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An overlooked component: (Z)-9-tetradecenal as a sex pheromone in *Helicoverpa armigera*

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ABSTRACT

The sex pheromone blend of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is a multi-component system, as is that of many other moths, and (Z)-11-hexadecenal 90–99% + (Z)-9-hexadecenal 10–1% was recommended as a standard blend for attracting the species. However, this fails to account for the significance of other compounds that exist in the sex gland. The aim of the present study was to investigate the function of other compounds present in the female sex gland of *H. armigera*. Extract of female sex glands were analysed by GC–MS combined with GC–EAD. Total 10 compounds were identified, which two novel were reported in female sex gland: heptanal and nonanal, and some previously identified compounds were confirmed. We developed bioassays to evaluate the potential roles of these 10 compounds. In Y-tube bioassays, the gland constituents hexadecanal, (Z)-7-hexadecenal and (Z)-9-tetradecenal increased male attractiveness when added as a three-compound admixture to the standard blend. Field trapping tests showed that (Z)-9-tetradecenal doubled trap catch in comparison with the standard blend, but that the addition of (Z)-7-hexadecenal and hexadecanal did not significantly increase trap catch. These results indicated that while (Z)-7-hexadecenal and hexadecanal function well only at short range, (Z)-9-tetradecenal plays a very important role at both short and long ranges. We suggest that that (Z)-9-tetradecenal as a previously overlooked sex pheromone component of *H. armigera*, it should be added to sex pheromone lure formulations to improve pheromone trap sensitivity and the efficacy of commercial mating disruption.

1. Introduction

Sex pheromones play key roles in insect courtship and mating behaviors (Shorey, 1973), and their impact has been studied for more than half a century, especially in the Lepidoptera (Karlson and Butenandt, 1959). This highly diverse order of insects, with more than 150,000 described species (Ando et al., 2004), developed more channels of communication than did smaller groups to achieve unique communication signals for individual species (Byers, 2006). More than half of the species in the Lepidoptera have adopted at least two sex pheromone components (Byers, 2006; Renou and Lucas, 1994). One or two of these pheromones are "major" components, which can be detected effectively at long distances and generate an initial attraction response in males. The remainders of the compounds are minor components, which have been suggested act once the males reach the periphery of the female and play important roles in approaches and courtship. These minor components function in combination with the primary components to evoke other aspects of the mating sequence (Baker et al., 1976; Cardé et al., 1975; Roelofs and Cardé, 1977). This model leads to the hypothesis that minor components do not participate in long range attraction and that they are important in short range interactions such as approach, landing, and courtship (Baker et al., 1976; Cardé et al., 1975; Roelofs and Cardé, 1977; Bradshaw et al., 1983). However, evidence has shown that minor components in isolation have no behavioral effects; when these components are part of the total pheromone blend, however, they act synergistically and generate powerful behavioral responses (Baker and Cardé, 1979). These results led to the alternative hypothesis that the entire blend acts as a unit to create optimal attraction for males over the entire response range at the lowest response thresholds (Baker and Cardé, 1979; Linn et al., 1986, 1987). Regardless of the conflict between these hypotheses, a growing body of evidence suggests that minor components are able to increase attraction as part of a pheromone blend (Downham et al., 2003; Gries et al., 1996; Teal et al., 1986; Trimble and Marshall, 2008). Additionally, some minor components have recently been shown to inhibit attraction (Bosa et al., 2006; Curkovic, 2007; Mazor and Dunkelblum, 1992). Some components may function variably as attractants or antagonists depending on their proportions in the blend (Baker, 2008). Further evidence is required to understand fully the role of minor components in pheromone blends.

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The cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is an important pest worldwide (Fitt, 1989) and has a wide range of host plants, including peanuts, corn, tomato, pigeon pea, cotton, sorghum and cowpea (Wu and Guo, 2005). *H. armigera* uses complex pheromone blends, as do many other moths (Witzgall et al., 2010). One of the most important pheromone components for the species is (Z)-11-hexadecenal (Z11–16:Al); this was not only the first component described for *H. armigera* (Piccardi et al., 1977) but also the first and only major long distance pheromone discovered for the species (Nesbitt et al., 1979; Roelofs and Cardé, 1977). Z11–16:Al was later shown to be one of the most common sex pheromones, employed by 119 moth species (Byers, 2006). Many other components were also discovered in *H. armigera*, including tetradecanal (14:Al), (Z)-11-tetradecenol (Z11–14:OH), (Z)-9-tetradecenal (Z9–14:Al), hexadecanal (16:Al), (Z)-7-hexadecenal (Z7–16:Al), (Z)-9-hexadecenal (Z9–16:Al), 1-hexadecanol (16:OH), (Z)-9-hexadecenol (Z9–16:OH), (Z)-11-hexadecenol (Z11–16:OH), (Z)-11-tetradecenal (Z11–14:Al), and (Z)-11-hexadecenyl acetate (Z11–16:Ac) (Witzgall et al., 2010). Z9–16:Al was found to be an important minor component and has been recommended as an optimal ingredient to blend with Z11–16:Al to attract males; the ratio of Z9–16:Al to Z11–16:Al can vary from 1:99 to 10:90 (Kehat and Dunkelblum, 1990; Kehat et al., 1980; Nesbitt et al., 1980). Variations in this ratio may be influenced geographically (Tamhankar et al., 2003). The function of Z7–16:Al is not certain, but when combined with the basic blend it may either enhance or inhibit attraction depending on the ratio. For e.g., 1% Z7–16:Al added to 2 mg of the basic blend reduces copulatory response, but the same proportion added to 10 μg of the binary mixture increases copulatory response (Kehat and Dunkelblum, 1990). In field tests, Z7–16:Al has not shown any significant effect when added to the basic blend; adding 2.3% Z7–16:Al to the mixture did not enhance or reduce attraction (Kehat et al., 1980). The function of Z9–14:Al is also uncertain; this component was shown to increase attraction in Australian populations (Rothschild, 1978), but in Israeli populations it showed no or even inhibitory effects either alone or in combination with other stimulatory compounds (Gothilf et al., 1978; Kehat and Dunkelblum, 1990). 16:OH and Z11–16:OH are attraction inhibitors when added to attraction blends (Kehat and Dunkelblum, 1990; Nesbitt et al., 1980; Wu et al., 1997). In addition, the alcohols are believed to function not only as pheromone components but also as biosynthetic precursors for the aldehydes (Teal et al., 1986). Z11–14:Al caused sexual excitation at dosage of 1 and 10 μg in caged moths but did not show any effect when present at <1 μg (Gothilf et al., 1978). 16:Al elicited electroantennographic responses (Nesbitt et al., 1979), but it does not elicit behavioral responses in flight tunnel experiments (Kehat and Dunkelblum, 1990). Moreover, when combined with the standard blend, 16:Al (4–7%) caused an increase in attraction over the standard blend in field testing, but this increase effect was not significant (Wu et al., 1997). The functions of several compounds which Z9–16:OH, 14:Al, Z11–16:Ac and Z11–14:OH were not clearly tested by bioassays (Wang et al., 2005; Lu et al., 2001; Konyukhov et al., 1983). More appropriate bioassay tests may have been necessary to interpret the function of these compounds.

