Autodetection in *Helicoverpa armigera* (Hubner)

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Autodetection is an olfactory behavioural process where the females of some species respond to their own pheromonal blends. Through electroantennogram studies it has been proved that the gravid females of *Helicoverpa armigera* (Hubner) respond to their pheromone blend consisting of Z-11-hexadecenal and Z-9-hexadecenal in the ratio 97 : 3. Male antennae respond more strongly than virgin female antennae. However, antennae of gravid females elicit strong response than unmated males. Also, males showed strong responses in cross-wind flying in wind tunnel experiments, when sex pheromone blends were used. Virgin females and gravid females showed poor response in wind-tunnel studies. The ovipositional experiment where gravid females were allowed to oviposit in the presence and absence of pheromone odours indicated that there was no difference in the number of eggs laid. Through morphological studies, it has been proved that the females also possess sensilla trichoidea, destined to perceive the pheromone blends, though lesser in number than the males. These results support the hypothesis that autodetection of sex pheromones exists in females of *H. armigera* and is thought to function as a mechanism to induce dispersal under high population densities.

Keywords: Autodetection, electroantennogram, *Helicoverpa armigera*, oviposition, synthetic sex pheromone.

THE bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), is a serious polyphagous pest of many cultivated crops in India. Among the management options, monitoring the *H. armigera* populations using pheromone as an integrated pest management (IPM) tool is greatly appreciated in several countries. Piccardi *et al.*[1] identified the major component of the female sex pheromone of *H. armigera* as Z11-16 : Ald, but in further publications by Nesbitt *et al.*[2,3] three compounds, i.e. Z11-16; OH, Z9-16 : Ald, and 16 : OH were described based on analysis of insect materials from Malawi, Sudan and India. Nevertheless, (Z)-11-hexadecenal and Z-9-hexadecenal were identified as the major and minor functional components. The species being an important pest of several crops in the old world, blends of different combinations are being recommended. In many countries, including India, a binary mixture of (Z)-11-hexadecenal and (Z)-9-hexadecenal in the ratio 97 : 3 is being recommended as the most common blend for monitoring *H. armigera* populations. Monitoring of *H. armigera* populations using pheromone traps is a common tool in several crops such as chickpea[4], cotton[5], carnation[6] and tomato[7]. A formulation containing 5% loading of pheromone in the ratio 97 : 3 Z11-16 : Ald : Z9-16 : Ald was prepared by Chamberlain *et al.*[8] in an attempt to demonstrate mating disruption to control the pest on *Gossypium hirsutum* var. CIM 240. A high degree of trap 'shut down' was observed in the pheromone-treated plot, suggesting that communication disruption had been achieved. Mahmudunnabi *et al.*[9] have developed a integrated pest management package which involves mass trapping of adults.

Mated male moths do not respond to the sex pheromone till their reproductive organs are ready[10,11]. Generally, female moths of several species are considered as anosmic. However, autodetection also known as 'antennal sensitivity of female moths to their sex pheromone components' does exist in several species of moths[12,13]. Schneider *et al.*[14] defined autodetection as 'antennal detection by a female of her own pheromone plume or plumes from neighbouring females'. In moth species only a few examples of autodetection have been recorded, viz. *Choristoneura fumiferana* (Clemens)[15], *Spodoptera littoralis* (Boisduval)[16], *Cydia* spp.[17] and *Panaxia quadripunctaria* (Poda)[14,18]. To know whether *H. armigera* females also respond to their pheromone blends consisting of Z-11-16-Ald and Z-9-16-Ald, we conducted a study using electrophysiological, behavioural and morphological tools to identify the functional significance of autodetection in the bollworm.

Materials and methods

Insect rearing

The cultures of *H. armigera* used in the present study have been maintained in our laboratory (National Germplasm registration no. NBAII-MP-NOC-01). The larvae of *H. armigera* were reared on artificial diet[19] in the laboratory

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consisting of kabuligram (105 g), methyl-4-hydroxy benzoate (2 g), sorbic acid (1 g), yeast (10 g), ascorbic acid (3.25 g), agar–agar (12.75 g), multivitamin capsules (four), vitamin E capsules (two), streptomycin (0.25 g), 10% streptomycin (0.25 g) and 10% formalin (5 ml).

