. 2c; Table 1). Based on the lack of sensitization elicited by the latter compound, we decided not to test the other minor component *Z*9-12:OAc, which had been noticed to induce responses also on SlitOR3, but lower than its analog *Z*9-14:OAc (de Fouchier et al. 2015).

Analysis by LRIRM showed a significant effect of the dose in both minor components although not in the major compound, which, in turn, displayed a significant effect when responses at different times were compared to that at $T = 0$ (Table 2). The interaction between the two covariates (dose and time) was found not significant, although a trend towards significance ($0.05 < p \leq 0.10$) was detected at 10 µg and 33 min for the two dienic acetates.

Experiment II: effect of pre-exposure to low doses of the major pheromone component

After perceiving that pre-exposure to doses of 1 and 10 µg of *Z*9,*E*11-14:OAc increased the sensitivity to subsequent stimulus, we focused on the search of the lowest threshold dose of the major pheromone component to induce a significant sensitization effect. A pre-stimulus with 0.1 ng of *Z*9,*E*11-14:OAc did not induce any change in the subsequent response to 1 µg of the major component after 5 and 15 min in comparison to naive antennae (control) (Fig. 4a, b; Table 3). Pre-exposure to 1 and 10 ng of the acetate induced, however, a significant increase of the response after 5 min (58.3% at 1 ng) and after 5 and 15 min at 10 ng (38.9 and 25.1%, respectively). No effect was observed by pre-exposure of 1 ng after 15 min (Fig. 4b; Table 3). Illustrative EAG responses of both types of antennae (naive and pre-exposed to the major pheromone compound) are shown in Fig. 5.

Analysis by LRIRM revealed that neither the covariates antenna (pre-exposed or not) nor dose showed a significant effect (Table 4). A significant first order interaction between the covariates antenna and dose was detected, although the second order interaction was only close to significant (Table 4).

Discussion

Behavioral responses in insects may vary according to several factors, such as physiological stages, biotic and abiotic environmental factors, and previous experience (Gadenne et al. 2016). In *S. littoralis*, for instance, the response of males to the sex pheromone can vary as a function of the circadian rhythm (Merlin et al. 2007) and mating status (Kromann et al. 2015). This behavioral plasticity acts as an adaptive mechanism, enabling the insects to cope with a wide range of stimuli in a changing environment. Previous studies in males have shown that prior experience of

Table 2 Effect of dose and time on the antennal response of *S. littoralis* males to different pheromone components (Experiment I)

	*Z*9,*E*11-14:OAc		*Z*9,*E*12-14:OAc		*Z*9-14:OAc	
Number of observations	108		84		91	
Number of insects	27		21		23	
Observations per insect	4		4		Min = 3, max = 4 (mean = 3.957)	
Response variable						
Log (depolarization mean, mV)						
Fixed part[a]	Coef.	(95% CI)	Coef.	(95% CI)	Coef.	(95% CI)
Dose (1 µg)						
10 µg	0.220	(−0.055; 0.496)	1.098*	(0.589; 1.606)	0.530*	(0.087; 0.972)
Time (0)						
11 min	0.348*	(0.209; 0.486)	0.295	(−0.062; 0.653)	−0.089	(−0.366; 0.189)
22 min	0.491*	(0.352; 0.629)	0.352	(−0.005; 0.710)	−0.095	(−0.373; 0.182)
33 min	0.578*	(0.440; 0.716)	−0.080	(−0.437; 0.277)	−0.111	(−0.388; 0.167)
Dose by time interaction (10 µg, 0 min)						
10 µg, 11 min	−0.105	(−0.332; 0.122)	0.107	(−0.387; 0.600)	0.112	(−0.257; 0.482)
10 µg, 22 min	−0.090	(−0.317; 0.138)	0.060	(−0.433; 0.554)	0.207	(−0.162; 0.576)
10 µg, 33 min	−0.211°	(−0.438; 0.017)	0.576°	(−0.082; 1.069)	0.122	(−0.251; 0.495)
Constant	−0.113	(−0.280; 0.055)	−1.313*	(−1.682; −0.945)	−0.568*	(−0.901; −0.235)
Random part						
Estimate of the random-intercept (insect level or level-2) standard deviation ($\sqrt{\sigma_u^2}$)	0.286		0.433		0.433	
Estimate of the residual (observation level or level-1) standard deviation ($\sqrt{\sigma_e^2}$)	0.206		0.407		0.317	
Derived estimates						
ρ	0.659		0.530		0.652	
R^2	47.57%		55.68%		24.03%	