To study the significance of the sex pheromone compounds in the Lepidoptera, we used *H. armigera* from China as test subjects. Ten compounds were identified in extracts from female sex glands and then were tested in a series of blends, both in the laboratory and the field, to determine the role of every compound. Our results will not only be useful to better understand the role of compounds present in sex glands, but they will also prove key to improving current blend formulations for management and control of the cotton bollworm.

2. Materials and methods

2.1. Insects

A laboratory population of *H. armigera* was established using wild moths collected in Henan, China. Larvae were fed an artificial diet (wheat germ 94 g, canned tomato paste 45 g, yeast 35 g, methyl parahydrobenzoate 1.6 g, sorbic acid 0.8 g, ascorbic acid 2.6 g, agar 11 g, linoleic acid 1 ml and distilled water 750 ml) (Wu and Gong, 1997). Adults were fed a 10% sugar–water solution. Individuals were kept in environmental chambers at 25 ± 1 °C under a 16 h light/8 h dark photoperiod. Before eclosion, pupae were sexed and placed individually in petri dishes. The emerged moths were collected daily when scotophase finished, and designated as "age 1".

2.2. Pheromone gland extraction and chemical analysis

Pheromone glands were excised from 3 to 4 days old virgin females. To excise the glands, virgin females were observed during 30-min intervals under red light in a dark room (red light did not inhibit calling behavior). When the pheromone glands were everted, they were immediately excised with a small blade and immersed in 10 μl hexane in individual conical glass vials for 10 min at room temperature. The hexane extract was then transferred into a different clean conical glass vial and maintained at −20 °C for posterior analysis. A total of 28 female glands were analyzed. Extracts were concentrated by gently blowing nitrogen on the vials before analysis. Extracts were analyzed by gas chromatography–mass spectrometry (GC–MS) using an Agilent 5973 N mass selective detector coupled with an Agilent 6890 N network gas chromatograph (GC) system, which was equipped with a high-polar capillary column (HP-88, 60 m × 0.25 mm × 0.2 μm). The column oven was maintained at 45 °C for 1 min, and then the temperature was increased to 120 °C at 8 °C/min. Temperature was then increased to 160 °C at 3 °C/min where it was held for 7 min, and finally increased to 250 °C at 5 °C/min and held for 5 min. Helium was used as the carrier gas at a constant flow rate of 1 ml/min. In addition, the column was displaced by a mid-polarity column (DB-35, 30 m × 0.25 mm × 0.25 μm) and a polarity column (DB-WAX 30 m × 0.25 mm × 0.25 μm) to further identify extract compounds. All capillary columns were provided by Agilent Technologies, Santa Clara, CA, USA.

Identification of compounds was conducted by comparing the mass spectra of the chromatographic peaks with entries in the NIST08 database using the data acquisition software MSD Chemstation G1701EA E.01.00.237 (Agilent, USA). All volatile compounds showing mass spectra with match factors ⩾90% were put on a **"positive list"** of tentatively identified substances, which were then compared against mass spectra of commercial standards. Finally, confirmation of compound identities was ensured by comparisons to the retention times of standards on three columns.

2.3. Chemicals

Chemicals used in identification, electrophysiological analyses and bioassays are shown in Table 1.

2.4. Electrophysiological analyses

Gas chromatography–flame ionization detection coupled with electroantennographic detection (GC–EAD) was used to identify electrophysiologically active compounds in male antennae. The column and temperature program of the GC was identical to that of the GC–MS analysis previously described. Each antenna was prepared by cutting both extremes and was immediately mounted

Table 1
List for purity and source of chemical standards.