Agar–agar was boiled in a separate beaker with a small quantity of water and homogenized with 780 ml of water in a blender and transferred to vials of 13 ml capacity. The first instar larvae were released individually on the vials and the opening was closed with sterilized absorbent cotton plug. The fully developed pupae were collected and kept in a cage made of cloth and in a tray of the moisture chamber. The males and females were separated using the standard pupal identification features and kept individually for adult emergence. The females and males were allowed to mate in small plastic jars of 1 litre capacity. Once the adults started laying eggs, the females were considered as gravid females. Virgin and unmated males of the same age (7 days) were used for the study.

Electroantennogram studies

Electroantennogram (EAG) recordings were made as described by Cork *et al.*[20] using up to one-week-old unmated males, virgin and gravid females of *H. armigera*. A microelectrode puller (Narashige, Japan, model PC-10) was used to etch the glass microelectrodes (3 cm length, tip diameter 20–50 μm) from the capillary tubing (borosilicate glass capillaries, 2.0 mm outer diameter, 1.16 mm inner diameter). Once cooled, the micro-electrodes were filled with NaCl (8.0 g/litre) solution isotonic with the insect haemolymph[21]. This saline solution in the microelectrode would provide electrical contact between the antennal haemolymph and the silver/silver chloride junction of the recording apparatus once inserted into an antenna. We evaluated maximum amplitude of the EAG during a 1s stimulation. The preparation was placed in a filtered air stream passing through a glass tube Pasteur pipette of 130 mm length and 6 mm outer diameter. Whatmann filter papers cut into small size (3 mm breadth × 40 mm length) were impregnated with the pheromone blend 100 μl placed in glass Pasteur pipettes. Stimulation of the antennal preparation was carried out by means of controlled airflow (300 ml/min) through the pipette with the filter paper. The pheromone samples used for the study were purchased in pure form as Z-11-hexadecenal and Z-9-hexadecenal from Pheromone Chemicals, Hyderabad and mixed before use to get the ratio of 97 : 3. To measure stimulus–response characteristics, the test stimuli were successively given with control stimulations interspersed between stimuli. Between stimulus presentations, purified air was passed over the antennal preparation for at least 30 sec. The amplitude for each stimulus was measured using the SYNTECH electroantennogram software.

Behavioural studies using wind tunnels

Wind tunnel consists of a cylindrical tunnel of 1.0 m length and 15 cm diameter with the bait chamber (11 cm length) and test chamber (11 cm length) connected on both the ends. The whole set up is made of 3 mm thick transparent, odourless and nonadsorbent acrylic sheet. The wind tunnel was cleaned with a wet cloth and dried in open air for 2 h before the start of the experiment. A regulator-controlled fan fitted to the bait chamber was used to draw charcoal-filtered clean air from the bait chamber to the test chamber. Wind speed was maintained at the rate of 0.5 m/sec with an airflow of 85 l/min. The wind tunnel was divided into eight equal zones (each 11.2 cm apart) marked 1 to 8. Zone 1 was near the test chamber and zone 8 near the bait chamber. Pheromone blends, Z-11-hexadecenal and Z-9-hexadecenal (97 : 3), diluted with hexane were impregnated into the rubber septa. The septum was then allowed to evaporate at room temperature in a fume hood. It was ensured that each septum contained 1 mg of the pheromone compound.

A single test insect was released into the test chamber. Then freshly prepared pheromone septum was placed at the bait chamber and maximum distance (zone) that the test insect reached in 30 min was recorded. A minimum of 10 insects were used for each of the odours tested. A score of 8 was given for the insects which reached the bait chamber and a score of 1 to the insects which reached zone 1. The maximum score of 8 indicated the insect to be highly responsive.

