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Support for (*Z*)-11-Hexadecanal as a Pheromone Antagonist in *Ostrinia nubilalis*: Flight Tunnel and Single Sensillum Studies with a New York Population

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Abstract The flight-tunnel response of male Z-strain European corn borer moths (ECB), *Ostrinia nubilalis*, from a population in New York State (USA), was significantly antagonized by addition of 1% (*Z*)-11-hexadecanal (Z11-16:Ald) to their sex pheromone (a 97:3 mix of (*Z*)- and (*E*)-11-tetradecenyl acetate [Z/*E*11-14:OAc]). The level of antagonism was equivalent to that observed for the previously identified ECB antagonist, (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), and supports a recent report showing that Z11-16:Ald, a minor pheromone component of the Noctuid moth, *Sesamia nonagrioides*, caused antagonism of ECB pheromone communication in sympatric populations in the Iberian Peninsula. Single-sensillum recordings from ECB antennae, which included cross-adaptation experiments, showed that the same olfactory receptor neuron processing Z9-14:OAc inputs was responsible for detecting Z11-16:Ald, and that this neuron was not responsive to two other aldehydes, (*Z*)-9-tetradecanal (Z9-14:Ald) and (*Z*)-9-hexadecanal (Z9-16:Ald), found in other moth sex pheromones. Our results show that the antagonism is not confined to one geographic region, is specific for Z11-16:Ald, and that antagonist pathways might have the potential for processing a number of structurally similar compounds.

Keywords Sex pheromones · European corn borer · *Ostrinia nubilais* · Mediterranean corn borer · *Sesamia nonagrioides* · Behavioral antagonist · Z11-16:Ald · Flight tunnel · Single sensillum recordings · Cross adaptation

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Introduction

Mate location in many insect species is mediated by female-released sex pheromones. In almost all cases, these have proven to be mixtures, with blend quality and quantity providing for varying degrees of signal specificity (Linn and Roelofs 1995). Signal specificity is also facilitated by sensitivity to pheromone components of related species that function as behavioral antagonists (Cardé and Haynes 2004). When added in very small amounts to the sex pheromone of a particular species, the new mixture results in arrestment of pheromone-mediated flight. For example, different pheromone strains of the European corn borer moth (ECB), *Ostrinia nubilalis* (Crambidae), utilize mixtures of (*Z*)-11- and (*E*)-11-tetradecenyl acetates (*Z*11/*E*11-14:OAc) in their sex pheromones (Roelofs et al. 1985, 1987). However, males are also sensitive to a non-pheromonal compound, (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), and possess a separate antennal olfactory pathway for processing this compound (Hansson et al. 1987). As little as 1% Z9-14:OAc added to the Z/E11-14:OAc mixture dramatically reduced the number of males completing upwind oriented flight in a wind tunnel (Glover et al. 1989).

A study by Eizaguirre et al. (2002) showed, in pheromone mating disruption tests for the Mediterranean corn borer, *Sesamia nonagrioides* (Noctuidae), that larval counts were significantly reduced for the target species and for ECB. Combining pheromone blends of both species in the same dispenser showed further that there was mutual antagonism for both species. Subsequently, Gemeno et al. (2006) showed that the antagonism was caused specifically by addition of (*Z*)-11-hexadecanal (Z11-16:Ald) to the ECB pheromone, with as little as 1% reducing upwind flights by 83% and trap capture by 90%. The compound Z11-16:Ald is a minor pheromone component of *S. nonagrioides* and is not part of the ECB pheromone. Here, we provide results of flight tunnel and single-sensillum recording experiments to address the following questions: 1) are ECB from a population in New York State antagonized by addition of Z11-16:Ald to their pheromone blend? 2) is the sensory input for Z11-16:Ald on the same olfactory receptor neuron (ORN) as for Z9-14:OAc?, and 3) do other aldehyde components, such as Z9-14:Ald and Z9-16:Ald, that are pheromone components in several heliothine moth species (Lee et al. 2006), also act as antagonists for ECB?

Methods and Materials

Insects A colony of the univoltine Z-strain of ECB from New York State was maintained in a walk-in environmental chamber. The Z-strain was used in our experiments because it is the predominant population in the Iberian Peninsula, the origin of moths used in the studies by Eizaguirre et al. (2002) and Gemeno et al. (2006). Mating and larval rearing conditions were as described in Roelofs et al. (1987) at a constant 25°C, 16:8 L/D photoperiod. Pupae were sexed, and the male pupae were placed on a layer of vermiculite in plastic and screen emergence cages inside a walk-in environmental chamber (25°C; 16:8 L/D photoperiod). Cages of adults were separated daily so that individuals of known age could be used for flight tunnel tests.