Chemical standards	Abbreviations	Purity	Source
Heptanal	7:Al	95	J&K (China)
Nonanal	9:Al	95	J&K (China)
Tetradecanal	14:Al	90	Shenzhen Guanya Hengfa Trade (China)
(Z)-9-tetradecenal	Z9–14:Al	93+	Shin Etsu Chemical Co. Ltd. (Japan)
Hexadecanal	16: Al	92+	Shin Etsu Chemical Co. Ltd. (Japan)
(Z)-7-hexadecenal	Z7–16:Al	96	Shin Etsu Chemical Co. Ltd. (Japan)
(Z)-9-hexadecenal	Z9–16:Al	95+	Shin Etsu Chemical Co. Ltd. (Japan)
(Z)-11-hexadecenal	Z11–16:Al	95+	Shin Etsu Chemical Co. Ltd. (Japan)
1-hexadecanol	16:OH	96+	West Long Chemical Co. Ltd. (China)
(Z)-11-hexadecenol	Z11–16:OH	92+	Shin Etsu Chemical Co. Ltd. (Japan)

between two Kaissling saline-filled Ag/AgCl electrodes. The electrode at the distal end of the antenna was connected via an interface box to a signal acquisition interface board (IDAC; Syntech, Netherlands) connected to a personal computer (PC). EAD signals and flame ionization detector (FID) responses from the GC were simultaneously recorded by the PC using AutoSpike software (Syntech, USA). A chemical standard blend containing each compound (7:Al, 9:Al, 14:Al, Z9–14:Al, 16:Al, Z7–16:Al, Z9–16:Al, Z11–16:Al, 16:OH and Z11–16:OH) at a concentration of 20 ng/μl, except for 14:Al (at 12 ng/μl), was tested.

2.5. Laboratory bioassay

H. armigera male responses to synthetic chemical blends were tested in a glass Y-tube olfactometer. The olfactometer consisted of a stem (90 cm long, 6 cm diameter) with two arms (45 cm long, 5 cm diameter) at a 120° angle. Unidirectional airflow was maintained downwind at 1.5 l/min by connecting the olfactometer arms to a vacuum pump. Air was charcoal-filtered before entering the system. A pair of prepared well rubber septa was placed at the end of the Y-tube arms. In each trial, a single male moth was placed at the base of the olfactometer stem and its behavior was observed for 5 min under a 0.6 lx red lamp in a dark room. A choice was scored positive when the male walked upwind towards the septum and either remained adjacent to the septum or paced up and down within 30 cm of the septum during the 5 min period. Trials in which the males either walked back to the main stem or chose a different arm were excluded. Y-tubes were cleaned with acetone and oven dried at 100 °C for at least 2 h before each trial. Preliminary Y-tube tests showed no difference in the responses when the choices were two septa with no chemicals, and B (standard blend) = Z11–16:Al (98.6%) + Z9–16:Al (1.4%) attracted more males than Z11–16:Al alone.

The blends (B, C, D, E, F, G, H, I and J) were prepared for the bioassays according to the natural proportions that were extracted from the sex gland. Every standard blend was dissolved with hexane at 0.1 μg /μl, 100 μl of each blend was loaded onto gray rubber septa (The West Company, Phoenixville, PA) for a total of 10 μg for each septa. The blends were tested in the following pair combinations: (1) B vs. C, (2) B vs. D, (3) B vs. E, (4) B vs. F, (5) B vs. G, (6) B vs. H, (7) B vs. I, and (8) B vs. J. Blend compositions were as follows: Standard blend B = Z11–16:Al 98.6% + Z9–16:Al 1.4%; C = Z11–16:Al 98.1% + Z9–16:Al 1.4% + 7:Al 0.5%; D = Z11–16:Al 94.1% + Z9–16:Al 1.3% + 9:Al 4.9%; E = Z11–16:Al 95.1% + Z9–16:Al 1.3% + 16:Al 3.6%; F = Z11–16:Al 97.6% + Z9–16:Al 1.4% + Z7–16:Al 1%; G = Z11–16:Al 97.4% + Z9–16:Al 1.4% + 16:OH 1.2%; H = Z11–16:Al 94.5% + Z9–16:Al 1.3% + Z11–16:OH 4.2%; I = Z11–16:Al 97.9% + Z9–16:Al 1.3% + 14:Al 0.8%; J = Z11–16:Al 98.3% + Z9–16:Al 1.4% + Z9–14:Al 0.3%. Every experiment was tested with at least 30 individuals, and each male was tested for 5 min.

2.6. Field trapping experiments

Field trapping experiments were designed according to the results of the Y-tube bioassays. A new blend (Z = Z11–16:Al 94% + Z9–16:Al 1.2% + 16:Al 3.5% + Z7–16:Al 1% + Z9–14:Al 0.3%) was included in these experiments. Blends (B, E, F, J or Z) of chemicals were dissolved with hexane at 5 μg/μl and loaded onto gray rubber septa (The West Company, Phoenixville, PA) at a dose of 500 μg/septa; septa with only 100 μl hexane were used as a control (CK), and all septa were sealed and stored under −20 °C refrigerated conditions. All of the trials were started on the evening of August 24th, the third day after the preparation of the septa, and the trials were terminated after 18 days. Septa were changed with a freshly prepared well from the refrigerator every week during the test period. Traps were plastic basins (24 cm in diameter and 10 cm in height) filled with water and baited with dispensers. Six different treatment traps (CK, B, E, F, J and Z) were fixed on bamboo poles and spaced in a line with separation of at least 30 m between each trap; the traps were kept uncovered by leaves and were almost as tall as the surrounding crops. There were 9 replicates of each treatment: 2 in a corn field neighboring grass, 2 in corn field neighboring the green house, 1 in cotton field neighboring the green house, 2 in a cotton field neighboring peanuts and 2 in cotton field neighboring fruit tree orchard mixed with grass. The position of each treatment in the line of traps was randomized to minimize marginal effects. Although many crops were involved, we did not consider the influence of different crops among replicates, but we did ensure that the eco-environmental conditions were identical for every treatment in the same replicate. Captured moths were recorded and removed daily.

