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The Role of Heliothine Hairpencil Compounds in Female *Heliothis virescens* (Lepidoptera: Noctuidae) Behavior and Mate Acceptance

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Abstract

Studies on numerous insect species suggest that male-produced sex pheromones play a role in attracting females; as aphrodisiacs, making females more quiescent; or as a means of inhibiting competing males. Male heliothine moths display abdominal hairpencils during courtship, but the specific effects of the odors released on female behavior have not yet been elucidated. This study investigates the role of male hairpencil compounds in female *Heliothis virescens* mating behavior. Female *H. virescens* were exposed to filter paper loaded with hairpencil extracts of male *H. virescens*, *Heliothis subflexa* and *Helicoverpa zea*, and observed for behavioral responses to odors. Single synthetic compounds found in the *H. virescens* hairpencil blend were also tested. In mating assays between single male and female *H. virescens* it was found that: (i) antennectomized females mated less frequently than sham-operated females; (ii) females mated less frequently with males whose hairpencils had been surgically removed; (iii) females mated with males with ablated hairpencils if a filter paper loaded with one male equivalent of *H. virescens* hairpencil extract was presented simultaneously; and (iv) this effect was species-specific, as presentation of *H. subflexa* or *H. zea* hairpencil extracts did not restore mate acceptance. This study suggests that odors released by male hairpencils are important in mate acceptance by female *H. virescens*, and may play a role in mate choice and species isolation.

Key words: aphrodisiac, courtship, mating behavior, pheromone, species isolation, tobacco budworm

Introduction

Male pheromone production has been studied in several insect species. Male insects often possess scent-releasing organs in the form of hairpencils, coremata or androconial scales (Birch, 1970a,b, 1975; Baker *et al.*, 1981; Birch and Hefetz, 1987; Fitzpatrick and McNeil, 1988; Birch *et al.*, 1990; Heath *et al.*, 1992). In the Lepidoptera, studies have identified hairpencil secretions produced by several species (Phelan *et al.*, 1986; Teal and Tumlinson, 1989; Heath *et al.*, 1992; Thibout *et al.*, 1994; Huang *et al.*, 1996). The behavioral role of these secretions is not well understood, but most often these odors have been deemed important in courtship behavior.

Male heliothine moths display abdominal hairpencils during courtship yet the specific effects of hairpencil odors upon female heliothine mating behavior has not been clearly determined. Studies on numerous insect species suggest that male-produced sex pheromones may play a role in attracting females (Heath *et al.*, 1992) and/or other males (Takayoshi and Hiroshi, 1999); as aphrodisiacs, making females quiescent and more likely to accept a courting male (Teal *et al.*, 1981); as a means of mate recognition and sexual selection in females (Jacquin *et al.*, 1991); or as a means of inhibiting competing males, either by direct repulsion through hairpencil odors or by discontinuing female 'calling' (Hendricks and Shaver, 1975; Huang *et al.*, 1996).

Heath *et al.* (1992) demonstrated that male *Trichoplusia ni* Hübner release sex pheromone which is attractive to females, inducing upwind flight in wind tunnel bioassays. The yellow peach moth, *Conogethes puncitferalis* Guenée, produces tiglic acid, which increased mating success and also attracted conspecific males (Takayoshi and Hiroshi, 1999). Teal *et al.* (1981) hypothesized that male *Heliothis virescens* F. hairpencil odors are important in mating success and female acceptance. Male sex pheromone of *Mamestra brassicae* L. also increased mate acceptance and was believed important in the recognition of conspecific sexual partners (Jacquin *et al.*, 1991). Male *Helicoverpa armigera* Hübner produce (*Z*)-11-hexadecen-1-ol (*Z*-11–16:OH), which inhibits male upwind flight in wind tunnel assays, thus preventing conspecific males from competing for a single female (Huang *et al.*, 1996).

Species isolation in heliothine moths is accomplished through selective attraction of males to conspecific female pheromone blends (Roelofs and Cardé, 1974; Tumlinson *et*

al., 1975; Tingle *et al.*, 1978; Klun *et al.*, 1979, 1980a,b; Teal *et al.*, 1981, 1984, 1986; Pope *et al.*, 1982, 1984; Vetter and Baker, 1983, 1984; Heath *et al.*, 1990; Vickers, 2002). Males are both attracted to conspecific females through reception of attractive compounds in the female pheromone blend and repelled from females of other species through the action of antagonists which inhibit upwind flight (Vickers *et al.*, 1991; Vickers and Baker, 1997; Fadamiro *et al.*, 1999; Quero and Baker, 1999). Species isolation is important in these species as forced mating under laboratory conditions resulted in sterile male hybrids between *H. virescens* and *Heliothis subflexa*, and was fatal between *Heliothis zea* and *H. virescens* (Laster, 1972; Goodpasture *et al.*, 1980).

