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**Host Plant Chemical Mediation of Heliothis
Reproductive Behavior**

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FINAL REPORT OF BARD PROJECT US-2217-92

Host Plant Chemical Mediation of *Heliothis* Reproductive Behavior

Introduction

Phytophagous insects respond to chemicals from their host plants in a number of ways, including orientation or attraction in response to volatiles produced by plants. Orientation to odors from host plants may occur in order to locate food, mates, or oviposition sites. A detailed understanding of these behaviors and of the chemical stimuli evoking them may provide useful means for attracting and trapping insect pests of crop plants.

Heliothine moths (*Helicoverpa* and *Heliothis* herein) include a number of major pests of cultivated crop plants throughout the world. In North America, these include *Heliothis virescens* and *Helicoverpa zea*. In much of Eurasia (including Israel) Australia, and Africa, these include *Helicoverpa armigera* and *Helicoverpa peltigera*.

These 4 species of concern all are attracted to odorants from host plants (Tingle and Mitchell 1992, Mitchell et al 1991, 1992 BARD feasibility study report). Host plant chemicals also play a role in the sexual behavior of *Helicoverpa* species. Synthesis and possibly release of sex pheromone in *H. zea* and *H. phloxiphaga* is stimulated by kairomones from hosts plants (Raina 1988, 1992). Pheromonal scent marking on host plants also occurs in *H. virescens* and *H. zea*.

Studies of several other insects, including the cabbage looper *Trichoplusia ni*, have indicated a variety of other behaviors may occur in association with host plants, including the use of plants as sexual rendezvous sites and of direct involvement of plant chemicals in sexual behavior. Some pest species of moths also may use host plants as adult food sources.

These studies were undertaken to develop a more thorough understanding of how *Heliothis/Helicoverpa* moths use host plant odorants to locate and select foods, mates, and oviposition sites. We used *Heliothis virescens* and *Helicoverpa zea* in Florida, and *Helicoverpa armigera* and *Helicoverpa peltigera* in Israel as objects of study because of their pest status. It is

hoped that such an understanding will provide direction for work to discover and develop novel means to control these pests through behavioral manipulation. The specific objectives of the proposal were to 1) identify host odor affects on known Heliothine sexual behavior, 2) identify novel sexual behavior that is host dependent, 3) isolate and characterize host kairomones important to pest Heliothine host and mate-location behavior, and 4) investigate female attraction to males.

General Methods

Rearing: In Israel, *Helicoverpa armigera* and *Heliothis peltigera* moths were reared on an artificial diet. At the beginning of the project, a locally mixed bean diet was used which was replaced by a commercial US Premix mix. Moths were sexed at the pupae stage and were held separately under a reversed 14:10 hour light-dark cycle at $25 \pm 2^\circ \text{C}$. The quality of the laboratory reared moths was monitored by attraction of male moths to virgin females in the wind tunnel. The rearing was occasionally improved by introduction of feral larvae. Mated females were obtained by keeping males and females together. When mating was observed, the females were separated and used before laying eggs.

In Florida, *Heliothis virescens* and *Helicoverpa zea* were obtained initially as larvae on corn and tobacco plants, from which colonies were established. In the laboratory, these insects were reared on an artificial diet (Heliothis Premix, Stonefly Industries, P. O. Box 4264, Bryan, Texas 77805). Eggs for rearing and mated females for experiments were obtained by placing 2 to 5 pair of moths in a 4 liter cardboard bucket with a cloth lid and supplied with sugar water. Females used in attraction assays were subsequently dissected to verify the presence of a spermatophore. All insects were kept on a 14:10 light dark cycle, at 24° and 45-65% RH. Males and females (pupae and adults) were kept in separate environmental chambers prior to use in experiments.

Plants and plant material: In Israel, various whole plants and plant parts were tested for attractancy. Plant extracts were prepared by immersing the relevant plant material in

dichloromethane for 10-30 min. Extract was filtered to remove solids, and was concentrated to contain of 1 gr material per 100 ml of solution. Volatiles were collected in an air flow system and trapped on Porapak Q filters. The trapped material was eluted with hexane. All samples were kept in the refrigerator at 4° C .

In Florida, tobacco plants were grown in field plots and cotton (Germaine 510) was grown in a greenhouse. *Desmodium tortuosum*, Florida beggarweed, was field collected as needed from roadsides and old fields.

Wind tunnel assays: In Israel and in Florida, moths were tested for attractancy during the fifth through eight hours of a 10 hour scotophase (reverse phase) using a clear Plexiglas flight tunnel (0.5 x 0.5 x 2 meters). The temperature was maintained at 24-26° C and 50-60% humidity, wind velocity was ca. 0.5 meter/sec. Moths were tested individually for response to whole plants, plant extracts or volatile collections; to live moths, moth extracts, and pheromone, and to food materials. Moths were placed in small cylindrical screen vials (5 cm in diameter and 8 cm height) on a stand near the center of the downwind end of the flight tunnel. Moths tested for attractiveness (pheromone release), were placed in 15 x 15 x 15 cm screen cages near the center of the upwind end of the tunnel. Plants were similarly tested. Solutions containing plant or moth extracts or volatile collections were placed on a piece of muslin cloth. The cloth was secured over the open end of a glass cylinder (ca. 3 cm) with a rubber band. Air was passed through the cylinder at a rate of 2.5 liter/min to provide a continuous flow of odor through the tunnel. At the beginning of a trial, the cage or vial containing the individual moth was opened and the moth was given 2 min. to take flight. Moths were positively scored for reaching the cloth. Each responding moth was removed after 5 minutes. Moths were tested in the wind tunnel in groups of 5 or 10 and each group served as a replicate. Data were subjected to regression analysis or analysis of variance, with treatment means separated using Duncan's Multiple Range Test ($p < 0.05$).