Chemicals Z11-14:OAc, E11-14:OAc, and Z9-14:OAc were obtained from the Pherobank http://www.pherobank.nl). Z11-16:Ald, Z9-14:Ald, and Z9-16:Ald were obtained from Bedoukian Research, Inc. (Danbury, CT, USA). Purity of each compound was verified as >95% by gas chromatography (GC). Stock solutions were prepared in high-performance

liquid chromatography-grade hexane (HPLC-grade hexane). Solutions were applied to red rubber septa (Thomas Scientific, Swedesboro, NJ, USA; Glover et al. 1989) for flight-tunnel tests, and to filter paper strips for electrophysiological measurements (see below).

Flight Tunnel Adults were tested in the sustained-flight tunnel during their second to third night as adults, under standard conditions used in previous studies of *O. nubilalis* (Glover et al. 1989): 20–21°C, 60–65% relative humidity, 0.50 m/sec air flow, and illumination of 11 lx of red light at the tunnel floor, during the third to the sixth hour of scotophase. Adults were taken to the room housing the flight tunnel 1 hr before the start of scotophase and placed individually in screen release cages so they could acclimate to the flight tunnel room environment. The temperature during the 1-hr period of acclimation, was 25°C, then dropped after lights-off to 20–21°C. Adult moths were tested individually, and males were scored for three behaviors in the flight sequence: taking flight (TF) from the release cage; initiation of upwind flight (UP) a minimum of 20 cm toward the source; and source (SC) contact after completing upwind flight to the rubber septum.

Two experiments were conducted to study the effects of Z11-16:Ald on ECB pheromone-mediated flight behavior. The first was designed to determine whether Z11-16:Ald arrested the upwind flight behavior of males, and to compare the level of antagonism with Z9-14:OAc (Glover et al. 1989). Males were tested to four treatments: 1) the Z-strain 97:3 Z/E11-14:OAc blend (30 μg); 2) the Z-strain blend (30 μg)+1% Z9-14:OAc; 3) the Z-strain blend (30 μg)+1% Z11-16:Ald; and 4) the Z-strain blend (30 μg)+10% Z11-16:Ald. The second experiment was designed to determine the specificity of the antagonist effect of Z11-16:Ald by testing two additional, structurally related aldehydes found in other pheromone systems, Z9-14:Ald and Z9-16:Ald (Lee et al. 2006). Males were tested to three treatments: 1) the Z-strain 97:3 Z/E11-14:OAc blend (30 μg); 2) the Z-strain blend (30 μg)+10% Z9-14:Ald; and 3) the Z-strain blend (30 μg)+10% Z9-16:Ald. Over the course of both experiments, 30 males were tested to each treatment. Males were tested only once, with each of the treatments of an experiment used on each day of testing.

For each flight tunnel experiment, the number of moths in each treatment that exhibited each behavior in the upwind flight sequence was combined for all testing periods and converted to a percentage value for graphical display. *Post hoc* pairwise comparisons with Fisher's exact tests were implemented according to the JMP statistical analysis program for Macintosh ($P<0.05$).

Electrophysiology With excised antennae, ORN recordings were made by using the cut sensillum technique (Kaissling 1974; van der Pers and den Otter 1978). The basal end of each antenna was placed in a saline-filled electrode and positioned with a micromanipulator such that a single trichoid sensillum would be cut between a vertical tungsten knife and horizontal glass knife. The cut sensillum was then contacted with a saline-filled glass micropipette containing a Ag recording electrode. The AC signal passed through the built-in amplifier (DAM50, World Precision Instruments, Sarasota, FL, USA) of a portable recording unit into a computer and was recorded with Syntech software (Autospike v.32; Syntech, Kirchzarten, Germany).

A stream of purified, humidified air blew continuously over the antenna (10 ml/sec) through a 25-cm long glass tube (8 mm ID), the end of which was placed 2 cm from the antenna. With a stimulus flow controller device (SFC-2, Syntech), a 50-msec air pulse at 40 ml/sec flow rate was injected through the odor cartridge and into the air stream. Linear flow through the air stream was ~0.3 m/sec. Cartridges were created from dilutions (0.1, 1, and 10 μg/μl) of Z11-16:Ald and Z9-14:OAc, and from 10 μg/μl of Z11-14:OAc, E11-14:

OAc, Z9-14:Ald, and Z9-16:Ald in HPLC-grade hexane. For each concentration, 10 μl was pipetted onto a 0.5×2.0 cm filter paper strip held in a 15-cm-long Pasteur pipette odor cartridge. The filter paper loadings thus included 1, 10, or 100 μg for the various compounds.