REML estimates derived from the linear random-intercept model. Estimated regression coefficients and their 95% confidence intervals (CI) are reported under "fixed part" and the standard deviations under "random part"

ρ conditional intraclass correlation coefficient, R^2 determination coefficient

* $p \leq 0.05$; °$0.05 < p \leq 0.10$

[a] Reference categories in parenthesis

the sex pheromone increases the sensitivity to this stimulus at central and peripheral levels (Anderson et al. 2003, 2007; Guerrieri et al. 2012; Quero et al. 2014). Here, we present evidence that a brief exposure to the major pheromone component *Z*9,*E*11-14:OAc and to the minor *Z*9,*E*12-14:OAc on excised antennae of *S. littoralis* males induces higher responses to successive stimuli not only to the major compound, as reported previously (Quero et al. 2014), but also on the diene minor component. No sensitization effect, however, was observed on the monoene minor component *Z*9-14:OAc. Three functional classes of sensilla have been identified so far on male antennae of *S. littoralis*: a long trichoid sensilla (LT1) housing one OSN population tuned to the major pheromone component (Ljungberg et al. 1993; Quero et al. 1996); another less-abundant type (LT2) housing two OSNs, one of them tuned to the diene minor component and to the behavioral antagonist *Z*9-14:OH (Ljungberg et al. 1993); and a third long trichoid sensilla (LT3), located on the distal part of the antennae, which housed one OSN responding to the minor diene acetate and to the minor monoene acetates *Z*9-14:OAc and *Z*9-12:OAc (de Fouchier et al. 2015). The short-term peripheral sensitization observed in our EAG experiments was triggered by two doses of the major component (1 and 10 µg) and by the highest dose (10 µg) of the minor component *Z*9,*E*12-14:OAc. This difference in sensitivity is probably related with the higher abundance of pheromone receptors (PRs) responding to the major component relative to the minor compound

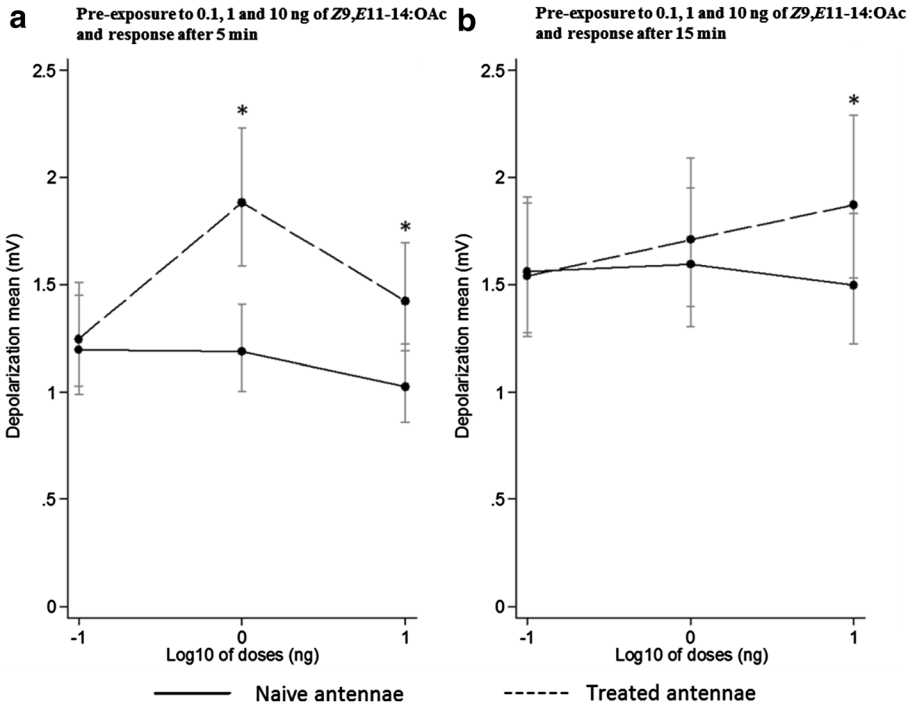

Fig. 4 Mean electroantennographic response (mV ± 95% CI) of naive and treated antennae of *S. littoralis* males to 1 µg of *Z*9,*E*11-14:OAc after being previously stimulated with different amounts of the chemical. *Asterisks* within each dose denote statistically significant differences between the responses of both types of antenna ($p < 0.05$)