GC-linked to EAD analysis

To confirm the results obtained from electroantennogram experiment, gas chromatography coupled electroantennogram detector (GC-EAD) analysis was carried out using a Syntech Electroantennogram detector coupled to an Agilent GC with flame ionization detector (FID; model Agilent 7890 A GC System) fitted with in-built outlet for the EAD with temperature control program for the effluents and splitter with make-up gas.

The electroantennogram unit consists of the EAG amplifier (model AM-02), stimulus controller (model CS-05) and IDAC (intelligent data acquisition controller) card of type-AM 02, and effluent assembly with temperature control (Syntech, Germany). The effluent assembly was fitted to the wall of the GC through a prefabricated hole and the glass column from the splitter assembly was allowed to pass through the effluent assembly and open at the stimulus delivery tube.

The samples were injected through a split injector with the split ratio of 50 : 1 at a temperature of 250°C. An HP 5% phenyl methyl siloxane capillary non-polar column (30 m × 0.25 mm × 0.25 μm) was used for the separation of compounds and FID (250°C) was ignited using

high-purity hydrogen gas (99.999%) and zero air (99.999%) as reference gas. Helium (99.999% purity) was used as carrier gas at a pressure of 0.5 kg cm^{-2}. The oven temperature program was set at 70°C min^{-2} with 2 min hold and a ramp of 10°C min^{-1} till 260°C and held for 5 min with a column flow of 1 ml/min. The transfer temperature line (Syntech Laboratories, Germany) between GC and EAD was maintained 2°C above the maximum temperature to avoid condensation. The effluents emerging from the column were split using Agilent electronic splitter with make-up gas with a flow rate of 80 cm/s, and the effluents were sent to the EAD and FID (30 : 1) through a glass wool column of 0.25 mm thickness for recording the response of antenna to the effluents and for the analytical detection respectively.

The antenna from gravid females was gently excised and fitted in the micromanipulator assembly of the electroantennogram in the glass capillary electrodes filled with 0.8% NaCl solution in deionized water. The antenna was kept at close proximity to the stimulus delivery tube described earlier, so that the effluents from GC were gently blown onto the antenna after eluting through the GC columns. One microlitre of the sample was injected into the injector and traces from FID and EAD were plotted, and the matching peaks along with their retention times were measured using Syntech GCEAD software version 1.1 (2010). Each analysis was repeated at least thrice to draw conclusions.

Influence of pheromone on ovipositional response of H. armigera

A transparent chamber of 15 cm length, 10 cm width and 20 cm height made of glass was cleaned well and a black cloth was fixed on the inner wall of the chamber. Eight gravid females were released into the chamber and air at 3.5 l/min was pumped into the chamber using an aerator. Initially the pump was run for 30 min without any odour. Later, activated charcoal-filtered air was passed through a cylindrical glass tube with the undiluted pheromone blend of Z-11-16-Ald and Z-9-16-Ald (97 : 3) kept in an adsorbent septum. The experimental set-up was maintained for 24 h and the number of eggs laid on the black cloth were counted after the experiment. Control experiment was conducted in the same manner, except that air was pumped directly without the pheromone blend. The experiment was replicated for 12 times to draw conclusions.

Morphological studies on the sensilla of male and female H. armigera

The antennae from both the male and female were dissected along with the basal segment and slides were prepared using a modified method of instructions for slide-mounting scales and mealybugs (www.ars.usda.gov/sp2userfiles/place/12754100/IDservice/aphidslides.pdf). The antennae were boiled in 90% ethanol for 5 min and then transferred to 10% KOH and boiled again for 10 min. Then, the antennae were transferred to absolute alcohol for removing KOH. They were kept in a solution of acid fuschin overnight and later transferred to clove oil. The cleared specimens were mounted on a glass slide. The number of sensilla trichodea was counted on the both male and female segment-wise and recorded. At least five antennae were used for each sex.