By using 24 antennae from 18 moths, dose–response curves were obtained for Z11-16:Ald and Z9-14:OAc. A balanced design with respect to the order of application of treatments was employed to allow a more accurate comparison of the relative responsiveness of the ORNs to these compounds. For the first 12 antennae, recordings were performed with 1, 10, and 100 μg loadings of Z11-16:Ald in the first sensillum, whereas recordings to the same range of dosages of Z9-14:OAc were performed on a second sensillum. For the remaining 12 antennae, Z9-14:OAc was used before Z11-16:Ald. At least 30 sec was allowed to elapse between all stimulations. Spikes were counted from within 300 msec of the initiation of neuronal activity, which can occur between 50 and 200 msec after the stimulus is first applied (Fig. 1a). As previously reported, there was little spontaneous background activity (Cossé et al. 1995; Domingue et al. 2006), and the initiation of response was easily discerned by the first appearance of spikes. Analysis of variance (ANOVA) was performed by using the GLM procedure of SAS (Version 9.1), to test the effects of order of application and compound on response at the 100 μg loading. Responses at 1 μg were nearly always zero for both compounds. Responses at 10 μg to both compounds often occurred, but there were enough non-responses to cause non-normality of the data at this concentration (Wilks normality, α=0.05). However, by using the increase in spike frequency from 10+ to 100 μg, which is normally distributed, we performed a similar ANOVA.

Paired stimulations aimed at exploring cross-adaptation were also performed, in most cases by using additional sensilla from the antennae that were used to obtain dose–response curves. Air from two pheromone cartridges, with loadings of 100 μg, was pulsed as

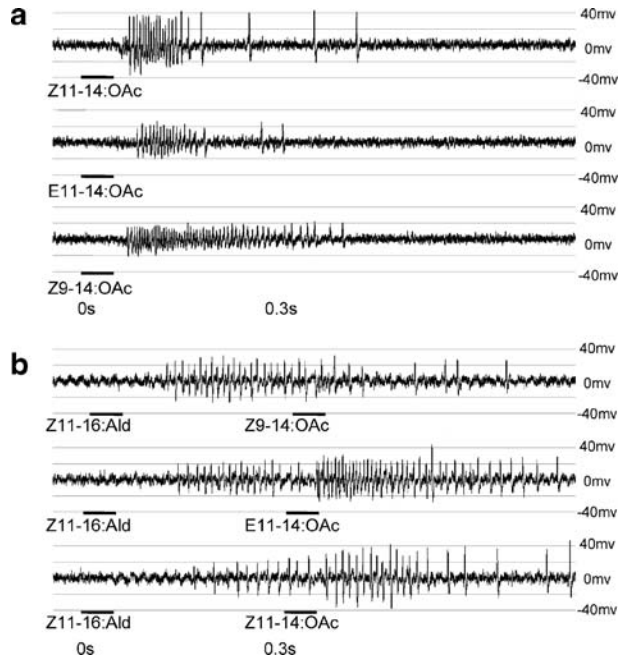

Fig. 1 Examples of ORN stimulation in Z-strain ECB in two sensilla from different individuals, using 100-μg loadings in each case: (a) Spike patterns in response to the known pheromone components Z11-14:OAc and E11-14:OAc and the antagonist Z9-14:OAc. (b) Cross-stimulation involving Z11-16:Ald with the antagonist Z9-14:OAc, the minor pheromone component E11-14:OAc, and the major pheromone component Z11-14:OAc. Horizontal bars indicate stimulus application and duration

described above, with an interval of 300 msec between the start of the pulses from the first and second cartridges. This method is similar to that employed for other moths (Ochieng et al. 2002) and was calibrated from unpublished data for this species such that for each ORN there is self-adaptation, marked by a lack of repeated stimulation by the same compound. To infer which ORN is sensitive to Z11-16:Ald, cartridge pairs used for cross-adaptation *versus* this compound included, Z9-14:OAc, Z11-14:OAc, and E11-14:OAc, which are, respectively, known to stimulate distinct ORNs (Hansson et al. 1987). It was readily apparent that cross-adaptation occurs between Z11-16:Ald and Z9-14:OAc. For comparative purposes, and to ensure the validity of our experimental technique, we also performed self-adaptation with Z9-14:OAc and Z11-16:Ald.

At least 10 replicates of all paired stimulations were performed. No more than three paired stimulations were performed per sensillum, with at most five sensilla examined per antenna. In many cases, there were two distinct bursts of neuronal activity with obviously different spike sizes (Fig. 1b). To assess the intensity of response to stimulation by the first compound, spikes were counted within 300 msec of the initiation of neuronal activity. For the second compound, initiation of the second response was noted if a different spike size from the first stimulus appeared, or if there was a period of inactivity of greater than 50 msec, which would normally not be observed in spike trains arising from a single stimulation. In a few rare cases, there were similarly sized action potentials in a continuously uninterrupted pattern for more than 300 msec after the initial response. Because the initiation of a second response was unclear in these cases, we assigned the counts for the first and second compounds to the respective periods between 0 and 300 msec and 300 and 600 msec after the first onset of neuronal activity.

To describe the process of cross-adaptation statistically we performed three separate ANOVA, considering pairings involving: (1) Z11-16:Ald by E11-14:OAc cross-adaptation; (2) Z11-16:Ald by Z11-14:OAc cross-adaptation: and (3) Z11-16:Ald by Z9-14:OAc cross-adaptation or self-adaptation of either compound. For this analysis, we discarded categorization of spike size and looked at the effect on spike frequency of being the first or second compound applied (Order), which would indicate adaptation. For all three analyses, the effect on spike frequency of compound identity (Compound) and the interaction between Compound and Order were also considered. For the third analysis only, the effect of cross-adaptation vs. self-adaptation (X-adapt) was also considered, along with its interactions with Compound and/or Order.