Table 3 Percent increase of the mean predicted response to 1 µg of *Z*9,*E*11-14:OAc of pre-exposed antennae at different times and doses of the major pheromone component

Pre-exposure dose (ng)	Time (min)	Increase of response (%)	(95% CI)[a]
0.1	5	4.2	(−13.16; 24.95)
	15	−1.5	(−18.57; 19.25)
1	5	58.3*	(34.77; 86.05)
	15	7.1	(−11.47; 29.66)
10	5	38.9*	(17.51; 64.21)
	15	25.1*	(3.41; 51.44)

Asterisks denote statistical significance at $\alpha = 0.05$ level

[a] 95% confidence interval

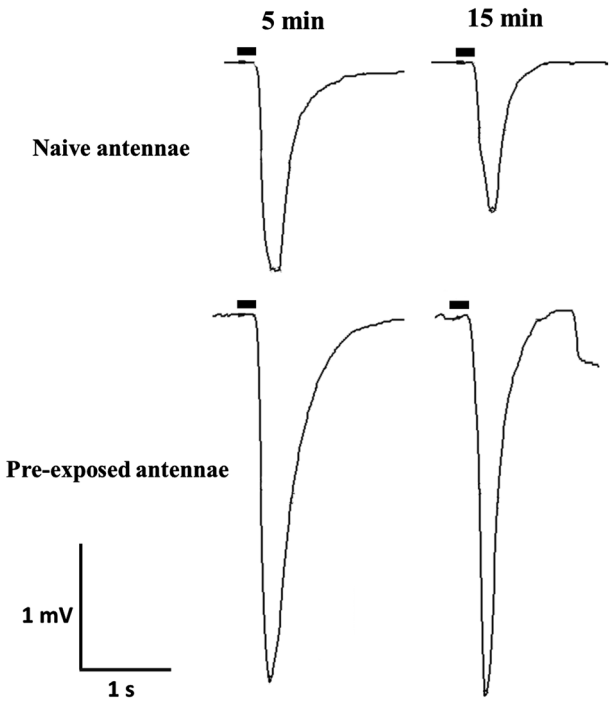

Fig. 5 Illustrative electroantennographic responses of naive antennae (*upper traces*) and pre-exposed antennae with *Z*9,*E*11-14:OAc (10 ng) (*lower traces*) to puffs of 1 µg of *Z*9,*E*11-14:OAc at 5 and 15 min. *Horizontal black bar* upon each trace denotes the duration of the puffed stimulus

(Ljungberg et al. 1993; Quero et al. 1996; de Fouchier et al. 2015).

Four possible PRs have been identified in the *S. littoralis* male transcriptome (Legeai et al. 2011). By expression in *Drosophila* OSNs, two of them, SlitOR6 and SlitOR13, have been found to detect minor pheromone components, being SlitOR6 highly sensitive to the diene acetate *Z*9,*E*12-14:OAc (de Fouchier et al. 2015). SlitOR13, in turn, was less sensitive and non-specific since it displayed similar responses to the minor diene and the monoene *Z*9-14:OAc in addition to smaller responses to *Z*9-12:OAc (de Fouchier et al. 2015). This receptor appeared to be housed in the third type of sensilla LT3 (see above). The lack of sensitization elicited by the monoene acetate *Z*9-14:OAc could be explained by the lower sensitivity displayed by SlitOR13. The absence of the sensitization effect would not be related either to the removal of the last 2–3 flagellomeres of the antenna because according to Binyameen et al. (Binyameen et al. 2012) the

Table 4 Effect of pre-exposure dose, recording time and treated/naive antenna on the antennal response to the major pheromone component of *S. littoralis* (Experiment II)