Preparation of Male pheromone: Glass flow systems (30 cm x 9 cm in diameter) were used to collect male deposited material for experiments with *H. armigera* and *H. peltigera*. In each experiment, 25-30 naive male moths were placed on a large filter paper, covering ca. 80% of the

glass surface. Charcoal filtered air was passed through the system at a rate of 2 liters/min and the volatiles were trapped on Porapak Q filters. Each run was conducted overnight. The filter paper was cut into small pieces (dark spots were discarded) and extracted with dichloromethane for 30 min. After filtration, the solution was concentrated to contain 1 male equivalent (MEq) in 10 ul. The Porapak Q filters were also eluted with dichloromethane. All samples were kept in the refrigerator at 4°C.

Field tests: IPS dry traps were used in all tests in Israel. Samples of active solutions (plant or male derived) were deposited on cotton wicks and tests were conducted for 1-2 nights in a cotton field. Groups of 10-15 females were used in attempts to attract females.

Chemical analyses: Plant derived volatiles were analyzed directly by capillary GC on 30 m x 0.25 DB5, RTX 2330 and HP225 columns or by GC-MS using the DB5 column. Male deposited pheromone was analyzed either directly on the same GC columns or was first submitted to TLC separation. Merok silica 2 mm thick plates were used and they were developed with a 4: 1 mixture of hexane and ethyl acetate. Each plate was loaded with 150-200 male equivalents. The separated bands were visualized either by a UV lamp or by iodine vapor (most of the plate was protected with aluminum foil from the iodine). Bands were cut out and the silica was extracted with dichloromethane. For further chemical analysis or bioassay, the concentration was adjusted to contain 1 male equivalent (Meq) in 10 ul of solvent. Compounds were identified by GC with synthetic standards for comparison and by GC-MS. Isopropyl myristate was prepared by acid catalyzed esterification of myristic acid with isopropanol and nonanal was prepared by oxidation from nonanol. Other compounds were obtained from commercial sources

EXPERIMENTS AND RESULTS:

1. RESPONSE TO PLANTS.

H. armigera attraction to plants: At the preliminary stage the response of males and females was tested to whole plants and parts of them (Table 1.) The purpose of the screening was to

select plants that evoked the highest response.

Table 1. Attraction of *Helicoverpa armigera* males and virgin females to different host plants without flowers in a wind tunnel bioassay.

Host plant	Females		Males	
	No. tested	% attracted	No. tested	% attracted
Tomato (Marmande)	200	36.1	240	44.9
Tomato (green fruit)	100	26.0	50	28.5
Tomato (pink fruit)	60	10.5		
Eggplant	130	9.5	50	2.5
Pepper	100	11.5		
Cotton	100	10.9	50	50.6
Tobacco	140	16.5	90	22.3

The most attractive plants (or parts) were tomato for females and cotton (Acala) for males. The response of males varied significantly and contrary to first screening (Table 1), was in other tests similar to that of females.

Response of *H. armigera* to extracts of plants The response of virgin females to all extracts was similar (10.9 to 18.1%). Males responded preferably to sunflower and corn silk and to tomato (Table 1).

Table 2. Responses of *Helicoverpa armigera* males and virgins females to host plant extracts.

Host plant extract*	Females		Males	
	No. tested	% attracted	No. tested	% attracted
Cotton flowers	440	10.9a	160	8.1b

Sunflower flowers	160	15.5a	270	15.5a
Chick pea	420	18.1a	120	0c
Corn silk	170	14.8a	60	18.0a

*To 3 gr material in 300, ul dichloromethane.

Comparison of response of virgin and mated females: Three of the host plants, tomato, chick pea and sunflower, were selected in order to compare the response of virgin and mated females (Table 3).

Table 3. Responses of *H. armigera* and *H. peltigera* virgins and mated females to host plants and volatiles collected on Porapak Q.

Host plant or volatiles*	Unmated females		Mated females	
	N	% attracted	N	% attracted
Tomato plant (Marmande)**	100	30.0b	20	60.0a
<i>armigera</i>				
Chick pea volatiles**	100	15.0b	50	34.5a
<i>peltigera</i>				
Sunflower volatiles**	30	19.5b	30	48.0a
<i>peltigera</i>				

* From 5 gr material collected on Porapak Q in 100 pI solvent.

**Statistics were performed on each host separately.

Results indicated that both *H. armigera* and *H. peltigera* mated females responded better than virgin ones.

Table 4: Attraction of *H. armigera* and *H. peltigera* virgin females to cultivated tomato (Maramande) and wild cross cultivar* or to related host-volatiles.

Host plant or volatiles **	<i>H. peltigera</i>		<i>H. armigera</i>	
	N	% attracted	N	% attracted
Tomato (Maramande)				
(whole plant)	90	20.5a	70	16.9a
Tomato (wild)				
(whole plant)	30	Ob	50	Ob
Tomato (Maramande)				
Volatiles	30	25.8a		
Tomato (wild)				
Volatiles	30	Ob		
Tomato (Maramande) +				
Tomato (wild) Volatiles	30	23.1a		

* A cross between the cultivated tomato *Lycopersicon esculentum* and the wild species *L. pennellii*.

**From 2 gr material collected on Poropack Q in 200 ul dichloromethane.

Response of *H. armigera* and *H. peltigera* to different cultivars of tomato plants (Table 4):

The results indicated that different varieties, whole plants or volatiles, may differ in their ability to attract females. Both *H. armigera* and *H. peltigera* females were attracted to the cultivated tomato (Marmande) plant, however, they did not respond to a wild tomato variety. A mixture of volatiles from the two tomato varieties evoked the same level of response as that from the cultivated variety alone, indicating that the wild variety did not contain any attractant, inhibitory or synergistic component.