The composition of male *H. virescens* hairpencils has been characterized by Teal and Tumlinson (1989) as a blend of 14, 16 and 18 chain saturated alcohols, acetates and carboxylic acids. Primarily: 2.7 ng tetradecanoic acid (14:OOH), 22.3 ng hexadecanol (16:OH), 22.3 ng hexadecanoic acid (16:OOH), 212.4 ng hexadecanyl acetate (16:OAc), 7.5 ng octadecanol (18:OH), 6.5 ng octadecanoic acid (18:OOH) and 14.2 ng octadecanyl acetate (18:OAc).

Two studies have investigated the potential effects of male hairpencil secretions on female *H. virescens* calling behavior. Hendricks and Shaver (1975) found that female sex pheromone emission was inhibited after exposure to a high level (50 male equivalents) of hairpencil extract in field trials. Raina and Stadelbacher (1990) also found that female *H. virescens* pheromone titer, calling behavior and receptivity to courting males was significantly reduced following mating by *H. virescens* males or backcrosses from *H. subflexa* × *H. virescens* hybrids. Each study investigated the potential effects of hairpencil secretions following a courtship encounter, but not the behavioral roles of these odors during courtship. In this study, we experimentally tested the effect of conspecific hairpencil odors on female *H. virescens* and their importance in courtship. We hypothesize that hairpencil display, and the specific chemical composition of conspecific hairpencil odor, are required for females to accept courting males.

Materials and methods

Moths

Virgin male and female *H. virescens* moths from the colony at the University of Utah were used in assays of female behavior and hairpencil extraction. Larvae were reared on a pinto bean diet (Shorey and Hale, 1965) until pupation. Pupae selected for experimentation were sexed and removed to an environmentally controlled chamber (Percival Scientific, Boone, Iowa) at 25°C, 60% relative humidity, set on a reversed light schedule (14L:10D) until eclosion. Male and female pupae were placed in cups of vermiculite (to maintain humidity) and were placed in separate environmental chambers to prevent pre-mating exposure to odors. Following eclosion, adult moths were provided access to sugar-water (92 g/l) *ad libitum* through a 1 oz. cup and a paper towel wick. Male and female moths aged 3–5 days were used in mating assays. Prior to all behavioral assays, moths were removed to the darkened room where experiments were to occur, and allowed to acclimate for 1 h. Assays were conducted between the 3rd and 5th hours of scotophase.

Behavioral assays

Observations of moth behavior were conducted in a dark room, illuminated with a single red incandescent light bulb controlled by a rheostat. Room temperature ranged from 20.6 to 22.0°C. Behavioral assays were conducted in a chamber constructed from two circular Plexiglas plates (2 mm thickness, 17.5 cm diameter, top and bottom) and steel screening for the wall (3 mm mesh size, 6 cm height; Figure 1). Three Plexiglas rods were placed on the outer edges of the cage to provide additional support, and a screw

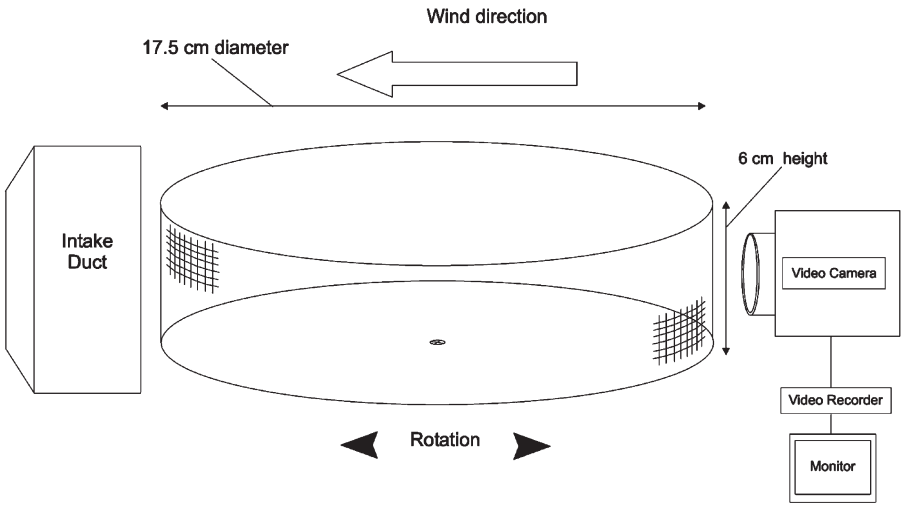

Figure 1 Assay chamber used for behavioral observations and mating trials.