To explore the sensitivity of Z-strain ECB ORNs to the aldehydes, Z9-14:Ald and Z9-16:Ald, an additional experiment was performed. Responses to Z9-14:Ald and Z9-16:Ald were assessed in comparison to a control consisting of Z11-16:Ald or Z9-14:OAc, with 24 antennae from different moths. In all cases, 100 μg cartridge loadings were used. On four successive sensilla from each antenna, one of the following sequences of stimulations was performed: A) Z9-14:Ald, Z9-16:Ald, and Z11-16:Ald; B) Z9-14:Ald, Z9-16:Ald, and Z9-14:OAc; C) Z9-16:Ald, Z9-14:Ald, and Z11-16:Ald; D) Z9-16:Ald, Z9-14:Ald, and Z9-14:OAc. Across 24 moths, each of the possible combinations of treatments A–D, with respect to sensillum order, was completed once. With this design, Z9-14:Ald and Z9-16:Ald were tested 96 times, and Z11-16:Ald and Z9-14:OAc 48 times. For this last experiment, we examined both the spike frequency and presence or absence of response for each test compound and the control with respect to sensillum order. For a small number of pairwise comparisons of interest, *t* tests or Fisher's exact tests were used depending on whether the spike frequency or presence of response was considered. Similarly, least-square or logistic regressions were performed when appropriate for either type of variable, by using Proc Reg or Proc Logistic in SAS.

Results

Flight Tunnel Experiment 1: There was no difference in the percentage of males taking flight (100%) among the four treatments (Z-strain blend alone,+1% Z9-14:OAc,+1% or 10% Z11-16:Ald, Fig. 2a). Significant decreases, however, were observed in the percentage of males initiating upwind flight with Z-strain blend and any of the added compounds, compared with the Z-strain blend alone. The greatest decrease occurred with 10% Z11-16:Ald treatment (0% UP vs. 100% for Z-strain alone; Fisher's exact $P<0.001$, $df=1$), followed by 1% Z9-14:OAc and 1% Z11-16:Ald (62 and 74%, respectively, vs. 100% for Z-strain alone; Fisher's exact $P<0.001$, $df=1$, for 100% vs. 74%). Significant decreases were further observed in the percentage of males reaching the source, with 0, 4, and 8% SC for 10% Z11-16:Ald, 1% Z9-14:OAc, and 1% Z11-16:Ald, respectively, compared with the Z-strain blend alone (97% SC; Fisher's exact $P<0.001$, $df=1$, for 100% vs. 8%).

Experiment 2: As with experiment 1, male Z-strain ECB exhibited high levels of upwind flight (UP, 100%) and source contact (SC, 96%) with the Z-strain blend alone (Fig. 2b). However, addition of 10% Z9-14:Ald or Z9-16:Ald did not affect the percentage of upwind

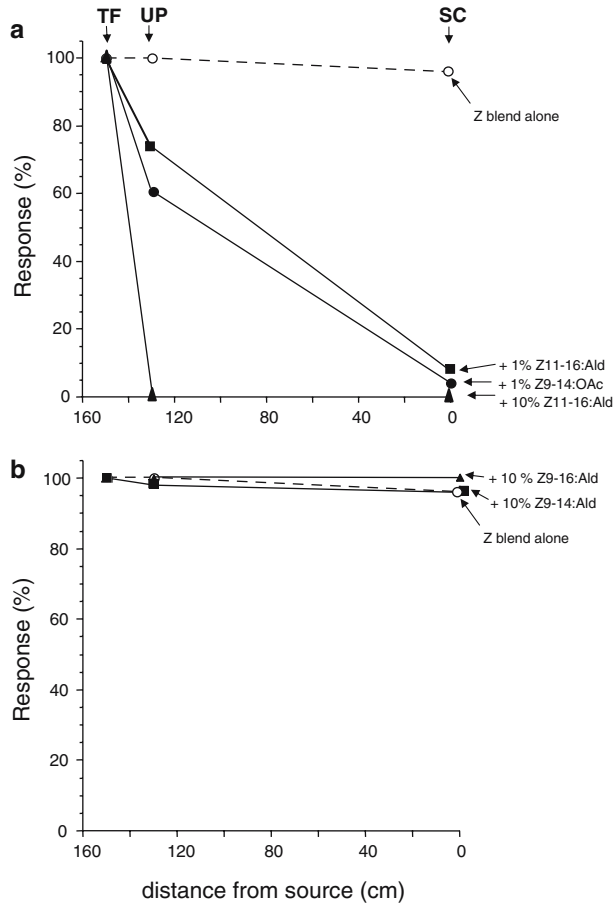

Fig. 2 Proportion of male ECB of the Z-strain exhibiting three behaviors in flight tunnel tests (taking flight, TF; upwind flight, UP; source contact, SC) to: (a) the Z-strain 97:3 Z/E11-14:OAc blend alone (30 μg), the Z-strain blend+ 1% or 10% Z11-16:Ald, or the Z-strain blend+1% Z9-14:OAc; (b) the Z-strain 97:3 Z/E11-14:OAc blend alone (30 μg), the Z-strain blend+10% Z9-14:Ald or 10% Z9-16:Ald. $N=30$ for each of the treatments. See text for statistical comparisons

flights and source contact observed with the Z-strain blend alone (100% SC with Z9-16:Ald, 96% SC with Z9-14:Ald).