Associative learning of host odor by female *Heliothis virescens*

Mated females and males were placed in a cage containing host plant foliage for one scotophase and were tested for attraction responses to a bouquet of host plant foliage in a flight tunnel during the following scotophase. Inexperienced moths were also tested as a control. This experiment was conducted using the host *Desmodium tortuosum* and cotton plants with squares. Females of *H. virescens* were significantly more likely to respond to *D. tortuosum* plants than were inexperienced females, indicating they may have learned the odor of the plant when contacting it during the previous scotophase (Figure 1, top). Both male and female *H. virescens* that were provided contact with cotton prior to bioassays were more likely to be attracted to cotton than were inexperienced moths (Figure 1, bottom).

Chemical analysis of plant volatiles: An attempt was made to identify components in the plant volatiles. Sunflower and cotton volatiles were analyzed by GC and GC-MS and compared to library data and available standards. As expected, numerous peaks were observed. Many of the components were monoterpene compounds. Several, like α - and β -pinene, myrcene and limonene were tested in the wind tunnel with *H. armigera* females. The response of the individual compounds was negligible and this line of research was not continued.

Development of controlled release system for testing plant odorants.

The development of host-based chemical attractants for monitoring pest moth species has been severely limited by the lack of suitable formulation systems. Putative insect attractants emitted from plants such as hexanol, ethyl hexanoate, ethyl octanoate, cineole, myrcene and other related terpenes are highly volatile. Currently available formulation systems such as rubber matrices, laminates, and polyvinyl polymers do not provide constant release of these types of chemicals over reasonable periods of time needed for bioassays and field tests. Formulation of highly volatile compounds using capillary tubes filled to variable heights have been used for laboratory bioassays. This formulation method will provide reasonable systems for use in laboratory bioassays over limited release rate ranges, but are unsuitable for field use due to the need to maintain the capillary in a

vertical position and protect the lure from adverse weather. Research was conducted to develop lures that would provide a constant release rate of a variety of chemicals over time. Of the systems investigated it was determined that membrane type systems were the most cost effective and user friendly compared to specialized systems such as laminates.

Initial investigations were conducted using 6 mill co-polymer of polyethylene as the membrane. Lures were constructed in a 1 cm X 3 cm shape having a thickness of approximately 4 mm. The exposed area of membrane was a circle having a 5 mm diameter. Separate lures were loaded with hexanol, ethyl hexanoate, ethyl octanoate, and cineole as the test chemicals and lures were prepared using a mixture of the four chemicals at equal proportions and as a ratio of 10:10:1:1 of cineole, ethyl octanoate, hexanol and ethyl hexanoate respectively. Release rates of emitted chemicals were determined using systems that have been previously described (Heath et al., 1992). Briefly this system consists of a glass chamber (25.7 cm long and 7.6 cm ID) constructed of Pyrex glass with a glass frit inlet and a ground-glass joint outlet and a multiport collector base to which the collector traps were connected. Collector traps used to trap organic volatiles were made from a 4.0 cm long by 4.0 mm ID piece of glass tubing and contained 50 mg of Super-Q® as the adsorbent. Two stainless steel frits were used to contain the adsorbent. The collector traps were connected to stainless steel tubing using 0.64 cm unions and 0.64 cm ID Teflon® ferrules. These traps were cleaned by soxhlet extraction using methylene chloride for 24 h and dried in a fume hood prior to use. Volatiles from the lures collected on the traps were eluted using 100 µl of 100 µl of high purity methylene chloride. An internal standard was added prior to analysis.

Gas chromatographic analyses were conducted using a Hewlett-Packard Model 5890A Series II gas chromatograph, equipped with a cool on-column capillary injector (septum injector) and flame ionization detector. Helium was used as the carrier gas at a linear flow of 18 cm/sec. The chromatographic data was collected and processed using the Perkin-Elmer Nelson Turbochrom® III software running on an IBM-type 386-PC system under MS-Windows® 3.0. Capillary gas chromatography (CGC) analysis of the terpenes was done using a retention gap column prior to the capillary column. This system permitted

the injection of samples without concentration in 5-100 μ l of solvent (Murphy et al., 1993). A combination of three fused silica columns connected in series using GlasSeal® connectors (Supelco Inc., Bellefonte, PA) was used. The primary deactivated fused silica column, 8.0 cm long by 0.5 mm ID, was connected between the injector and the retention gap column. This primary column permitted the use of 0.4 mm OD stainless steel needles with a septum injector for on-column injections. The retention gap columns used were 10 m by 0.25 mm ID deactivated fused silica and the analytical column used for analysis was a 30 m by 0.25 mm ID (0.25 μ m film) SE-30. Confirmation of compound identity was obtained using mass spectroscopy. Mass spectra were obtained using the capillary columns, operated as described above, coupled to a Finnigan Ion Trap® mass spectrometer in either electron impact (EI-ITDMS) or chemical ionization (CI-ITDMS) mode. The reagent gas used for CI was isobutane.

As shown in Figure 2 the membrane type lures released the four test chemicals in a zero-order rate having varying slopes and release rates. The utility of this formulation is obviously limited. Additional research was conducted to determine if a mechanical negative release modulator (NRM) could be employed to provide a more stable type of formulation. The results of the inclusion of a NRM on the release rates of the test compounds is shown in figure 3. The release rates of the four compounds are similar and offered a unique system for further investigation. Because plant extracts often occur as a blend of chemicals lure were loaded with the four test compounds in equal proportion. As shown in figure 4 the release rates of the chemicals were similar and the release of the chemicals was reasonably constant over a 30 day period. Additional tests were done to determine if various ratios of the emitted chemicals could be provided. Figure 6 shows the release rate over time from lures containing a mixture of the four chemicals loaded at a ratio of 10:10:1:1 of cineole, ethyl octanoate, hexanol and ethyl hexanoate respectively. The percent of release of the chemicals over a 30 day period was constant (figure 6).

2) RESPONSE OF FEMALES TO MALES AND MALE DEPOSITED PHEROMONE

Female attraction to live males.