was placed in the center of the bottom plate. The top of the cage was removable to exchange/add moths. The mating chamber was mounted to a bench top, 2 cm in front of an intake duct (7 × 20 cm opening). Suction air flow was generated from the duct at a rate of 0.20–0.40 m/s. On the opposite side of the assay chamber, a video camera (Panasonic© WV-BP330 CCTV camera, with vari-focal lens) was positioned to record all moth behavior. The screw-mount on the cage allowed for rotation of the cage to ensure that: (i) the female moths were upwind from males at start of courtship; and (ii) moths remained in a plane of focus to record observations.

Previous work by Teal *et al.* (1981) has documented the reproductive behavior of *H. virescens* and indicated that the males display abdominal hairpencils during courtship of the female. Mating observations were carried out on normal pairs of male and female *H. virescens* to determine if the female engaged in stereotypical behaviors which might be associated with the male hairpencil display and/or odor constituents of the hairpencils. The ethology of the courtship was documented and analyzed according to the frequency and sequence of female behaviors.

Hairpencil compounds

Synthetic hairpencil components: (14:OOH, 16:OH, 16:OAc, 16:OOH, 18:OH, 18:OAc and 18:OOH) were kept in storage at –20°C. Stock solutions were obtained from either James Tumlinson (Center for Medical, Agricultural and Veterinary Entomology, Agricultural Research Service, US Department of Agriculture Gainesville, FL), and/or purchased from Sigma Aldrich (St Louis, MO). Samples of stock solutions were confirmed at >95% purity by injection onto a Shimadzu GC 17A gas chromatograph equipped with a 30 m × 0.25 mm ID DB-5 capillary column. All solutions were diluted as a decade series to a concentration range of 10 ng/µl–10 µg/µl for each compound.

Hairpencil extracts and inter-specific discrimination

Female responses to extracts of male hairpencil were assayed independently of male presence to remove any stimuli other than odor which might influence female behavior. Hairpencil extracts from male *H. virescens*, *H. subflexa* and *H. zea* were produced by surgically removing hairpencils and placing into a volume of hexane such that one pair of hairpencils (1 ME) = 50 µl hexane (20 hairpencils/1000 µl hexane). Prior to behavioral assays, a 50 µl volume of extract (1 ME) was reduced by concentration under N_2 to a volume of 10–20 µl which was then loaded on a 1 cm diameter filter paper disc (Whatman No. 4), held by an alligator clip at the end of a wire post (23 cm total length). Hexane was allowed to evaporate from the filter paper in the fume hood. Between trials, the wire post and clip were rinsed with acetone to prevent cross-contamination of odor sources. Female responses to odors were recorded by placing a single female into the assay chamber, and placing the odor source immediately upwind (outside the cage, female facing upwind). Odor trials were conducted by exposing the odor source to the female for 30 s, removing for 30 s, repeated five consecutive times on each female (no physical contact was made between moths and the odor source). Control trials (n = 10) were conducted using 10 µl of hexane applied to filter paper. Behaviors exhibited by females during each odor stimulation were recorded, and analyzed according to frequency of response to hairpencil extracts with respect to normal female behavior during courtship. Principal behaviors recorded for females exposed to odor sources were: moving away, antennal flicking, wing fanning, ovipositor extension, abdominal extension and dragging (Table 1).

Table 1 Principal behaviors recorded from *Heliothis virescens* females during odor presentation and mating assay trials

Behavior	Description
Fly away	Female flew away from the odor source (also part of 'Move away' behavior)
Move away	Female actively avoided odor source, moving >40 mm away when exposed
Antennal flicking	Exposure to odor source increased flicking and cleaning of antenna
Wing fanning	Wing fanning was normally performed by 'calling' females which remained stationary, while vibrating the wings and exposing the ovipositor
Ovipositor extension (aka 'calling')	Exposure of the ovipositor from the abdominal tip. Included rhythmic pumping of the ovipositor in and out, or simply leaving the ovipositor exposed. This indicated that an individual female was emitting pheromone or 'calling'
Abdominal extension	The ovipositor was extruded fully and the abdomen was extended and curled to the substrate
Dragging	Exclusively followed abdominal extension. Involved extending the abdomen and ovipositor and pressing it to the substrate while walking
Abdominal curl	Different from abdominal extension, this was documented strictly as the curling of the abdomen toward the courting male, usually a precursor to clasping by the male and mating
Clasp	Successful mating indicated by clasping of male genitalia