Electrophysiology We were able to obtain dose–response curves to both Z11-16:Ald and Z9-14:OAc (Fig. 3a). From Fig. 3, it appears as if the sensilla are relatively more sensitive to Z11-16:Ald if it is applied first. However, statistical analyses did not confirm the significance of this pattern. At 100 μg, responses to Z9-14:OAc were greater than those to Z11-16:Ald (i.e., Compound is significant, Table 1). This result was not significantly influenced by which compound was applied first (i.e., Order and the interaction, Table 1). Similarly, for the increase in spike frequency between the 10 to 100 μg loadings, the effect of Compound was significant, but not Order or the interaction (Table 1).

In a sensillum not used for any statistical analysis (Fig. 1a), we demonstrated that ORN responses to Z11-14:OAc, E11-14:OAc, and Z9-14:OAc occurred with the same relative spike sizes as found previously (Hansson et al. 1987; Roelofs et al. 1987). It was apparent in cross-adaptation experiments that, based on spike size, Z11-16:Ald stimulated the same ORN as did Z9-14:OAc. This cross-responsive (i.e., cross-adapted) ORN was usually characterized by the smallest spike size (Fig. 1b). The ORN responding to E11-14:OAc was most often of intermediate spike size, being slightly smaller than that for Z11-14:OAc, which was always the largest (Fig. 1b).

These visual observations were supported by statistical analysis of the cross-adaptation data. When Z11-16:Ald was paired with E11-14:OAc or Z11-14:OAc, there was no effect of Order on spike frequency (Table 2, Fig. 4). Analysis of spike frequencies in stimulations involving Z11-16:Ald and Z9-14:OAc showed a strongly significant effect of Order (Table 2, Fig. 4), meaning that the second stimulation was generally inhibited by the first. In this analysis, there was also a significant effect of Compound (Table 2) demonstrating

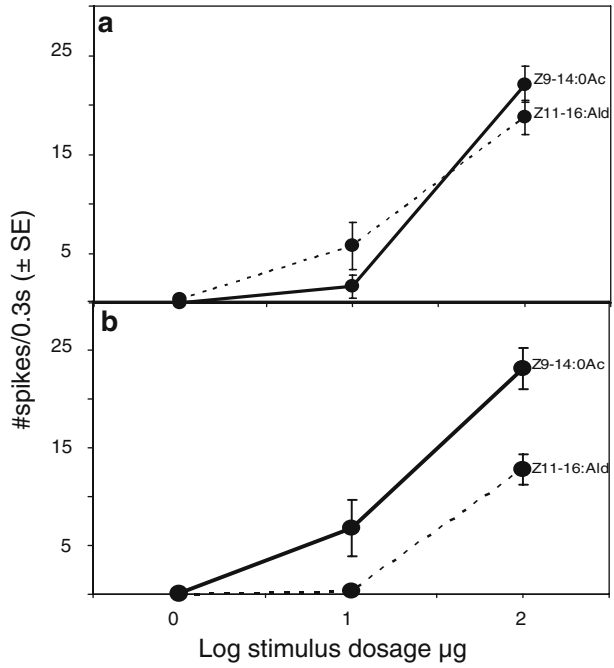

Fig. 3 Dosage-dependent response of male Z-strain ECB ORNs to Z11-16:Ald and Z9-14:OAc. (a) For $N=12$ antennae, dose-response testing was performed on the first sensillum to Z11-16:Ald, and on a second sensillum to Z9-14:OAc. (b) For another $N=12$ antennae, dose-response was performed first to Z9-14:OAc, and then to Z11-16:Ald

Table 1 Analysis of variance of spike frequency (spikes/0.3 sec) at a 100-μg loading and spike frequency increase from a 10- to 100- μg loading, both with respect to whether Z9-14:OAc or Z11-16:Ald is employed, and in which respective order per antenna these compounds were tested

Variable	Effect	*df*	*MS*	*F*	*P*
Spikes at 100 μg	Order	1	75.00	1.34	0.260
	Compound	1	560.33	9.98	0.005
	Order–Compound	1	147.00	2.62	0.120
	Error: Antenna (Order)	22	56.121		
Spike frequency increase (10 to 100 μg)	Order	1	65.33	0.84	0.370
	Compound	1	38.33	4.94	0.037
	Order–Compound	1	36.75	0.47	0.500
	Error: Antenna (Order)	22	78.07		

Order distinguishes whether the response was the first or second on an antenna. Compound distinguishes whether Z9-14:OAc or Z11-16:Ald is employed for a given stimulation. For both cases, we use the Antenna for the model error, which is nested in the specific Order treatment.

that over all treatments, the ORNs were more sensitive to Z9-14:OAc than to Z11-16:Ald. However, there was no significant effect of self- *versus* cross-adaptation (X-adapt) or any interaction effect (Table 2).