2.7. Statistical analyses

Chi-square tests were used to compare the proportion between the selected choices in the Y-tube experiments, with the null hypothesis that cotton bollworm moths showed no preference for either olfactometer arm with different blend probabilities. For the field trapping experiments, data were analyzed by analysis of variance (One-Way ANOVA) on $\sqrt{x}+0.5$ transformed data according to the Duncan multiple-comparison test at $P = 0.05$. All analyses were performed with the program SPSS.17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Chemical and electrophysiological analyses

Ten compounds of the extract blend elicited male antenna electrophysiological responses when detected by GC–EAD with the equipped capillary columns HP-88 and DB-WAX (Figs. 1 and 2 c). Comparison of the GC–MS retention times of these ten compounds

with chemical standards showed that the peaks, from 1 to 10, were 7:Al, 9:Al, 14:Al, Z9–14:Al, 16:Al, Z7–16:Al, Z9–16:Al, Z11–16:Al, 16:OH, and Z11–16:OH (Table 2, Figs. 1 and 2). Further identification of these ten compounds based on GC–MS equipped with column DB-35 showed that the retention times of the chemicals standard and the extracted compounds were consistent. A slight change in peak order was observed, with 16:Al present after Z7–16:Al and Z9–16:Al; the resulting order, from 1 to 10, was 7:Al, 9:Al, 14:Al, Z9–14:Al, Z7–16:Al, Z9–16:Al, 16:Al, Z11–16:Al, 16:OH, and Z11–16:OH (Table 2 and Fig. 3). Figs. 1a, 2a and 3a show the total ion current chromatogram from synthetic standards. Figs. 1b, 2b and 3b display the total ion current chromatogram from pheromone gland extracts of female calling *H. armigera*. Mean percent compositions (calculated using the percent composition of each compound in the extracts, using the most prevalent component Z11–16:Al as 100%) of 7:Al, 9:Al, 14:Al, Z9–14:Al, 16:Al, Z7–16:Al, Z9–16:Al, Z11–16:Al, 16:OH, and Z11–16:OH were 0.56%, 4.92%, 0.81%, 0.30%, 3.77%, 0.96%, 1.37%, 100.00%, 1.27%, and 4.40%, respectively; mean qualities were 0.59, 0.85, 0.80, 0.90, 1.27, 0.91, 0.95, 16.65, 1.93, and 1.42 ng, respectively (Table 3). Quality was calculated by creating an external standardization equation (Ese) (Table 3), using a series of standards with known concentrations (0.2, 1, 2, 10, 20, 30, 40, and 50 ng).

3.2. Laboratory bioassays

Male responses to the various blends are shown in Fig. 4 and were as follows: B vs. C ($\chi^2 = 0.000$; $df = 1$; $P > 0.05$), B vs. D ($\chi^2 = 1.000$; $df = 1$; $P > 0.05$), B vs. E ($\chi^2 = 6.533$; $df = 1$; $P = 0.011$), B vs. F ($\chi^2 = 11.645$; $df = 1$; $P = 0.001$), B vs. G ($\chi^2 = 0.125$; $df = 1$; $P > 0.05$), B vs. H ($\chi^2 = 3.667$; $df = 1$; $P > 0.05$), B vs. I ($\chi^2 = 2.133$; $df = 1$; $P > 0.05$), and B vs. J ($\chi^2 = 6.533$; $df = 1$; $P = 0.011$). Three (E, F and J) of the experimental blends were significantly more attractive than the standard blend B, and no significant increases or inhibitions of attraction were detected in the other blends.

3.3. Field trapping test

No moths were attracted to septa with hexane (CK). Both blends J and Z were significantly more attractive than the other blends (F, E, and B), but there were no significant differences either between blends J and Z or among blends F, E, and B (Fig. 5).

4. Discussion

We detected 10 compounds, 7:Al, 9:Al, 14:Al, Z9–14:Al, 16:Al, Z7–16:Al, Z9–16:Al, Z11–16:Al, 16:OH and Z11–16:OH, that elicited electrophysiological responses in the male antennae of *H. armigera*, and we confirmed that Z11–16:Al and Z9–16:Al are the essential components of the sex pheromone blend for the species (Kehat and Dunkelblum, 1990; Kehat et al., 1980; Nesbitt et al., 1980; Tamhankar et al., 2003).

Remarkably, when Z9–14:Al was added to the standard blend according to the natural proportion, more than twice the males were captured in comparison with the standard blend alone in both Y-tube tests and field trapping experiments. A similar result