To determine if there were behavioral effects due either to the quality of the odor blend or from a single compound in the blend, 1 ME of *H. virescens* hairpencil extract was tested and the female behavioral response compared to a synthetic blend (5 ng 14:OOH, 20 ng 16:OH, 20 ng 16:OOH, 200 ng 16:OAc, 10 ng 18:OH, 10 ng 18:OOH, 15 ng 18:OAc; Teal and Tumlinson, 1989) and the single compounds at concentrations found in the naturally occurring blend. Each compound was loaded onto a filter paper disc, and exposed in the same manner as the extract trials. Five females were tested (exposed five times each) per compound. Control trials were conducted using 10 µl of hexane.

Individual hairpencil compounds were also tested for female behavioral responses by applying 10 µl of solution to a 1 cm diameter disc filter paper. Single compounds were exposed to female *H. virescens* using the same protocols as extracted hairpencil solutions (expose for 30 s, remove for 30 s, repeat five times). Five females were tested (exposed five times each), per concentration level (10, 100, 250 and 500 ng), per compound (14:OOH, 16:OH, 16:OAc, 16:OOH, 18:OH, 18:OOH, 18:OAc), or 20 trials per odorant. Individual females were exposed to only a single odorant at a single concentration. Control trials were conducted using 10 µl of hexane applied to filter paper. Female behaviors were recorded and analyzed as in tests of hairpencil extracts according to frequency of response at various concentrations.

Hairpencil and antennal ablation assays

A series of hairpencil and antennal ablation trials were conducted to determine if odors present in male *H. virescens* hairpencils and detection by the antennae were important in mate acceptance by *H. virescens* females. Three different experiments were conducted: (i) antennectomized females with normal males; sham-operated females with normal males; (ii) hairpencil ablated males with normal females; sham-operated males with normal females; (iii) hairpencil-ablated males plus an odor source of either male *H. virescens*, *H. subflexa* or *H. zea* hairpencil extract (1 ME) with females; and hairpencil-ablated males plus a control odor source (hexane) with females. All ablation surgeries were conducted before scotophase (reversed light cycle) on the day of experimentation, and moths were returned to the environmental chamber until 1 h before the experiment (when they were removed for acclimation to room conditions).

Experiment 1: female H. virescens *antennectomy*

Antennectomy surgery consisted of excising each antenna at the scape with irridectomy scissors. Sham-operated female moths were manipulated in a similar manner to feign a 'mock surgery'. Male moths were not manipulated prior to mating assays with ablated females.

Experiment 2: male H. virescens *hairpencil ablations*

Male hairpencil surgery was conducted by gently squeezing the abdomen to expose the hairpencils, which were then trimmed to remove as much of the hairpencils (Figure 2) as possible. Mock surgery on male sham-operated moths involved squeezing of the abdomen, with no removal of hairpencils. Female moths were not manipulated.

Experiment 3: odor replacement

For odor replacement trials, male and female *H. virescens* were treated as in experiment 2. During odor replacement trials, an odor source (1 cm diameter filter paper) with 1 ME of *H. virescens*, *H. subflexa* or *H. zea* hairpencil extract was introduced upwind from the female moth while the ablated male was courting. The control for this experiment was a filter paper with only hexane applied to it.

For all courtship assays, female moths were first added to the assay chamber and observed for calling behavior. Only those females which were observed to be consistently 'calling' (stationary, with wings fanning and ovipositor