Number of observations		136
Number of insects		68
Observations per insect		2
Response variable		
Log (depolarization mean, mV)		
Fixed part[a]	Coef.	(95% CI)
Dose (0.1 ng)		
1 ng	−0.007	(−0.263; 0.249)
10 ng	−0.155	(−0.416; 0.105)
Time (5 min)		
15 min	0.266^{o}	(−0.012; 0.544)
Dose by time interaction (0.1 ng, 15 min)		
1 ng, 15 min	0.028	(−0.355; 0.411)
10 ng, 15 min	0.113	(−0.273; 0.499)
Antenna (naive)[b]		
Treated	0.041	(−0.141; 0.223)
Antenna by dose interaction (treated, 0.1 ng)		
Treated, 1 ng	0.419*	(0.176; 0.662)
Treated, 10 ng	0.288*	(0.041; 0.535)
Antenna by time interaction (treated, 5 min)		
Treated, 15 min	−0.056	(−0.319; 0.208)
Antenna by dose by time interaction (treated, 0.1 ng, 15 min)		
Treated, 1 ng, 15 min	−0.335^{o}	(−0.698; 0.028)
Treated, 10 ng, 15 min	−0.049	(−0.415; 0.317)
Constant	0.180^{o}	(−0.012; 0.371)
Random part		
Estimate of the random-intercept (insect level or level-2) standard deviation ($\sqrt{\sigma_u^2}$)	0.241	
Estimate of the residual (observation level or level-1) standard deviation ($\sqrt{\sigma_e^2}$)	0.218	
Derived estimates		
ρ	0.55	
R^2	20.8%	

REML estimates derived from the linear random-intercept model. Estimated regression coefficients and their 95% confidence intervals are reported under "fixed part" and the standard deviations under "random part"

ρ conditional intraclass correlation coefficient, R^2 determination coefficient

* $p \leq 0.05$; $^{o}0.05 < p \leq 0.10$

[a] Reference categories in parenthesis in fixed part

[b] Antenna covariate (treated/naive) refers to whether antenna was pre-exposed or not

distal part of the antenna would comprise at least up to 15 flagellomeres.

The sensitization evoked by a minor component such as *Z*9,*E*12-14:OAc is remarkable because it may convey implications of ecological relevance. As in many moths, *Spodoptera* spp. shares the same components in their sex pheromone bouquets (Guerrero et al. 2014). This overlapping in pheromone composition could induce interspecific interactions, including olfactory-guided heterospecific attraction and cross mating, with severe ecological and evolutionary consequences (Groning and Hochkirch 2008; Burdfield-Steel and Shuker 2011). For instance, the pheromone blend of the sibling species *S. litura* includes the dienes Z9,*E*11-14:OAc and *Z*9,*E*12-14:OAc and the monoenes *Z*9-14:OAc and *E*11-14:OAc, all of them also found in *S. littoralis* (Guerrero et al. 2014; Saveer et al. 2014) although the unconjugated diene acetate has only been reported in some strains and in low relative amounts

(Nesbitt et al. 1973; Tamaki and Yushima 1974; Dunkelblum et al. 1982; Saveer et al. 2014). The role of *Z*9,*E*12-14:OAc in field trapping has yielded controversial results as the presence of only 0.5% combined with the major component *Z*9,*E*11-14:OAc was highly attractive to males (Kehat and Dunkelblum 1993), whereas catches were reduced significantly when present at 5–10% of the pheromone lure (Kehat et al. 1976). These results highlight the importance of the amount of *Z*9,*E*12-14:OAc in mediating attraction to the pheromone source since ratios out of the optimum range may lead to an undesired antagonistic effect. It would be possible that an increased responsiveness to the unconjugated acetate after a first pre-exposure might facilitate a more accurate discrimination of the airborne pheromone bouquets for finding conspecific mates, thus avoiding the encounter with heterospecific females.