Heliothis virescens were tested in a flight tunnel for preliminary evidence of attraction to male pheromone, using methods reported in Landolt and Heath (1989) for the cabbage looper moth *Trichoplusia ni*. In the first experiment, both male and female *H. virescens* were tested for attraction responses to a screen cage containing fifteen males at the upwind end of a flight tunnel. Five to seven moths were tested at each bihourly interval for the entire scotophase, and the entire day long test was conducted over five different days for male attraction to males, and over another 5 days for female attraction to males. Both sexes of *H. virescens* exhibited some attraction response to the cage of 15 males (Figure 7), but at low response rate and with no strong periodicity to the response through the scotophase. From 4 to 10% of females responded, while 5.5 to 18% of males were attracted to the caged males.

As expected, males of *H. armigera* were highly attracted to virgin females (64.5%), but females were also moderately attracted to live males. Male attraction to males and female attraction to males occurred only at a very low level. An attempt to capture females in trap's baited with live males failed, indicating that attractancy may be only at a short distance.

Table 5. Attraction of *H. armigera* virgin females and males to males and females (10-15 moths in a cage).

Host	Females		Males	
	N	% attracted	N	% attracted
Females	120	8.5c	200	64.5a
Males	200	27.5b	70	9.5c

Female attraction to male extract. A hexane extract of male *H. virescens* terminal hairpencils was tested over a range of dosages for attractiveness to conspecific females in a flight tunnel. Twenty five unmated females were tested to each of 5 different dosages of

extract applied to filter paper (0.05, 1, 2, and 5 male equivalents). This test was conducted over 5 different days, during the 3rd to 5th hours of the scotophase. No females were attracted to the extract at any dose tested. A preliminary test of female attraction to a methylene chloride extract of males of this species showed some attraction at the 1.0 male equivalent dosage.

Additional flight tunnel experiments were conducted to attempt to partially isolate the source of the male produced pheromone of *H. virescens* that is attractive to females. The purpose of the study was to locate the origin of the pheromone in the male body and to produce purer extracts for bioassays. Five tests were conducted in a series. First, a comparison was made of dosages of solvent extracts of male *H. virescens*. A whole body extract was made by immersing groups of 50 males in a beaker of 100 ml solvent. Solvent was then removed, and concentrated under a stream of nitrogen (methylene chloride) or in a rotary evaporator (methanol) to a concentration of 5 male equivalents per 200 ul solvent. Dosages of extract were tested on filter papers placed in a flight tunnel. Five moths were tested to a dosage on a filter paper and 5 dosages were so tested in a series (0, 0.2, 1, 2, 5, and 10 male equivalents). This test was conducted 5 times using methylene chloride and 5 times using methanol. Response rates were consistently low (4 to 8 percent) for extracts using both methylene chloride and methanol with no indication of a dose response relationship. Second, methylene chloride extracts were made of male heads with thoraces and of male abdomens and were tested for attractiveness to females. All extracts were tested in a flight tunnel at a 5 male equivalent dosage in 100 ul solvent on filter paper. Five females were tested per application of extract to a filter paper and the two treatments were compared 10 times. Response rates again were low (Figure 8, top), but female attraction to extract of male abdomens (18 percent) was significantly greater than attraction to extract of head and thorax (2%) ($t=2.61$, $p=0.018$, $df=18$). Third, a comparison was made of female *H. virescens* attraction to extract of male abdomens, male abdominal hairpencils, and male abdomens minus the hairpencils. All extracts were made with methylene chloride and were tested at 5 male equivalents in 100 ul solvent on a filter paper. Five females were tested per application to a filter paper; all three treatments and a control were compared daily for 6

days, using a flight tunnel bioassay. A total of 30 moths were tested to each extract treatment. Female attraction response rates were 0 to the solvent control, 3.3 ± 3 percent to extract of male hairpencils, 13.3 ± 7 percent to extract of the whole male, and 16.7 ± 3 percent to the extract of the male abdomen minus the hairpencils (Figure 8, bottom). Fourth, a comparison was made of female attraction to dosages of methylene chloride extract of male abdomens. Treatments tested were 0, 1, 3, 5, and 10 male equivalents per 100 μ l solvent applied to a filter paper. Five females were tested per application of a treatment to a paper and the experiment (five moths tested to 5 treatments) was conducted 5 times. Response rates to all dosages were low with no clear dose response relationship (Figure 9).

Effects of feeding on female attraction to males.

An experiment was conducted to determine if female TBW attraction to males is affected by adult feeding on sugar solutions. Other studies on the cabbage looper, *Trichoplusia ni*, indicated that starved moths are attracted to male pheromone. Two groups of female TBW were evaluated; moths held in cages for 3 to 5 days with water and no sugar-honey mixture (starved), and moths held in cages for 3 to 5 days with water and a solution of sucrose and honey on cotton balls (fed). All females were tested for attraction to a whole body methylene chloride extract of males. For each assay, five fed females were tested for attraction responses in a flight tunnel to a 5 male equivalent dosage of extract on a filter paper and 5 starved females were tested for attraction responses to another 5 male equivalent dosage of extract on a filter paper. This assay was repeated 12 times over 6 work days. Response rates were similar to previous bioassays, with 16.7 % of fed females attracted and 8.3 % of starved females attracted to male pheromone.

Influence of male age on female attractancy (Table 6): Differences in female response to material obtained from 2-5-old males were not significant. Males 3-5-days-old consistently evoked a higher response than older ones. This age group was, therefore, used for further experiments.

As in part 1, most of the experiments were performed with *H. armigera*. Preliminary tests indicated that most of the male activity was found in the deposited material on filter paper. Material obtained by solvent elution of the Porapak Q traps evoked very low and inconsistent female response and, therefore, all further tests were conducted with male deposited material recovered from filter paper extracts. Dose of pheromone was measured in amount deposited by one male and considered as male equivalent (MEq).