Results

Male moths generally showed high electrophysiological response to the pheromone blends of conspecifc females. The blend of synthetic compounds Z-11-hexadecenal and Z-9-hexadecenal elicited good electrophysiological response of male *H. armigera* (Table 1). Highest amplitude of 2.218 mV was noticed for the synthetic blend followed by pheromone extract and better than honey in unmated males. In case of virgin females, response to the synthetic pheromone was comparatively lesser than the males. However, gravid females showed more amplitude (4.45 mV) than the males, probably due to higher response elicited by some individual females as reflected by higher critical difference (CD) values. Virgin females showed significantly less response than the gravid females. The abdominal extracts elicited lesser response than the synthetic compounds for all categories studied. As practised normally, the amplitudes are computed in relation to the ultimate odour response (in this case honey) to compensate the gradual death of the antenna, and expressed in per cent. Gravid females showed more response (402.49%) than unmated males (206.75%), confirming highest response by the gravid female.

Gas chromatography coupled electroantennogram studies with gravid females also confirmed the response of female antenna at 15.8 min of retention time with some minor response to other compounds (Figure 1). The response to other compounds (such as Z-9-hexadecenal) was less compared to Z-11-hexadecenal.

In behavioural studies, using zone analysis, the highest response was noticed by unmated males, while both the virgin and gravid females showed least response (Table 2). This study indicates that though the virgin and female moths of *H. armigera* elicit electrophysiological response, they do not move towards the pheromone source as noticed in the case of unmated males.

Ovipositional studies with the gravid females indicated that the number of eggs laid by them on constant exposure to pheromone was higher (193.7 ± 15.29) compared to that by unexposed females (99.93 ± 15.929) (n = 12). The present study indicates that gravid females continue to lay eggs, undeterred by the presence of pheromone

Table 1. Electroantennogram response (mV) of *Helicoverpa armigera*

Treatment	Unmated males		Virgin females		Gravid females	
	Mean amplitude (mV)	% Amplitude	Mean amplitude (mV)	% Amplitude	Mean amplitude (mV)	% Amplitude
Air	0.115	9.98	0.021	2.093	0.11	9.26
Honey	0.788	76.71	0.837	116.907	1.06	86.67
Pheromone blend (97 : 3)	2.218	206.75	1.731	223.450	4.45	402.49
Pheromone extract	1.836	169.5	1.411	186.976	1.99	175.32
Hexane	1.494	138.43	1.256	165.927	1.47	130.83
Honey	0.516	100.00	0.504	100.00	0.153	100.00
SED	0.2441	27.4023	0.1543	60.487	0.4789	53.65
CD (0.01%)	0.6713	75.35	0.4243	166.340	1.3168	147.54
CV%	23.78	16.17	35.362	4.0164	23.032	15.282