Responses by the smallest-spiking (antagonist) ORN to two other aldehydes, Z9-14:Ald and Z9-16:Ald, were generally negligible in single stimulations, yielding at most 2.4 spikes per 300 msec when Z9-14:Ald was puffed on the first sensillum of a particular antenna (Fig. 5a). It did not appear that these responses occurred in the E11- or Z11-14:OAc-tuned ORNs. On the first sensillum, when either Z9-14:Ald or Z9-16:Ald had equal opportunity to be puffed first, there was a 25% success rate of getting a response to Z9-14:Ald, (Fig. 5b).

Table 2 Analysis of variance of spike frequency (spikes/0.3 sec) for the cross-stimulation experiments grouped by the pairing of compounds with respect to Z11-16:Ald

Experiment	Effect	*df*	*MS*	*F*	*P*
E11-14:OAc vs. Z11-16:Ald	Order	1	0.319	0.01	0.935
	Compound	1	1139.147	24.26	<0.001
	Order–Compound	1	195.669	4.03	0.054
	Error: Pair(Order)	21	46.92		
Z11-14:OAc vs. Z11-16:Ald	Order	1	27.225	0.56	0.462
	Compound	1	133.225	2.76	0.114
	Order–Compound	1	0.225	0.00	0.946
	Error: Pair(Order)	18	48.225		
Z9-14:OAc vs. Z11-16:Ald	X-adapt	1	173.852	2.50	0.121
	Order	1	7896.071	113.72	<0.001
	Compound	1	775.013	11.16	0.002
	X-adapt–Order	1	20.885	0.30	0.586
	Order–Compound	1	49.613	0.71	0.403
	X-adapt–Compound	1	78.013	1.12	0.295
	Error: pair (X-adapt–Order)	42	69.437		

Order distinguishes whether a stimulus was first or second in the paired stimulation. Compound distinguishes which stimulus was provided for a given stimulation. X-adapt distinguishes whether a stimulus occurred within a cross-stimulated pair, or if the same stimulus was repeated. In each case, we use the unit of the unique paired stimulation event, Pair, as the error, which is nested in the specific Order or X-adapt treatment performed.

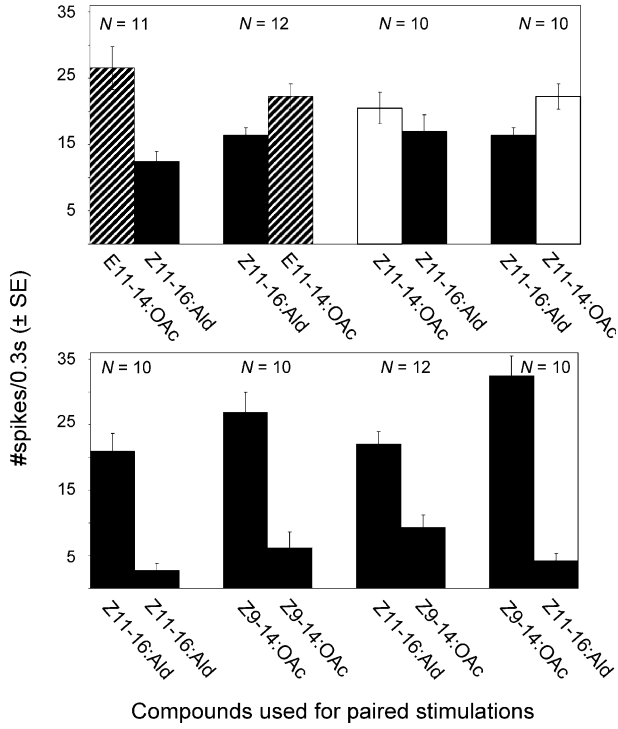

Fig. 4 Number of spikes per 300 msec for cross-stimulation involving Z11-16:Ald, Z11-14:OAc, E11-14:OAc, and Z9-14:OAc at 100-µg loadings. Differences in shading indicate ORNs with distinct spike sizes: a large spike size for Z11-14:OAc, an intermediate spike size for E11-14:OAc, and a small spike size for Z11-16:Ald and Z9-14:OAc