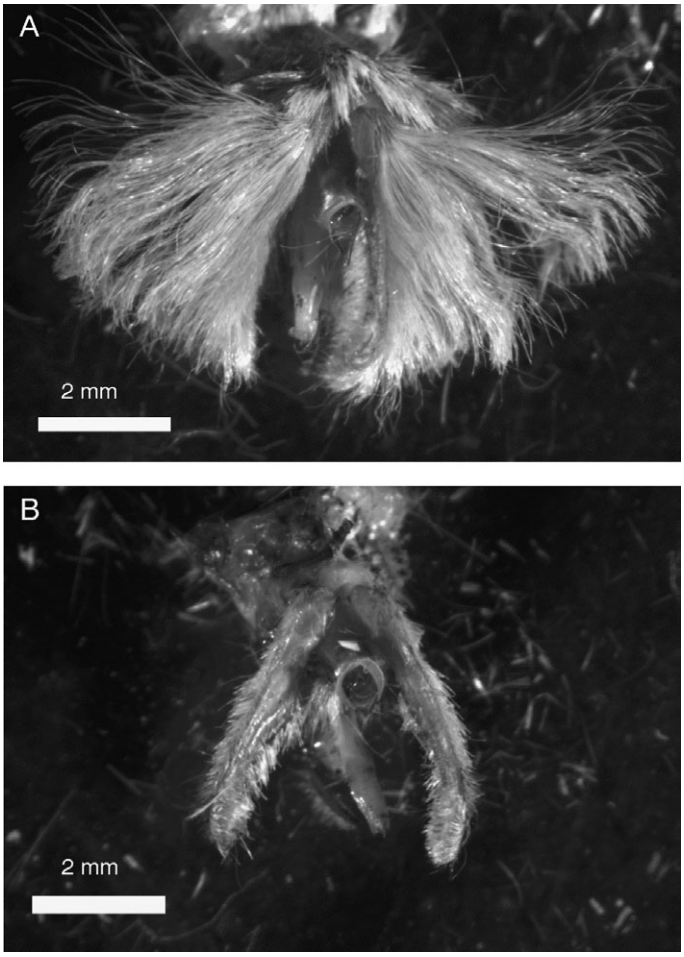

Figure 2 Male *Heliothis virescens* genitalia **(a)** before removal of hairpencils, **(b)** following removal of hairpencils.

exposed) were used for experimentation (Table 1). Calling females generally remained on the walls of the chamber, and were positioned by rotation, to the most upwind position, ventrally oriented to the video camera. Single males were then added to the downwind portion of the cage (all ablation experiments were performed only on single pairs of males and females). Behavior of moths was observed for 5 min. If, after 5 min, no interaction was observed (e.g. male unable to locate female), then the trial was ended by removing both moths. If courtship was initiated during the initial five min of observation, the trial was allowed to continue for an additional 5 min from the beginning of courtship. Behaviors of female moths were recorded in response to specific male courtship attempts, along with number of mating attempts and success/failure to copulate (as indicated by successful clasping of genitalia). For mating assays, antennal flicking was not recorded, additional female (and male) behaviors recorded during courtship were abdominal curling, and clasping (Table 1). The total number of mating attempts by each male was also recorded.

Behaviors observed in female assays of hairpencil compounds were analyzed using a one-way Analysis of Variance (ANOVA) to determine significant differences in behavioral frequency between inter-specific hairpencil extracts and hexane controls. Effects of treatment and concentration of single hairpencil compounds on female behavioral frequencies were analyzed using a two-way nested ANOVA, with concentration nested within treatment. Mating assays were analyzed by one-way ANOVA comparing treatment protocols, and differences in either behavioral frequencies or frequency of successful mating. Where an ANOVA was significant, the Fisher's least significant difference (LSD) test (Sokal and Rohlf, 1995) was used to separate means ($P < 0.05$). All analyses were conducted using a general linear model function in STATISTICA©.

Results

Hairpencil extracts and inter-specific discrimination

Sixty-six females were tested for their behavioral responses to hairpencil extracts (Figure 3). Extracts of *H. subflexa* and *H. zea* significantly increased moving away, whereas *H. virescens* extract increased abdominal extension relative to the *H. subflexa* extract and the hexane control. Flying away was only observed in response to the *H. subflexa* and *H. zea* hairpencil treatments, and occurred significantly more often in response to *H. subflexa* extract.

Comparison between singly presented hairpencil compounds at the concentrations in the male hairpencil blend,