Table 6: Attraction of *H. armigera* virgin females to male pheromone extracts

Age of males (days)		Females	
Pheromone*	No. tested	% attracted	
2	70	26.7 a	
3	50	34.6 a	
5	30	30.0 a	
7	40	17.5b	

*Equivalent to pheromone emitted by 3 males in 300 ul solution.

Effect of the dosage of male deposited pheromone on female attractancy (Table 7): The results indicate that a dosage equivalent to material deposited by 2-4 males gave the best response. The response to dosages below 1 male equivalent (MEq) was significantly weaker. In further tests 2 or 3 MEq were used.

Table 7: Effect of male pheromone dose on attraction of *H. armigera* virgin females.

Male pheromone (MEq)*	Females	
	No tested	% attracted
4.0	60	38.2 a
3.0	100	36.7 a
2.0	40	26.3 a
1.0	100	16.5 b
0.5	40	11.5 b

* 1 MEq in 100 ul solution.

Effect of female sex pheromone on production of male pheromone: The influence of female sex pheromone on the release of male pheromone was evaluated by introducing different dosages (50 to 1000 ug adsorbed on rubber septa or cotton dental wool) of the female pheromone into the male chamber. No stimulation or inhibition by the female pheromone was observed as shown by a representative test with 1000 ug (Table 8).

Table 8. Effect of *H. armigera* female sex pheromone on production of male pheromone as indicated by attraction of virgin females.

Male pheromone*	Attraction by females	
	N	% attracted
Stimulated by female pheromone **	250	18.9a
Not stimulated	250	19.5a

* Dosage of 2 MEq in 200 μ l solution. ** 1000 μ g of female pheromone dispensed in rubber septa were used to stimulate male pheromone production.

Cross attraction of two Heliothine virgin females to males: Response was tested to males of *H. armigera* and *H. peltigera* (Table 9). Females of each species were attracted, at about the same level, to conspecific and heterospecific males. This may indicate that male produced pheromones are not entirely species specific.

Table 9: Cross-attraction of *H. armigera* and *H. peltigera* females to 15 live males in a cage.

Attracting males	Attracted virgin females			
	<i>H. peltigera</i>		<i>H. armigera</i>	
	N	% attracted	N	% attracted
<i>H. peltigera</i>	60	41.9a	200	31.8a
<i>H. armigera</i>	60	27.6a	60	25.5a

Isolation and separation of male deposited pheromone

Most of the male pheromone activity was obtained from material deposited on filter paper and extracted with dichloromethane. This material was subjected to TLC separation and six bands were discerned by UV and Iodine detection (Figure 10). The bands were removed and extracted and concentrated to a volume that contained 1 MEq in 10 μ l of dichloromethane.

Bioassay of fractions in the wind tunnel: Results, presented in table 12 indicate that almost all activity is located in fraction 4.

Table 10. Attraction of *H. armigera* virgin females to TLC fractions of male pheromone.

Fraction No.	No. of MEq	Volume ul	No. tested	% attracted
1	5	50	30	5.5c
2	5	50	30	0
3	5	50	30	0
4	5	50	60	34.4a
5	5	50	40	10.3bc
6	5	50	30	0

Comparison of activity of the male pheromone before TLC with that of fraction No. 4: The results (Table 13) indicated that almost no material was lost during the TLC separation.

Table 11. Attraction of *H. armigera* virgin females to male pheromone before TLC as compared to fraction 4.

Pheromone	No. of MEq	Volume pl	No. tested	% attracted
Before TLC	5	50	90	37.7a
FractionNo. 4	5	50	90	26.3a
Fraction No. 4	10	100	90	31.1 a

Chemical analysis of active fraction 4 and bioassay of components: The active fraction No.4 was analyzed on different GC columns, compared with available standards and subjected to GC-MS analysis. The ratio of components varied in the different samples,

however some of the compounds were identified and subsequently bioassayed in the wind tunnel (Table 14). All were commercial materials with the exception of nonanal and isopropyl myristate.

Table 12. Attraction of *H. armigera* virgin females to compounds identified in male deposited pheromone, each 100 ug in 100 ul dichloromethane.

Compound	No. tested	% attracted
Nonanal	70	0
Benzaldehyde	90	7.6b
15:AC	70	0
16:AC	70	0
16:0H	90	10.7b
17:0H	70	0
18:AC	70	0
18:0H	150	20.5a
Isopropyl myristate	150	19.5a

The results in Table 14 indicated that almost all active compounds are relatively high boiling or polar (e.g. alcohols) which may explain the fact that they were readily absorbed on the filter paper in the flow system. The most active compounds were octadecanol (18:0H) and isopropyl myristate. The latter is novel as a male pheromone component. Some of the presently found alcohols and acetates were recently identified in male *H. armigera* hairpencil extracts in China (Huang et al., 1996).

Response of *H. armigera* virgin females to mixtures and to fraction No. 4: In the last two tests, a quaternary mixture (Mix 1) of the most active compounds: isopropyl myristate, octadecanol, heptadecanol and benzaldehyde and a ternary mixture (Mix 2) of: isopropyl myristate, octadecanol and heptadecanol were bioassayed and compared with the active fraction No. 4 (Tables 15 and 16).

Table 13 Attraction of *H. armigera* virgin females to Mix 1, Mix 2 and fraction No. 4.

Attractant	Amount	No. tested	% attracted
Mix 1: Isopropyl myristate + 18:0H + 16:0H + Benzaldehyde (4: 2: 1: 1)	50 ^u _p g	45	26.6a
Mix 2: Isopropyl myristate + 18:0H + 16:0H (4: 2: 1)	50 ^u _p g	40	17.5a
Fraction No. 4	5 Meq	70	28.8a
Fraction No. 4	10 Meq	70	35.9a

Table 14. Attraction of *H. armigera* virgin females to different dosages of Mix 1 and Mix 2 and fraction No. 4.