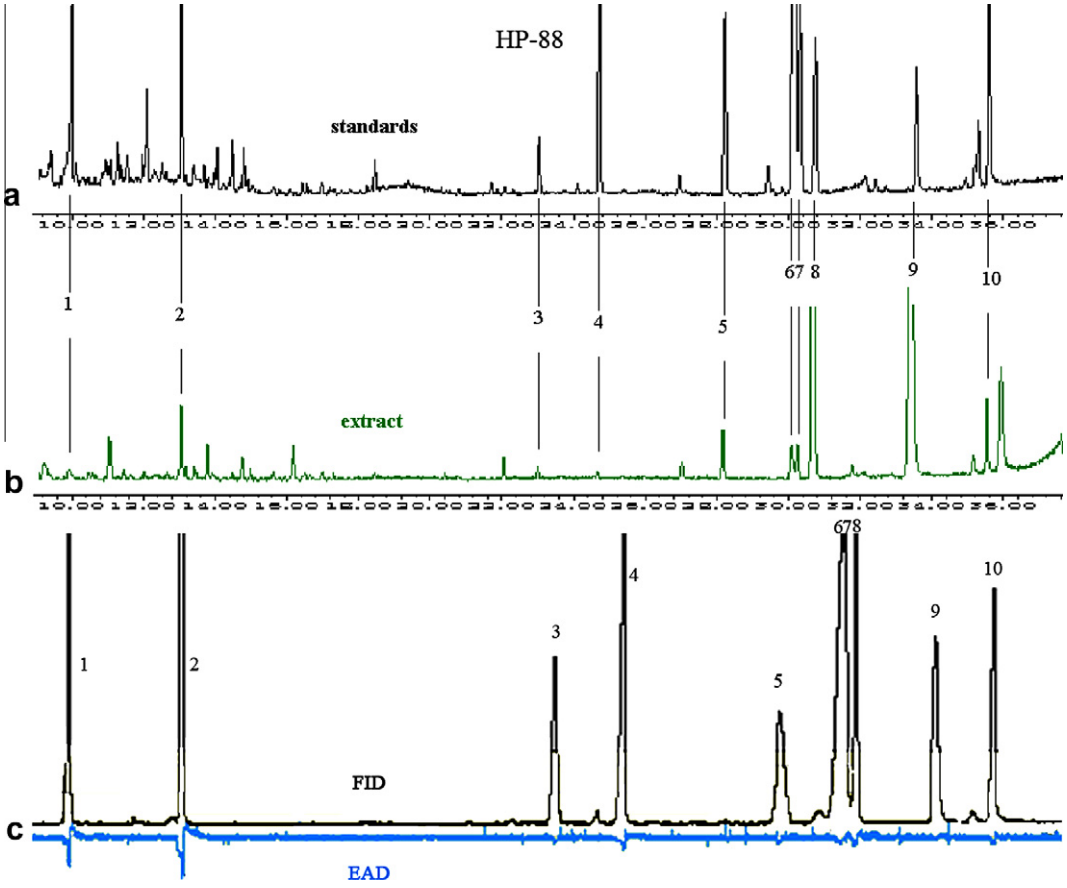

Fig. 1. Identified gland extracts of calling females by GC–MS and GC–EAD equipped with capillary column HP-88. (a) Total ion current chromatogram from synthetic standards. (b) Total ion current chromatogram from pheromone gland extracts of female calling *Helicoverpa armigera*. (c) Electroantennographic responses of *H. armigera* male antenna when exposed to synthetic chemical blends. Peaks from 1 to 10 are heptanal, nonanal, tetradecanal, (Z)-9-tetradecenal, hexadecanal, (Z)-7-hexadecenal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, 1-hexadecanol, and (Z)-11-hexadecenol.