Our results also show that exposure to sub-threshold amounts (doses that do not evoke reliable and significant EAG responses relative to control) of the major pheromone component significantly increases the male response to the following stimulations of the chemical. This increase is closely related to the pre-exposure dose and the time span between pre-exposure and subsequent recordings. When searching for a potential mate, a male will encounter scarce and patchy pheromone-plumes mixed with a wide array of odors from the environment. Therefore, it is crucial for males to discriminate the relevant stimuli from the background. Under this context, a short-term sensitization induced by minor amounts of the major pheromone compound would increase the sensitivity of the peripheral nervous system for the sex pheromone molecules, allowing the male to orient towards the emitting source more efficiently and reducing energy costs.

In summary, for the first time we report a sensitization effect at the peripheral level elicited by a short pre-exposure stimulus on *S. littoralis* isolated antennae to high doses of the major and minor sex pheromone components *Z*9,*E*11-14:OAc and *Z*9,*E*12-14:OAc, respectively. The effect was also noticed by pre-exposure of the antennae to sub-threshold amounts of the major pheromone compound, pointing out to a higher sensitivity at the peripheral level of the pre-exposed antennae when compared to the naive ones. The sensitization effect displayed by sex pheromone components may represent an additional tool that males rely on for a more effective discrimination of the olfactory bouquets present in the environment, particularly of those pertaining to closely related species, with the aim of finding conspecific mates more efficiently.

Acknowledgements We gratefully acknowledge MINECO for financial support (project AGL2015-66469-R) with assistance from the European Regional Development Fund.

References

Abrieux A, Mhamdi A, Rabhi KK, Egon J, Debernard S, Duportets L, Tricoire-Leignel H, Anton S, Gadenne C (2016) An insecticide further enhances experience-dependent increased behavioural responses to sex pheromone in a pest insect. PLoS One 11(11):e0167469. doi:10.1371/journal.pone.0167469

Acín P, Rosell G, Guerrero A, Quero C (2010) Sex pheromone of the Spanish population of the beet armyworm *Spodoptera exigua*. J Chem Ecol 36(7):778–786. doi:10.1007/s10886-010-9817-z

Anderson P, Sadek MM, Hansson BS (2003) Pre-exposure modulates attraction to sex pheromone in a moth. Chem Senses 28(4):285–291. doi:10.1093/chemse/28.4.285

Anderson P, Hansson B, Nilsson U, Han Q, Sjoholm M (2007) Increased behavioral and neuronal sensitivity to sex pheromone after brief odor experience in a moth. Chem Senses 32:483–491. doi:10.1093/chemse/bjm017

Anfora G, Frasnelli E, Maccagnani B, Rogers LJ, Vallortigara G (2010) Behavioural and electrophysiological lateralization in a social (*Apis mellifera*) but not in a non-social (*Osmia cornuta*) species of bee. Behav Brain Res 206(2):236–239. doi:10.1016/j.bbr.2009.09.023

Bartell RJ, Lawrence LA (1973) Reduction in responsiveness of males of *Epiphyas postvittana* (Lepidoptera) to sex pheromone following previous brief pheromonal exposure. J Insect Physiol 19(4):845–855. doi:10.1016/0022-1910(73)90156-X

Bernays EA, Chapman RF (1994) Effects of experience. In: Miller TA, van Emden HS (eds) Host-plant selection by phytophagous insects, contemporary topics in entomology, vol 2. Springer, New York. pp 206–229. doi:10.1007/b102508

Binyameen M, Anderson P, Ignell R, Seada MA, Hansson BS, Schlyter F (2012) Spatial organization of antennal olfactory sensory neurons in the female *Spodoptera littoralis* moth: differences in sensitivity and temporal characteristics. Chem Senses 37:613–629

Burdfield-Steel ER, Shuker DM (2011) Reproductive interference. Curr Biol 21(12):R450–R451. doi:10.1016/j.cub.2011.03.063

Daly KC, Figueredo AJ (2000) Habituation of sexual response in male *Heliothis* moths. Physiol Entomol 25(2):180–190. doi:10.1046/j.1365-3032.2000.00184.x

Das S, Sadanandappa MK, Dervan A, Larkin A, Lee JA, Sudhakaran IP, Priya R, Heidari R, Holohan EE, Pimentel A, Gandhi A, Ito K, Sanyal S, Wang JW, Rodrigues V, Ramaswami M (2011) Plasticity of local GABAergic interneurons drives olfactory habituation. Proc Natl Acad Sci USA 108(36):E646–E654. doi:10.1073/pnas.1106411108

de Fouchier A, Sun X, Monsempes C, Mirabeau O, Jacquin-Joly E, Montagné N (2015) Evolution of two receptors detecting the same pheromone compound in crop pest moths of the genus *Spodoptera*. Front Ecol Evol 3:95. doi:10.3389/fevo.2015.00095