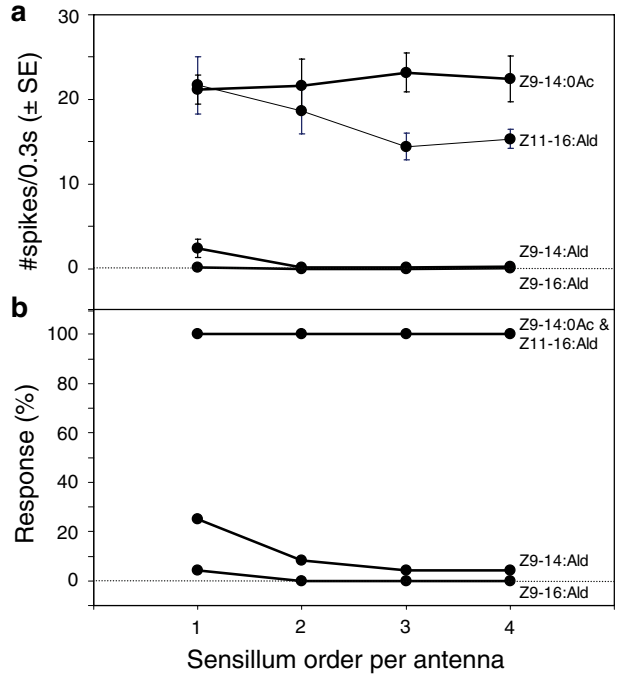

Fig. 5 Sensitivity of Z-strain ECB ORNs to Z9-14:Ald, Z9-16:Ald, Z11-16:Ald, and Z9-14:OAc in each successive sensillum tested per antenna, measured with respect to: (a) the mean (± SE) number of spikes stimulated over 300 msec at 100-µg loadings and (b) the percentage of sensillae responding. For Z9-14:Ald and Z9-16:Ald N=24 for each data point, whereas for Z11-16:Ald and Z9-14:OAc N=12

This is statistically different from the 100% response to the control compounds (Fisher's exact test, $P<0.001$). Under the same conditions, there was only a 4% success rate of this ORN responding to Z9-16:Ald on the first sensillum, which is marginally different (Fisher's exact test, $P=0.098$) from the 25% rate for Z9-14:Ald (Fig. 5b). Logistic regression for likelihood of response to Z9-14:Ald showed a significant effect of sensillum order for Z9-14:Ald ($P=0.026$), but not Z9-16:Ald ($P=0.918$).

Although designed primarily to explore the relative sensitivity of the antagonistic ORN to Z9-14:Ald and Z11-16:Ald, the final experiment also provided the opportunity to examine how the response to Z11-16:Ald and Z9-14:OAc changed with the use of multiple sensilla per moth. There was no significant difference between spike frequency for the two compounds at the first sensillum ($P=0.889$). However, a least-squares regression for Z11-16:Ald, had a significant negative slope ($P=0.031$), whereas the slope for Z9-14:OAc was not different for a similar regression involving Z9-14:OAc ($P=0.633$).

Discussion

Effects of Z11-16:Ald on Male ECB Flight Behavior Our results with males from a New York state ECB population support the finding by Gemeno et al. (2006) that Z11-16:Ald, when added to the ECB pheromone blend, can significantly arrest upwind flight behavior of male ECB. Addition of 1% Z11-16:Ald to the ECB Z-strain 97:3 Z/E11-14:OAc mixture reduced the proportions of males initiating upwind flight by 25% and the proportion reaching the source by 88%. These values compare well with the 26 and 83% reductions observed in flight tunnel tests reported by Gemeno et al. (2006). Our results show further that the antagonism by Z11-16:Ald is not restricted to the area of the Iberian Peninsula where *O. nubilalis* and *S. nonagrioides* are in sympatry, but also extends to ECB populations in North America. A third finding from our studies is that the effect appears to be specific to Z11-16:Ald because the related Z9-14:Ald and Z9-16:Ald, known pheromone components of other moth species, did not affect male flight behavior.

The effect of Z11-16:Ald was characteristic of other known pheromone behavioral antagonists (Linn and Roelofs 1995), evidenced by (1) a pronounced reduction in behavioral response when very low levels (1%) of the compound were added to the sex pheromone, and (2) significant reductions in the number of males completing upwind flights to the pheromone source (SC in Fig. 2). As for other behavioral antagonists, there was less of an effect on earlier behaviors such as taking flight or initiating upwind flight in ("locking onto") the pheromone plume (Fig. 2). Our anecdotal observations, with the 1% Z11-16:Ald treatment, showed that males typically exhibited upwind zig-zag flights, characteristic of pheromone-mediated flight, from one-half to two-thirds of the distance to the source, then began casting across wind, or hovered narrowly in place for several seconds, before exiting the pheromone plume. This flight pattern has been characterized as "arrestment" of upwind flight and is typical of the effect of behavioral antagonists (Baker 1989).

Olfactory Neuron Responses to Z11-16:Ald Gemeno et al. (2006) reported results from EAG experiments suggesting that Z11-16:Ald was not interacting with ORNs specific for the ECB pheromone components. EAG cross-adaptation experiments, however, did suggest the possibility that Z11-16:Ald and Z9-14:OAc were detected by the same ORN, one that previously had been shown to be specific for the latter compound (Hansson et al. 1987). In our study, single-sensillum recordings confirmed, via spike amplitude-matching, that a type

of ORN within the trichoid sensilla on ECB antennae is tuned to respond to both the Z11-16:Ald behavioral antagonist (Gemeno et al. 2006; this paper) and another antagonist Z9-14:OAc (Glover et al. 1989). There was negligible activity from this ORN in response to Z9-14:Ald or Z9-16:Ald, both of which were behaviorally inert. That the responses to Z11-16:Ald occurred on the same ORN that detects the presence of Z9-14:OAc was also supported by cross-adaptation studies.