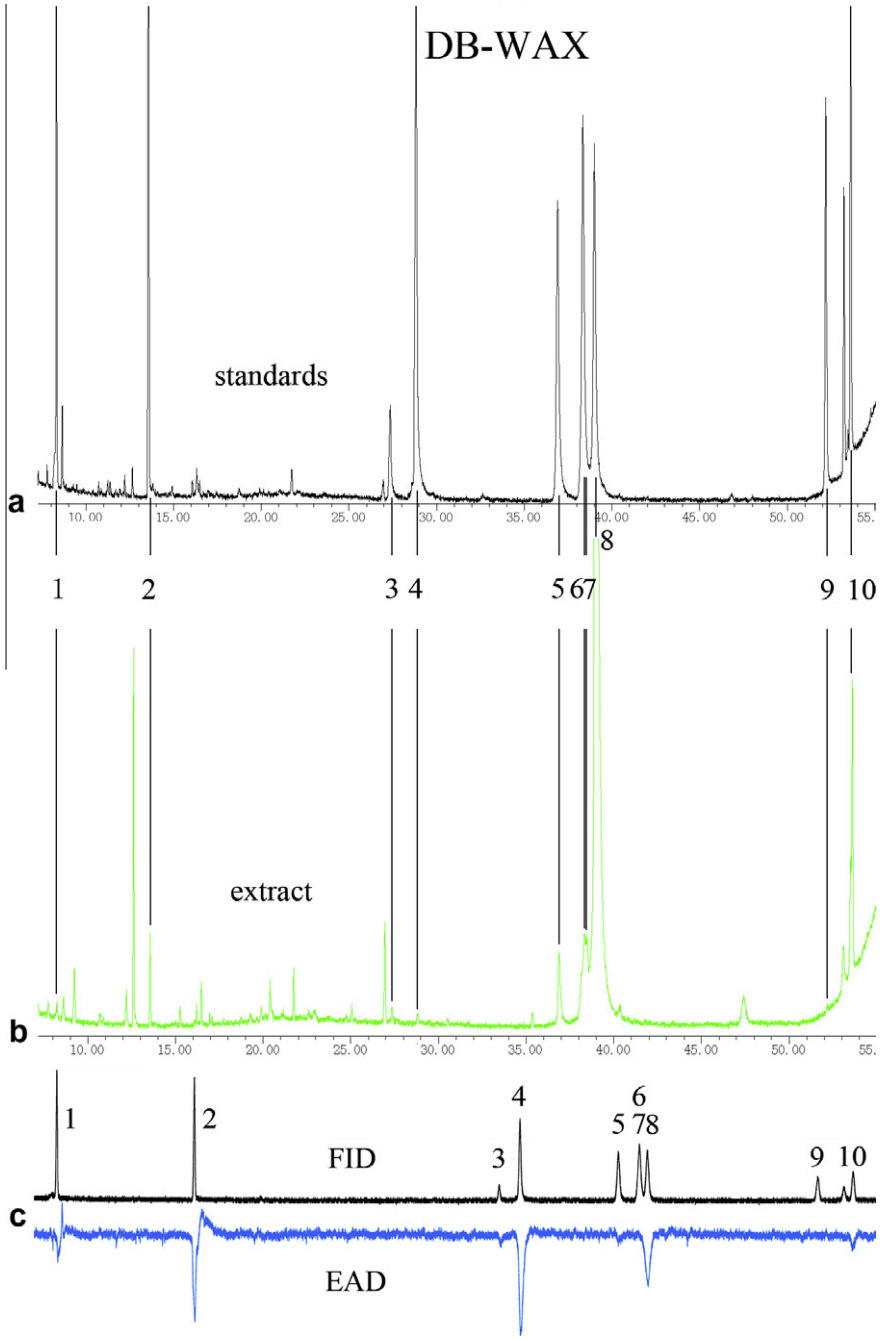

Fig. 2. Identified gland extracts of calling females by GC–MS and GC–EAD equipped with capillary column DB-WAX. (a) Total ion current chromatogram from synthetic standards. (b) Total ion current chromatogram from pheromone gland extracts of female calling *Helicoverpa armigera*. (c) Electroantennographic responses of *H. armigera* male antenna when exposed to synthetic chemical blends. Peaks from 1 to 10 are heptanal, nonanal, tetradecanal, (Z)-9-tetradecenal, hexadecanal, (Z)-7-hexadecenal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, 1-hexadecanol, and (Z)-11-hexadecenol.

was obtained with blend Z, which also contained Z9–14:Al. Only 0.3% Z9–14:Al was contained in both blends J and Z, which indicates that the compound is a powerful minor component that enhances attraction. The same trend was observed in Australian *H. armigera*, for which field traps using a blend that contained Z11–16:Al and Z9–14:Al, particularly in the ratios of 30:1 to 70:1, proved significantly more attractive (Rothschild, 1978). Thus, Z9–14:Al should be considered as an important component of the sex pheromone mixture. However, Z9–14:Al has also been shown to have inhibitory effects in experiments employing *H. armigera* populations from Israel (Gothilf et al., 1978; Kehat and Dunkelblum, 1990). These conflicting results for the role of Z9–14:Al may be influenced by differences in its proportions across different tests. Sex pheromone components can act antagonistically to reduce attraction if they are emitted individually or at excessively high or low rates (Baker, 2008). Geographical variation is another important reason for the conflicting roles of Z9–14:Al in sex pheromone composition; the effects of geography have been documented previously in *H. armigera* and other species of Lepidoptera (Kawazu et al., 2000; Tamhankar et al., 2003). Geography and proportion may be the major factors that have caused Z9–14:Al to be overlooked as a sex pheromone component in *H. armigera*. Here we suggest that Z9–14:Al should be considered as a component of the sex pheromone mixture of *H. armigera*, and

Table 2
Retention time of extract compounds peaks and chemical standards on GC–MS with different equipped columns.

Compounds	Retention time (min)					
	Column Standards	HP-88 Extracts	Column Standards	DB-Wax Extracts	Column Standards	DB-35 Extracts
7:Al	9.977	9.930	8.310	8.310	9.492	9.477
9:Al	13.057	13.048	13.556	13.556	15.344	15.346
14:Al	23.030	22.992	27.352	27.352	29.185	29.176
Z9–14:Al	24.720	24.682	28.827	28.819	29.269	29.241
16: Al	28.223	28.166	36.894	36.853	35.932	35.988
Z7–16:Al	30.148	30.072	38.328	38.378	–	35.770
Z9–16:Al	30.326	30.232	–	38.381	35.815	35.870
Z11–16:Al	30.739	30.673	38.986	38.975	36.097	36.147
16:OH	33.575	33.509	53.226	53.219	37.853	37.903
Z11–16:OH	35.594	35.538	53.610	53.600	38.048	38.097