Attractant	Amount	No. tested	% attracted
Mix 1: Isopropyl myristate + 18:0H + 16:0H + Benzaldehyde (4: 2: 1: 1)	50 ug	45	24.4a

ibid	150 ug	40	25.0a
Mix 2: Isopropyl myristate + 18:0H + 16:0H (4: 2: 1)	50 ug	40	17.5a
ibid	150 ug	40	20.0a
Fraction No. 4	5 Meq	70	32.9a

The results in Tables 14, 15 and 16 indicate that there is no synergism among the 3 or 4 active components. The activity of isopropyl myristate or octadecanol was in the same range as either Mix 1 or Mix 2. Attempts to catch females in the field in IPS traps baited with either MIX 1 or Mix 2 (1 or 2 mg each on dental cotton wicks) failed. This result is in line with the observation that live males did not attract females in the field.

3. HOST PLANT ODOR EFFECTS ON SEX ATTRACTIVENESS.

Response of Heliothine males and females to pheromone and host-plant: Two experiments were carried out with a mixture of host-plant material and male pheromone in order to evaluate possible synergistic effects between the two. Live males or male pheromone from *H. armigera* were used. In the first experiment, the response of the males and females to live moths and to tomato plants or combination of plant and moths was tested (Table 10). Results indicated that both sexes responded to the tomato plant, but no synergism was observed.

Table 15. Attraction of *H. armigera* virgin females and males to tomato plant, the opposite sex and a combination of host plant and opposite sex.

Stimulus	Females*		Stimulus	Males*	
	N	% attracted		N	% attracted
Tomato	40	27.4a	Tomato	50	48.6a
Males	40	22.0a	Females	80	74.4a

Tomato + Males 40 24.6a Tomato + Females 70 64.6a

* Ten moths were used in each run.

Response of *H. armigera* and *H. peltigera* 4-day-old virgin females to *H. armigera* male pheromone and cotton flower extracts: Results (Table 11) indicate that there was no synergistic effect for attraction. Interestingly, *H. peltigera* females were attracted to *H. armigera* male pheromone supporting the cross attraction results obtained with live males of the two species (Table 9). More experiments are required with different host-plant material in order to test this phenomenon and to assess its general character.

Table 16: Attraction of *H. peltigera* and *H. armigera* virgin females to cotton flower extract, male pheromone and combinations.

Bait *	Attracted virgin females *			
	<i>H. peltigera</i>		<i>H. armigera</i>	
	N	% attracted	N	% attracted
Cotton flowers extract	30	29.2a	100	9.5a
Male pheromone	30	30.4a	40	21.0a
Cotton flowers extract +				
Male pheromone	30	33.3a	100	23.3a

* 200 ul of plant extract solution (2 gr plant material equivalent) and 200 ul of *H. armigera* male pheromone solution (2 MEq).

Four experiments were conducted evaluating the possibility of host odor enhancing sex attraction in *Heliothis virescens*. First, males and females were tested for attraction to a cage containing 15 males and a bouquet of cotton foliage in a flask of water. Either 5 males or 5 females were tested bihourly throughout the scotophase of the light cycle, on each of 10

different days, providing 25 of each sex tested for each time period. As in the test of attraction to males alone, there was a small percentage response by either sex at each time period tested (Figure 11). There is some indication again, that male attraction responses to males may be greater than female responses to males, at least within the protocols used in these tests.

In a second test, male *Heliothis virescens* were tested for attraction responses to 5 females alone in cages or held with a cut tobacco plant in a cage, at hourly intervals throughout the scotophase. Twenty five males were tested per treatment per time period, over 10 days of testing. Male attraction responses to females with tobacco were slightly better than attraction to females without tobacco both early and late in the activity period (Figure 12). However, this was not significantly different by Duncan's Multiple Range test.

In a more limited format, a third test was conducted using a flight tunnel bioassay to look for host plant effects on female attraction to males and male attraction to females. Females were tested for attraction responses to males in a cage, a cut tobacco plant in a cage, or the combination of males and tobacco together. This was conducted as sets of 10 females tested per treatment per day, and replicated over 5 days, providing 50 moths tested per treatment. This assay was done early in the scotophase when male attraction to females is naturally low. Numbers of females responding to the cage of males and tobacco was not greater than that to tobacco alone (Figure 13). The experiment was repeated but testing males attracted to females and tobacco. In this case, significantly greater numbers of males were attracted to the cage containing both males and tobacco, compared to males alone or tobacco alone (Figure 13).

This same test design was then used in the fourth experiment to evaluate host plant effects on female attractiveness to males, using cotton instead of tobacco foliage, with 50 moths tested per treatment. In this test the difference in results was dramatic, with nearly 90% of males attracted to females on cotton, compared to cotton alone or females alone (Figure 14).

4. ATTRACTION TO ADULT FOODS.

Experiments were conducted to determine if moths are attracted to fermented sweet baits, because moths may be attracted to plants in part to obtain sugars. Extrafloral nectaries and

flowers may be a source of carbohydrates for moths attracted to plants.

Both sexes of *H. virescens* were attracted to an aged (fermented) solution of palm sugar, using the flight tunnel assay. Twenty-four percent of females and 27 % of males tested were attracted to the 200 ml of bait placed in a pan in the flight tunnel, while none were attracted to the water control. Greatest attractiveness occurred when the bait was allowed to age up to 24 days (Figure 15).

EVALUATION OF ACHIEVEMENTS

Four specific objectives were given in the original research proposal. These were 1) identify host odor effects on known sexual behavior, 2) identify novel sexual behavior that is host dependent, 3) isolate and characterize host plant kairomones important to Heliothine host and mate location behavior, and 4) investigate female attraction to males for the development of attractants for females.