Duerr J, Quinn W (1982) Three *Drosophila* mutations that block associative learning also affect habituation and sensitization. Proc Nat Acad Sci USA 79(11):3646–3650

Dunkelblum E, Kehat M, Gothilf S, Greenberg S, Sklarsz B (1982) Optimized mixture of sex pheromonal components for trapping of male *Spodoptera littoralis* in Israel. Phytoparasitica 10:21–26

Figueredo AJ, Baker TC (1992) Reduction of the response to sex pheromone in the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae) following successive pheromonal exposures. J Insect Behav 5(3):347–363. doi:10.1007/BF01049843

Gadenne C, Barrozo RB, Anton S (2016) Plasticity in insect olfaction: to smell or not to smell? Annu Rev Entomol 61:317–333. doi:10.1146/annurev-ento-010715-023523

Groning J, Hochkirch A (2008) Reproductive interference between animal species. Q Rev Biol 83(3):257–282

Grubb M, Thompson I (2004) The influence of early experience on the development of sensory systems. Curr Opin Neurobiol 14:503–512. doi:10.1016/j.conb.2004.06.006

Guerrero A, Malo E, Coll J, Quero C (2014) Semiochemical and natural product-based approaches to control *Spodoptera* spp. (Lepidoptera: Noctuidae). J Pest Sci 87:231–247. doi:10.1007/s10340-013-0533-7

Guerrieri F, Gemeno C, Monsempes C, Anton S, Jacquin-Joly E, Lucas P, Devaud J-M (2012) Experience-dependent modulation of antennal sensitivity and input to antennal lobes in male moths (*Spodoptera littoralis*) pre-exposed to sex pheromone. J Exp Biol 215(13):2334–2341. doi:10.1242/jeb.060988

Hansson BS (1995) Olfaction in lepidoptera. Experientia 51:1003–1027. doi:10.1007/BF01946910

Kehat M, Dunkelblum E (1993) Sex pheromones—achievements in monitoring and mating disruption of cotton pests in Israel. Arch Insect Biochem Physiol 22(3–4):425–431. doi:10.1002/arch.940220310

Kehat M, Greenberg S, Tamaki Y (1976) Field evaluation of the synthetic sex pheromone, as an attractant for males of the cotton leafworm, *Spodoptera littoralis* (Boisd.) in Israel. Appl Ent Zool 11(1):45–52

Kromann SH, Saveer AM, Binyameen M, Bengtsson M, Birgersson G, Hansson BS, Schlyter F, Witzgall P, Ignell R, Becher PG (2015) Concurrent modulation of neuronal and behavioural olfactory responses to sex and host plant cues in a male moth. Proc R Soc Biol Sci Ser B 282(1799):20141884. doi:10.1098/rspb.2014.1884

Kuenen LPS, Baker TC (1981) Habituation versus sensory adaptation as the cause of reduced attraction following pulsed and constant sex pheromone pre-exposure in *Trichopolusia ni*. J Insect Physiol 27:721–726

Legeai F, Malpel S, Montagné N, Monsempes C, Cousserans F, Merlin C, François M-C, Maïbèche-Coisné M, Gavory F, Poulain J, Jacquin-Joly E (2011) An expressed sequence tag collection from the male antennae of the noctuid moth *Spodoptera littoralis:* a resource for olfactory and pheromone detection research. BMC Genom 12(1):86. doi:10.1186/1471-2164-12-86

Ljungberg H, Anderson P, Hansson BS (1993) Physiology and morphology of pheromone-specific sensilla on the antennae of male and female *Spodoptera littoralis* (Lepidoptera, Noctuidae). J Insect Physiol 39(3):253–260. doi:10.1016/0022-1910(93)90096-a

Martínez T, Camps F (1988) Stimulation of sex pheromone production by head extract in *Spodoptera littoralis* at different times of the photoperiod. Arch Insect Biochem Physiol 9:211–220. doi:10.1002/arch.940090305