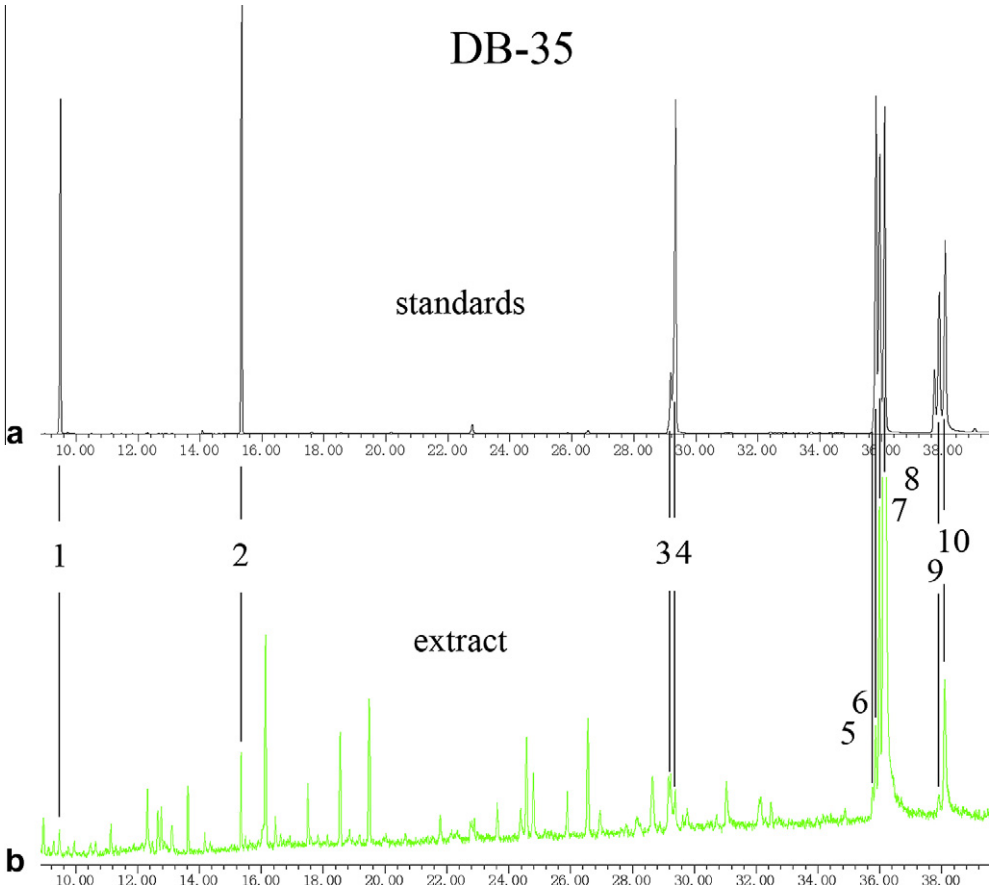

Fig. 3. Identified gland extracts of calling females by GC–MS equipped with capillary column DB-35. (a) Total ion current chromatogram from synthetic standards. (b) Total ion current chromatogram from pheromone gland extracts of female calling *Helicoverpa armigera*. Peaks from 1 to 10 are heptanal, nonanal, tetradecanal, (Z)-9-tetradecenal, (Z)-7-hexadecenal, (Z)-9-hexadecenal, hexadecanal, (Z)-11-hexadecenal, 1-hexadecanol, and (Z)-11-hexadecenol.

seriously consideration should be given to its inclusion in agricultural mating disruption programs in some regions.

Z7–16:Al, 16:Al and Z9–14:Al all elicited significant attraction in Y-tube experiments. While this study found that Z9–14:Al should be considered as an essential pheromone component of *H. armigera*, Z7–16:Al and 16:Al had no effects when added to the standard blend in the field tests. Z7–16:Al and 16:Al most likely play an important role in increasing attraction at short ranges, allowing males to detect females more efficiently in close interactions. Kehat et al. (1980) added Z7–16:Al to the basic blend and demonstrated no significant effect in the field. A flight tunnel experiment by Kehat and Dunkelblum (1990) showed that the function of Z7–16:Al is not certain, and the compound may either enhance or inhibit attraction depending on its emission rate. The emission rate is one of the important factors in the composition of the sex pheromone of any given species. Blend components are emitted at the right ratios to each other act to create "the pheromone" as a whole, the optimal odor that initiates and promotes optimal upwind flight attraction, and this particular blend ratio creates an optimal "balanced olfactory antagonism" (Baker, 2008). Previous tests showed that 16:Al elicits electroantennographic responses (Nesbitt et al., 1979), but the chemical neither elicits behavioral responses in flight tunnel tests (Kehat and Dunkelblum, 1990) nor significantly increases attraction in combination with the standard blend in field tests (Wu et al., 1997). Two major hypotheses exist regarding the function of minor

Table 3
Mean relative proportion and quality of compounds from sex gland of *Helicoverpa armigera* females.[a]

Compounds	Mean proportion (%)	Std. D	Ese	R^2	Quality (ng)
7:Al	0.56	0.4798	$Y = (x + 140{,}548)/274{,}169$	0.9930	0.59
9:Al	4.92	6.2818	$Y = (x + 159{,}161)/311{,}806$	0.9938	0.85
14:Al	0.81	0.4596	$Y = (x + 22972)/73964$	0.9870	0.80
Z9–14:Al	0.30	0.3846	$Y = (x + 318{,}004)/375{,}566$	0.9921	0.90
16:Al	3.77	1.6667	$Y = (x + 198{,}283)/303{,}826$	0.9937	1.27
Z7–16:Al	0.96	0.8642	$Y = (x + 263{,}928)/345{,}737$	0.9932	0.91
Z9–16:Al	1.37	0.6348	$Y = (x + 263{,}928)/360{,}769$	0.9932	0.95
Z11–16:Al	100.00	–	$Y = (x + 223{,}533)/315{,}749$	0.9936	16.65
16:OH	1.27	1.3794	$Y = (x + 283{,}925)/236{,}762$	0.9863	1.42
Z11–16:OH	4.40	3.0497	$Y = (x + 339{,}167)/282{,}342$	0.9812	1.93

[a] Mean proportion were calculated using the percent composition of each compound in the extracts, using the most prevalent component, Z11–16:Al, as 100%. The quality was calculated with an external standardization equation (Ese).