The major achievements of this project are 1) the demonstrations of attraction of Heliothine moths to plants, plant odors, and plant extracts, 2) the demonstration of female attraction to males, male extracts, and compounds identified from males, 3) the demonstration of *H. virescens* attraction to a fermented sweet bait, 4) the discovery that prior experience or learning affects attraction responses to host plant odors and, 5) the demonstration of host odor effects on attractiveness of females to males for *H. virescens*.

Attraction to plants .

The response of both *H. armigera* and *H. peltigera* to various whole plants and to plant extracts or volatiles showed moderate selectivity. Most of the tested plants exhibited some attractancy to both males and females. This observation could be anticipated due to the polyphagous behavior of the two moth species. Interestingly, females of the two Heliothine species, which were attracted to cultivated Marmande tomato variety, did not respond to a wild variety. This could be demonstrated with whole plants or with collected volatiles. The wild variety is not attractive, but it is not inhibitory as shown by the positive response to a mixture of volatiles from the two varieties. A similar selectivity of response of *H. virescens* females to a susceptible and a resistant tobacco variety was reported by Tingle et al., (1990).

Mated females responded significantly better to plants than virgin females do, indicating that mated females are using plant host kairomones for locating oviposition sites. A similar observation was made with *H. virescens* females (Tingle and Mitchell, 1992).

We identified several compounds from plant volatiles (cotton and sunflower) and some of them were tested individually in the wind tunnel. The response of the female moths was in all cases negligible. Attractancy of American Heliothine species to different plants and plant extracts has been demonstrated before (Mitchell et al., 1990; 1991; Tingle et al., 1989; Tingle and Mitchell, 1992). In all the reported studies, the active components were not characterized.

The results to date with plant odorants formulated to provide controlled release rates and ratios indicate that both laboratory and field tests of moth attraction to selected plant chemicals may now be feasible

Attraction to males

It is evident from these and previous studies (under a BARD funded feasibility study) that males of species of Heliothis produce and release odorants that are somewhat attractive to females. We do not know why the response rates observed in the bioassays are low compared to male responses to female pheromone in similar tests. This may be due to environmental and/or physiological factors not yet understood.

Female sex pheromones of Heliothine moths are species specific. We have previously characterized the pheromone complex of *H. armigera* and *H. peltigera* and identified the attractive and inhibitory components (Dunkelblum et al., 1989; Kehat and Dunkelblum, 1990). This was not the case with the male pheromones. Females of both species were cross-attracted to males of *H. armigera* and *H. peltigera*. Generally, the response of females to males or male deposited pheromone was much lower than that of males to female pheromone. The deposit of male pheromone was not synergized by female pheromone and its activity was not enhanced by host-plant odor. This observation is in contrast to that observed for the male pheromone of *Trichoplusia ni* (Landolt and Heath, 1990).

The male deposited pheromone from *H. armigera* was fractionated by TLC and most of the activity was concentrated in one band. Several compounds were identified in this band, mostly long-chain acetates and alcohols, similar to those extracted from hairpencils of *H.*

armigera Huang et al., 1996) and *H. virescens* (Teal and Tumlinson, 1989), but very different from those identified from *Trichoplusia ni* males (Landolt and Heath, 1990). One unusual compound found in the male pheromone of *H. armigera* was isopropyl myristate, which along with octadecanol were the most active individual pheromone components. Bioassays of mixtures of the most active components indicated that there is no synergism among them. Attempts to trap females in the field with the active components or mixtures of them failed.

Attraction to foods

We demonstrated that both sexes of *H. virescens* are attracted to fermented sweet baits. *Helicoverpa zea* also feeds on such materials and may be trapped with sugar baits (Ditman and Cory 1933). Because some host plants such as cotton possess extrafloral nectaries and other species may provide nectar in flowers, these moths may be attracted to odors from plants in part to obtain sugars as adult food. Populations of plant sucking insects such as aphids and scale may also produce sugars in honeydew that are attractive to heliothine moths. Isolation and identification of these attractive odorants should provide the source of useful lures for trapping moth sexes of these insects.

Learning Females of *H. virescens* were attracted more to the host *D. tortuosum* (Florida beggarweed) following exposure to the plant in the previous scotophase, indicating a positive effect of experience that may be interpreted as learning. The observed rates of attraction of moths to plants, plant odors, and plant extracts, in other experiments may then be low due to an absence of this affect in the laboratory trials. Additional work needs to be done to determine the affects of experience on moth responses to chemical blends.

Attraction to males and plants. Research conducted under this project has discovered host odor effects on sexual attraction of *H. virescens*, specifically on male attraction to females. Experiments were not concluded to determine if this enhancement is due to host odor stimulation of pheromonal calling in females or to synergism of female pheromone in stimulating a male response (see Landolt et al 1994 for similar work on cabbage looper moth). Experiments with female attraction to males and tobacco in the same species did not indicate any enhancement of sexual attractiveness. A more complete knowledge of preference for specific host plant odors by host-finding moths, as well as the role of learning in host plant

finding, may be necessary to conduct in-depth studies of host plant effects on sexual behavior, such as female attraction to male pheromone.

Both *H. armigera* and *H. peltigera* female moths were attracted to host-plants and to male deposited pheromone. The response was moderate but consistent. However, the activity, particularly as tested with the male pheromone, was limited to a short range in the wind tunnel. The mixtures of host-plant volatiles and of the male pheromone were complex, comprising of a large number of compounds. In view of these findings, the development of lures for females consisting of synthetic mixture based on these compounds seems very difficult. The basic information gathered in the present project is of interest and it confirms the importance of host-plants and male pheromone, in addition to the extensively studied female pheromones, in the reproductive complex of moths.

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DESCRIPTION OF COOPERATION

All four members of the research team (Landolt, Heath, Dunkelblum, Kehat) met in Gainesville, Florida on February 1994 to discuss the objectives and research approaches used in our respective laboratories. Recent research findings and improvements in techniques and experimental designs were shared, as were concerns and problems encountered. Most importantly, recent innovations in the technology used for collecting and analyzing plant odorants were reviewed and shared.