(Z)-11-Hexadecanal and Pheromone Behavioral Antagonism in ECB: Adaptation or Fortuitous Mimic? The importance of behavioral antagonists in pheromone communication systems is well established (Cardé and Haynes 2004). These compounds contribute to signal specificity by lowering the probability of cross-attraction between species that utilize common pheromone components. This antagonism might involve closely related species that have the potential to mate or species that are more distantly related. In either case, signal specificity is enhanced in a "noisy" chemical environment (Linn and Roelofs 1995). In most of the known cases, the antagonist chemicals are components involved in communication channels used by species that share common pheromone components, as a result of common lineage and use of similar biosynthetic pathways. Thus, for ECB, Z9-14:OAc is a potential by-product from its pheromone biosynthetic pathways and is also a pheromone component in two related *Ostrinia* species in Japan (*O. zealis* and *O. zaguliaevi*, Ishikawa et al. 1999). Phylogenetic analysis suggests that the latter species are ancestral to the group including ECB (Kim et al. 1999). Therefore, divergence might have involved a change in functionality for Z9-14:OAc. A second, well-studied example involves the heliothine moths, *Heliothis virescens* and *Helicoverpa zea* (Vickers et al. 1998), which utilize the same major pheromone component, Z11-16:Ald. In *H. virescens*, the compound Z9-14:Ald is a pheromone component, whereas in *H. zea* it acts as an antagonist. In both of these examples, males possess a separate, specialized, sensory pathway to process the antagonist input, suggesting that there has been selection for the character to enhance reproductive isolation or interference between species sharing common components (Löfstedt 1993; Linn and Roelofs 1995; Cardé and Haynes 2004).

The examples above illustrate the point that changes in odorant receptor (OR) expression or functionality of components in a common pathway are mechanisms that could give rise to new pheromone component blends, or behavioral antagonists. Antagonism could, however, also involve a chemical that is not a member of the suite of compounds that a group of species uses for its pheromones, if that chemical binds to an OR of an established antagonist pathway. We propose that this might be the case with Z11-16:Ald and ECB. This conclusion stems firstly from the observation that Z11-16:Ald is not involved in any known sex pheromone in an *Ostrinia* species (Ishikawa et al. 1999), and second, the aldehyde is structurally similar to Z9-14:OAc with respect to chain length and position of the double bond relative to the carbonyl group and the hydrocarbon end. Both behavioral antagonists might, therefore, bind to the same OR expressed in the ORN involved in antagonism, resulting in significant firing and a reporting of their presence to higher centers via the antagonist pathway. This explanation allows for the possibility that a small number of structurally similar compounds might exert an effect without having been under selection pressure to interfere with sex pheromone communication of two species sharing the same habitat. Gemeno et al. (2006), in fact, raised caution about the adaptive significance of their finding with respect to habitat-specific interactions between ECB and *S. nonagrioides*, noting that the antagonistic effect of Z11-16:Ald was initially observed as a result of combining pheromone blends in field trapping and pheromone mating disruption tests. The two species utilize the same host plants and have seasonal overlap, but are in different

families, differ in size, have no shared pheromone components, and mate at different times in the scotophase. In this scenario, the antagonist effect of Z11-16:Ald could be considered a coincidence of chemical structural similarity.

Alternatively, two different pheromone ORs could be expressed on the same dendrite, with one specifically binding Z9-14:OAc and the other, Z11-16:Ald. Although pheromone ORNs have generally been thought to express only one OR gene, recent studies on *Drosophila* (Goldman et al. 2005) as well as on *H. virescens* and *H. subflexa* (Baker et al. 2004) provide evidence for dual OR expression on a single ORN. In *O. nubilalis*, expression of two different ORs on the ORN involved in behavioral antagonism would imply an adaptive significance. This possibility would have been better supported if our analysis of variance for the paired stimulation experiments for these two compounds had shown a significant effect of cross- *versus* self-adaptation, or an interaction between cross- *versus* self-adaptation and the compound puffed. Thus, it appears that the neuronal adaptation caused by either of these compounds has an identical effect on subsequent stimulations, a pattern that would occur if both compounds bind to the same OR. However, such a pattern remains possible for a two-ORN model if recovery from adaptation is caused by factors other than OR saturation. Whether one or two ORs are involved in the behavioral antagonism observed in response to Z11-16:Ald and Z9-14:Ac, the result would be increased conspecific pheromone blend discrimination and, therefore, less cross-attraction in an environment occupied by other species that share a subset of the same compounds in their pheromone blends.

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