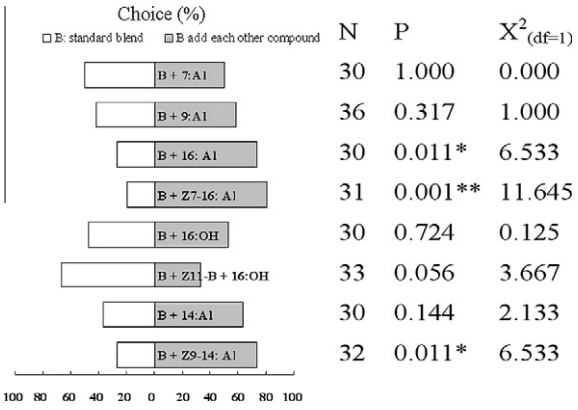

Fig. 4. Attraction responses of *Helicoverpa armigera* males when exposed to a standard pheromone blend (B) vs. an experimental blend simultaneously in a Y-tube olfactometer. Experimental blends were loaded onto rubber septa at 10 μg/septa according to the natural proportions that were extracted from the sex glands. Every experiment was tested with at least 30 individuals, and each male was tested for 5 min. B = Z11–16:Al 98.6% + Z9–16:Al 1.4%; C = Z11–16:Al 98.1% + Z9–16:Al 1.4% + 7:Al 0.5%; D = Z11–16:Al 94.1% + Z9–16:Al 1.3% + 9:Al 4.9%; E = Z11–16:Al 95.1% + Z9–16:Al 1.3% + 16:Al 3.6%; F = Z11–16:Al 97.6% + Z9–16:Al 1.4% + Z7–16:Al 1%; G = Z11–16:Al 97.4% + Z9–16:Al 1.4% + 16:OH 1.2%; H = Z11–16:Al 94.5% + Z9–16:Al 1.3% + Z11–16:OH 4.2%; I = Z11–16:Al 97.9% + Z9–16:Al 1.3% + 14:Al 0.8%; J = Z11–16:Al 98.3% + Z9–16:Al 1.4% + Z9–14:Al 0.3%. * Significant at $P < 0.05$; ** Significant at $P < 0.01$.

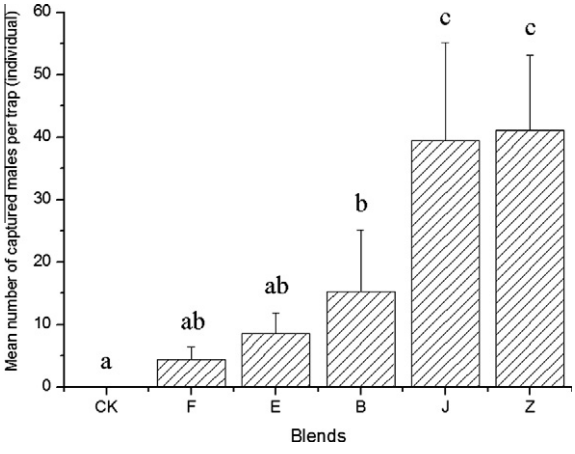

Fig. 5. Mean ± SE of *Helicoverpa armigera* males captured per trap with different blends. Bars with different letters represent significant differences at $P = 0.05$. Blends were loaded onto rubber septa at 500 μg/septa, and their compositions were as follow: F = Z11–16:Al (97.6%) + Z9–16:Al (1.4%) + Z7–16:Al (1%); E = Z11–16:Al (95.1%) + Z9–16:Al (1.3%) + 16:Al(3.6%); B = Z11–16:Al (98.6%) + Z9–16:Al (1.4%); J = Z11–16:Al (98.3%) + Z9–16:Al (1.4%) + Z9–14:Al (0.3%); Z = Z11–16:Al (94%) + Z9–16:Al (1.2%) + 16:Al (3.5%) + Z7–16:Al (1%) + Z9–14:Al (0.3%).

components. The first is that minor components do not participate in long range attraction, instead only combining with the major components to evoke other aspects of the mating sequence, particularly short range interactions such as approach, landing and courtship (Baker et al., 1976; Bradshaw et al., 1983; Cardé et al., 1975; Roelofs and Cardé, 1977). The second hypothesis is that the whole blend acts as a unit to achieve optimal attraction for males over the entire response range(Baker and Cardé, 1979; Linn et al., 1986, 1987). In consideration of our bioassays, we have synthesized these two seemingly conflicting hypotheses. Some minor components are always present and function only at short range (e.g., (Z)-7-hexadecenal and hexadecanal), alternatively, others (e.g., (Z)-9-tetradecenal) play important roles not only in short range interactions but also long distance communication. Different compounds each have different responsibilities to elicit specific activities, and all components must synergize to achieve their full function over the whole courtship and copulation process. This integrated hypothesis will help to guide the direction of future research.

Furthermore, we detected and identified two constituents in *H. armigera* female sex pheromone glands, 7:Al and 9:Al; to our knowledge, this paper is the first time that these compounds have been reported in Lepidopteran female sex pheromone glands (Witzgall et al., 2010). Both compounds elicited strong electrophysiological responses in male antennae but were behaviorally inactive. The proportion of 9:Al (4.92%) was second only to the component Z11–16:Al (100%), demonstrating that a high ratio of this compound was present in the sex pheromone gland. This result raises the question of how these behaviorally inert compounds could have been influenced by selection pressure. In our bioassay tests, 14:Al, 16:OH, and Z11–16:OH were shown to have no function, but 16:OH and Z11–16:OH have been shown to act as attraction inhibitors when added to attraction blends (Kehat and Dunkelblum, 1990; Nesbitt et al., 1980; Wu et al., 1997). Additionally, the alcohols have been implicated as biosynthetic precursors for the aldehydes (Teal et al., 1986). It is possible that the compounds have simply been conserved over evolutionary time and exist in the gland as behaviorally inert compounds; future investigations should focus on addressing these questions.

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