The effort to develop formulations suitable for the testing of many plant odor chemicals supported the research programs underway in all 4 laboratories, and is available for use in many planned lab experiments and field tests designed to develop attractants for females of species of Heliothis.

Communication was maintained and information was shared on experimental design and results with other research research directions. Continued work on plant chemical formulation and on technology to collect and analyze plant odorants is the most critical collaborative aspect of the project. The success of the Israeli lab with attraction of moths to plants and female attraction to male pheromone and the U.S. work on moth learning of host odor and female attraction to male pheromone was complementary, shared, and benefitted greatly from the work conducted at the sister laboratory. .

We expect this cooperation to continue well beyond the expiration of the BARD-funded project. There is mutual interest in several areas of research described herein, including the formulation of plant compounds for behavior studies, analysis of plant odors, the role of learning in moth host finding, and the chemistry and functions of moth male pheromones. The emphasis and expertise in the Israeli laboratory on responses of moths to plants and in chemical identifications, and the emphasis and expertise in the US laboratories on methods development and behavioral ecology are quite complementary and mutually beneficial.

LIST OF FIGURES

Figure 1. **Top.** Attraction of *H. virescens* moths to a bouquet of foliage of Florida beggarweed in a flight tunnel. Experienced males and females tested were placed in a cage containing *D. tortuosum* the night preceding bioassays. **Bottom.** Attraction of *H. virescens* moths to a bouquet of foliage of cotton in a flight tunnel. Experienced males and females tested were placed in a cage containing cotton foliage the night preceding bioassays. Inexperienced males and females tested had no prior contact with host plants.

Figure 2. Release rates of four plant compounds loaded in individual lures of a membrane-based formulation.

Figure 3. Release rates of four plant compounds loaded in individual lures of a membrane-based formulation containing a mechanical negative release modulator.

Figure 4 Release rates of four plant compounds loaded together in a single lure of a membrane system with a negative release modulator.

Figure 5. Release rates of four plant compounds loaded at a ratio of 10:10:1:1 from a lure of a membrane based formulation with a negative release modulator.

Figure 6. Release rates of four plant compounds from a lure of a membrane based formulation with a negative release modulator, over a 30 day period.

Figure 7. Percentage of unmated female (crosshatched bars) and male (slashed bars) *Heliothis virescens* exhibiting attraction responses (plume tracking) in a flight tunnel in response to a cage of 15 conspecific males, at bihourly intervals throughout the 10 hour scotophase of the light cycle.

Figure 8. Attraction of female *Heliothis virescens* to a methylene extract of (**Top**): the head and thorax combined or the abdomen of conspecific males, or (**Bottom**): the whole body of males, the

terminal hairpencils of males, or the abdomens of males with the hairpencils removed.

Figure 9. Attraction of female *Heliothis virescens* to a methylene chloride extract of males at dosages of 0, 1, 3, 5, or 10 male-equivalents applied to a filter paper.

Figure 10. Separation of *H. armigera* male-deposited pheromone by TLC.

Figure 11. Percentages of male (slashed bars) and female (crosshatched bars) *H. virescens* moths tested that were attracted to a cage containing 15 males and a bouquet of cotton foliage, at bi-hourly intervals through the scotophase.

Figure 12. Percentages of male *H. virescens* tested that were attracted to a cage containing 5 females or were attracted to a cage containing both 5 females and a cut tobacco plant, at hourly intervals through the scotophase of the photoperiod.

Figure 13. Percentages of moths tested that were attracted to the opposite sex, to tobacco, or to a combination of the opposite sex and tobacco.

Figure 14. Percentages of male *H. virescens* attracted to a cage of 5 females, a cage containing a bouquet of cotton foliage in a flask, or a combination of 5 females and cotton foliage.

Figure 15. Percentages of female *H. virescens* attracted to contact a pan containing 200 ml of palm sugar solution in a flight tunnel, for different ages of bait. The short series (solid bars) included 0, 3, 6, 9, and 12-day old bait, while the long series (cross hatched bars) included 0, 6, 12, 18, and 24 day-old baits.

PUBLICATIONS AND PATENTS.

None

TBW ATTRACTION TO D. TORTUOSUM EXPERIENCE AFFECTS HOST FINDING

TBW ATTRACTION TO COTTON EXPERIENCE AFFECTS HOST FINDING

Figure 1

HEXANOL ETHYL HEXANOATE CINEOLE ETHYL OCTANOATE

Figure 2

HEXANOL ETHYL HEXANOATE CINEOLE ETHYL OCTANOATE

Figure 3

MIX OF 4

Figure 4

HEXANOL ETHYL HEXANOATE CINEOLE ETHYL OCTANOATE

MIX OF 4 (RATIO 1:1:10:10)

Figure 5

HEXANOL ETHYL HEXANOATE CINEOLE ETHYL OCTANOATE

MIX OF 4

HELIOTHIS VIRESCENS ATTRACTION TO 15 CAGED MALES

HOUR OF SCOTOPHASE

ATTRACTION OF FEMALE H. VIRESCENS TO MALE BODY TAGMA EXTRACTS

ATTRACTION OF FEMALE H. VIRESCENS TO MALE BODY PART EXTRACTS

FEMALE TBW ATTRACTION TO MALE ABDOMINAL EXTRACT

Figure 1. Separation of *H. armigera* male deposited pheromone by TLC.

HELIOTHIS VIRESCENS ATTRACTION TO 15 MALES AND COTTON IN A CAGE

HELIOTHIS VIRESCENS MALE ATTRACTION TO 5 FEMALES AND A TOBACCO PLANT

HELIOTHIS VIRESCENS ATTRACTION TO OPPOSITE SEX AND TOBACCO IN CAGE

FIG. 13

HELIOTHIS VIRESCENS MALE ATTRACTION TO 5 FEMALES AND COTTON IN A CAGE