Merlin C, Lucas P, Rochat D, Francois MC, Maïbèche-Coisne M, Jacquin-Joly E (2007) An antennal circadian clock and circadian rhythms in peripheral pheromone reception in the moth *Spodoptera littoralis*. J Biol Rhythms 22(6):502–514. doi:10.1177/0748730407307737

Montagné N, Chertemps T, Brigaud I, Francois A, Francois MC, de Fouchier A, Lucas P, Larsson MC, Jacquin-Joly E (2012) Functional characterization of a sex pheromone receptor in the pest moth *Spodoptera littoralis* by heterologous expression in *Drosophila*. Eur J Neurosci 36:2588–2596. doi:10.1111/j.1460-9568.2012.08183.x

Mukunda L, Miazzi F, Sargsyan V, Hansson BS, Wicher D (2016) Calmodulin affects sensitization of *Drosophila melanogaster* odorant receptors. Front Cell Neurosci 10:28. doi:10.3389/fncel.2016.00028

Muñoz L, Rosell G, Quero C, Guerrero A (2008) Biosynthetic pathways of the Egyptian armyworm: an update. Physiol Entomol 33:275–290. doi:10.1111/j.1365-3032.2008.00645.x

Nesbitt BF, Beevor PS, Hall DR, Lester R, Poppi RG (1973) Sex pheromones of two noctuid moths. Nat New Biol 244:208–209

Poitout S, Bues R (1974) Élevage de chenilles de vingt-huit espèces de Lépidoptères Noctuidae et de deux espèces d'Arctiidae sur milieu artificiel simple. Particularités de l'élevage selon les espèces. Ann Zool Écol Anim 6:431–441

Quero C, Lucas P, Renou M, Guerrero A (1996) Behavioral responses of *Spodoptera littoralis* males to sex pheromone components and virgin females in wind tunnel. J Chem Ecol 22(6):1087–1102. doi:10.1007/BF02027947

Quero C, Vidal B, Guerrero A (2014) EAG responses increase of *Spodoptera littoralis* antennae after a single pheromone pulse. Nat Prod Commun 9(8):1099–1101

Rankin CH, Abrams T, Barry RJ, Bhatnagar S, Clayton DF, Colombo J, Coppola G, Geyer MA, Glanzman DL, Marsland S, McSweeney FK, Wilson DA, Wu C-F, Thompson RF (2009) Habituation revisited: an updated and revised description of the behavioral characteristics of habituation. Neurobiol Learn Mem 92(2):135–138. doi:10.1016/j.nlm.2008.09.012

Saïd I, Renou M, Morin JP, Ferreira JM, Rochat D (2005) Interactions between acetoin, a plant volatile, and pheromone in *Rhynchophorus palmarum*: behavioral and olfactory neuron responses. J Chem Ecol 31(8):1789–1805

Saveer AM, Becher PG, Birgersson G, Hansson BS, Witzgall P, Bengtsson M (2014) Mate recognition and reproductive isolation in the sibling species *Spodoptera littoralis* and *Spodoptera litura*. Front Ecol Evol 2(18):1–7. doi:10.3389/fevo.2014.00018

Snijders T, Boske RJ (2012) Multilevel analysis: an introduction to basic and advanced multilevel modeling, 2nd edn. Sage Publishers, London

StataCorp (2011) Stata statistical software: release 12. Stata Corp, College Station

Stelinski LL, Gut LJ, Miller JR (2003a) Concentration of air-borne pheromone required for long-lasting peripheral adaptation in the obliquebanded leafroller, *Choristoneura rosaceana*. Physiol Entomol 28(2):97–107. doi:10.1046/j.1365-3032.2003.00319.x

Stelinski LL, Miller JR, Ressa NE, Gut LJ (2003b) Increased EAG responses of tortricid moths after prolonged exposure to plant volatiles: evidence for octopamine-mediated sensitization. J Insect Physiol 49(9):845–856. doi:10.1016/S0022-1910(03)00136-7

Tamaki Y, Yushima T (1974) Sex pheromone of the cotton leafworm, *Spodoptera littoralis*. J Insect Physiol 20:1005–1014