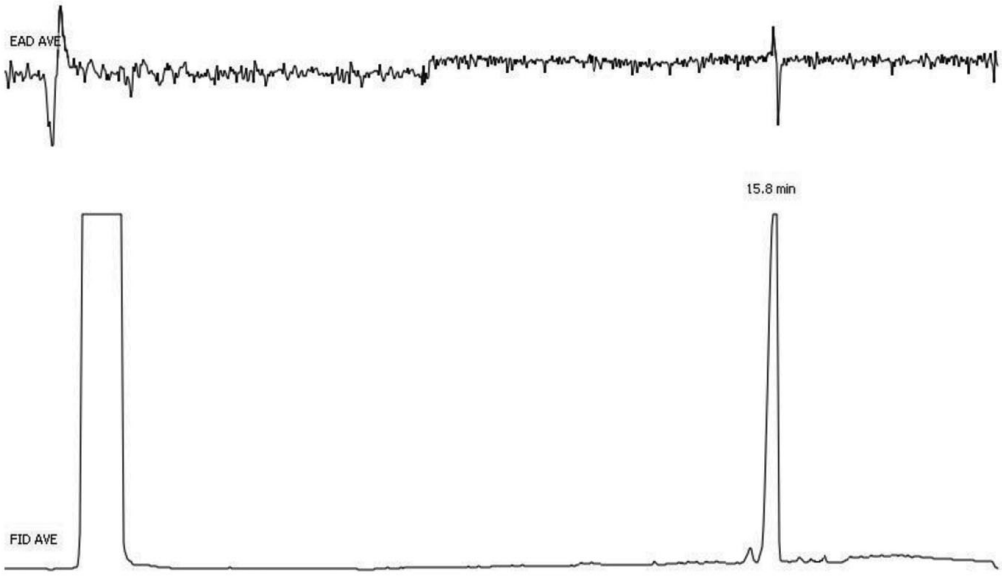

Figure 1. Gas chromatograph–electroantannogram detector traces of gravid females of *Helicoverpa armigera* to the pheromone blend of Z11-hexadecenal and Z9-hexadecenal in the ratio 97 : 3. The upper trace depicts antennal response, while lower one depicts the gas chromatogram traces. Note that the first antennal response is for the solvent (hexane), and the second response at a retention time of 15.9 min is for the pheromone blend.

Table 2. Orientation response of unmated males and virgin and gravid females measured in a wind tunnel

Sex of *H. armigera*	Zone values		
	Unmated males	Virgin females	Gravid females
Behavioural response	6.70	2.60	2.50
CD $P > 0.001$	1.82		
S Ed	0.63		
CV (%)	33.05		

blend of Z-11-hexadecenal and Z-9-hexadecenal under confined experimental conditions.

Morphological studies using a stereomicroscope indicated that females of *H. armigera* had 820.4 ± 79.3 (range 715–917) sensila trichodea in 77.1 ± 4.8 segments (range 74–85), while males had 1048.2 ± 250.5 (range 723–1361) sensilla trichodea in 79.4 ± 4.4 (range 74–85) segments ($n = 5$). The number of sensilla trichodea was more in males than that in females.

Discussion

Electrophysiological studies are excellent tools for measuring the response of adults of moths to various volatiles. Males of *H. armigera* were observed to respond more to the major compound Z-11-hexadecenal than the minor compound Z-9-hexadecenal[22]. However, in the present study, the gravid females were found to elicit good response to the pheromone blend of Z-11-hexadecenal and

Z-9-hexadecenal (97 : 3). No such response was noticed in any species belonging to the genera *Helicoverpa*, though Groot *et al.*[23] observed that females of *Heliothis subflexa* (Guenee) and *Heliothis virescesens* (Fab.) showed less response to the major sex pheromone compound (Z-11-hexadecenal) than the males. There are no earlier reports of *H. armigera* females showing EAG response to pheromone blends. However, the response of unmated males to pheromone blends has been well documented in earlier studies[24]. Autodetection was earlier reported from other moths such as *Panaxia quadripunctaria*[14], *Choristoneura rosaceana*[25] and *Grapholitha molesta* (Busck)[26].

The role of sensilla trichodea in the perception of sex pheromones of females in *H. armigera* and other moths was reported by earlier workers[27,28]. Morphological studies using a microscope have indicated the presence of sensilla trichodea in females too. Female antenna of *H. virescens* and *H. subflexa* responded to sex pheromone indicating that autodetection is possible in both the species[23]. Even in case of *H. armigera*, adults of both sexes showed four types of sensilla, viz. sensilla styloconica, sensilla chaetica, sensilla coeloconica and sensilla trichodea, and among these sensilla trichodea detected inter- and intraspecific communication signals. The length of the female sensilla trichodea was greater than that of male. The number of sensilla trichodea was estimated to be 7520 in females, and 6831 in males by Aliou diongue *et al.*[29]. Presence of sensilla trichodea was reported even in case of *H. assulta*[30]. Sexual dimorphism was noticed in the number of sensilla and in the subtypes of sensilla trichodea, which clearly indicated that sensilla trichodea is mostly involved in the perception of female-produced pheromone. Further, molecular studies showed that pheromone-specific receptor genes *HarmOR6*, *HarmOR11*, *HarmOR13*, *HarmOR14*, *HarmOR15* and *HarmOR16* were localized in the female antennae, though to a lesser extent than in the males[31].

In case of Oriental fruit moth, both virgin and mated females showed response to sex pheromone[26]. Three types of sensilla trichodea and two types of sensilla basiconica were identified on the antenna of *G. molesta* using scanning electron microscope. Males had a significantly greater number of sensila trichodea than females ($P < 0.001$). The increased number occurred mainly in the scale-covered regions of the antenna, where males had 2200 more sensilla trichodea (type A) than females as observed by George and Nagy[32]. This clearly shows that type-A sensilla trichodea is likely to be involved in pheromone perception. Further studies using single sensillum recording (SSR) need to be conducted to differentiate the response of sensilla, either individually to Z-11-hexadecenal, or Z-9-hexadecenal or to their blend.

Behavioural and ovipositional studies indicated that short-term effects of avoiding orientation to the pheromone sources may explain that gravid females use autodetection for locating conspecific females in the field, and accordingly move away from the fields where the females are located. However, spatial distribution between two gravid females in the same vicinity needs further study.

Recent studies[33] have shown that autodetection is employed not only for short-period behavioural responses but also for long-period responses, where the incidence of calling behaviour in *G. molesta* and *C. rosaceana* was advanced by 24 h following exposure to pheromone, apparently to have greater chances of mating. Autodetection was also considered as a mechanism to reduce competition between conspecific females[34].

Unmated males always showed greater response to pheromone blends in several species of moths in olfactometer experiments[22], confirming our studies of greater response of unmated males towards pheromone. Though electrophysiological response was more in gravid females, they did not show much orientation towards the pheromone source, posing a question as to how the electropysiological information is used. Do the gravid females which show strong electrophysiological response utilize the information to move away from the pheromone source, a method of spatio distribution pattern to orient in such a way to avoid the same host plants to forage for oviposition? needs to be studied, especially at the field level. In some insects such as *Adoxophyes orana* (Fisher von Rosslerstrm) and *Homona magnanima* (Diakonoff), the calling behaviour was delayed when females were exposed to pheromone[35]. But in some other insects, calling behaviour was advanced or the proportion of females showing calling behaviour increased on exposure to pheromone as observed in *C. fumifrana* and *G. molesta* (Busck)[36]. There were also instances when no changes were noticed in the behaviour, as in the case of *S. littoralis*[37].

In this study autodetection was found to induce disruption in orientation towards the pheromone sources. This may be a mechanism by which the adults distribute themselves within the fields, as proved by Stelinske *et al.*[26]. Studies are also necessary to document the dispersal pattern of adults of *H. armigera* when pheromone traps are used in higher numbers.

The functions of autodetection may vary such as establishment of societal contacts[38], jointly calling or spacing of food plants, control of timing of pheromone release[34] and spacing to avoid interference of pheromone plumes.

Exposure to pheromone-permeated ovipostional sites reduced egg laying in females of *C. rosaceana* and *Arytaenia velutinana*[25]. However, in the present study no change was observed in the oviposition behaviour of *H. armigera.* In fact, the number of eggs laid was significantly more when exposed to pheromone. However, the oviposition in experimental chambers in the presence of pheromones indicated that it is not aborted in confined situations. Palanaswamy and Seabrook[39] also observed

higher oviposition when exposed to pheromone. The question whether *H. armigera* females identify the volatiles and change their behaviour was answered by McCallum *et al.*[40], who observed that (S)- linalool acted as a potential repellent molecule enabling the gravid *H. armigera* to avoid plants which secrete higher levels of (S)-linalool.

To conclude, females of *H. armigera* are able to identify the presence of sex pheromone in the field and disperse within the same, though no difference in oviposition has been noticed in the presence/absence of pheromone under confined conditions. Thus further studies are needed to document the changes in dispersal at field or oviposition or secondary calling behaviour of females.

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