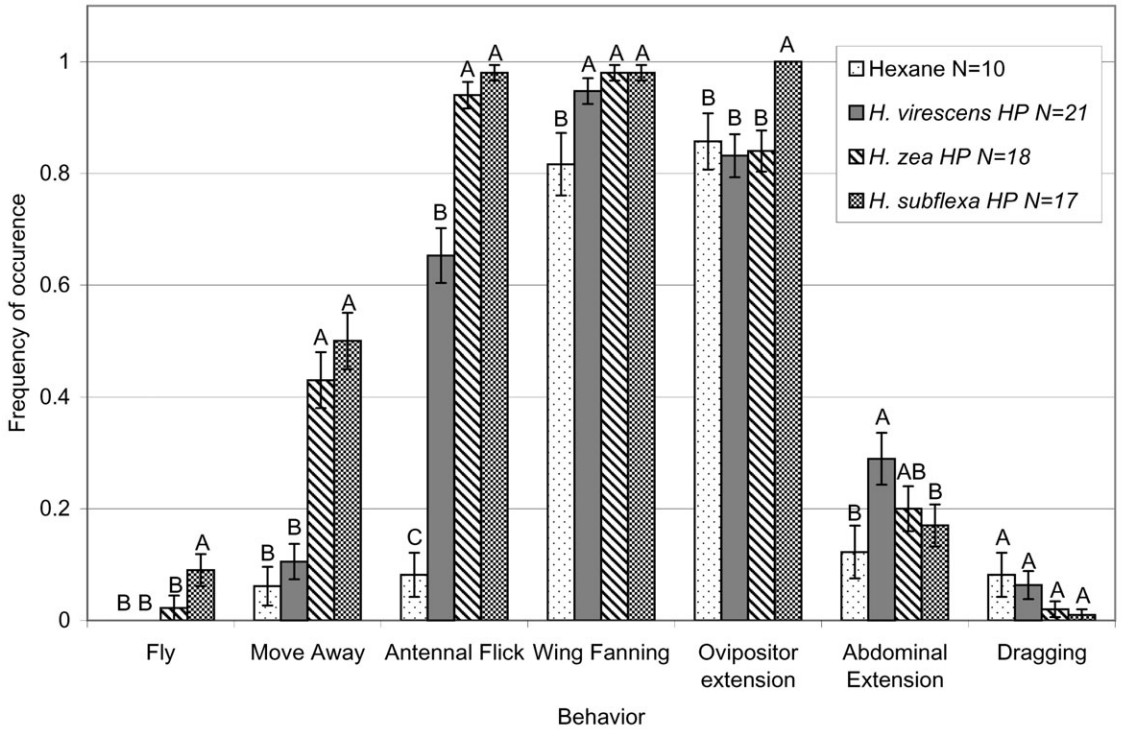

Figure 3 Occurrence of female *Heliothis virescens* behavioral responses during exposure to filter papers loaded with one male equivalent of conspecific (*H. virescens*) and inter-specific (*Helicoverpa zea* and *Heliothis subflexa*) hairpencil extracts. Control trials were conducted using hexane. Moving away significantly increased to extracts of *H. subflexa* and *H. zea* [$F(3,341) = 22.0$, $P < 0.001$] and abdominal extension increased to the *H. virescens* extract [$F(3,341) = 2.6$, $P < 0.05$]. The *H. subflexa* extract produced significantly more flying away [$F(3,341) = 3.6$, $P < 0.01$]. Means with no letters in common are significantly different from other extracts for a specific behavior.

and synthetic or natural blends indicated no differences between behavioral responses to a synthetic blend versus a 1 ME hairpencil extract (Table 2). Antennal flicking was reduced in all treatments involving compounds with a carboxylic acid terminal moiety, possibly due to the low volatility and concentrations of these odorants ($P < 0.001$). Both the male hairpencil extract and synthetic hairpencil blend produced a significant increase in abdominal extension ($P < 0.001$).

Trials on female responses to single hairpencil compounds compared occurrence of behaviors across all hairpencil compounds and between concentrations of compounds. For all odorants tested, there were significantly different behavioral responses ($P < 0.05$) from the hexane control, however this was not evident at all concentrations, nor was the effect necessarily concentration-dependent (Table 3). Abdominal extension was significantly increased in frequency with elevated concentrations of all compounds except 18:OAc. Dragging was only slightly increased in all treatments while ovipositor extension was significantly more frequent in the 14:OOH, 16:OAc and 18:OAc treatments relative to the hexane control. Octadecanyl acetate (18:OAc) significantly increased moving away from the odor source relative to other odorants which were not significantly different from the hexane control. Overall, concentration-dependent effects were found to significantly affect antennal flicking, wing fanning, abdominal extension and dragging (Table 3).

Hairpencil and antennal ablation assays

Antennectomized females mated significantly less frequently (33 ± 7.1% successful mating, $n = 45$) than sham-operated females (77 ± 7.6%, $n = 30$) when presented with normal male *H. virescens* [$F(1,74) = 17.1$, $P < 0.001$]. In antennectomized females, the frequency of abdominal extension and curling were significantly reduced, while moving away and flying significantly increased (Figure 4A). Overall mating attempts by males were similar between sham-operated (2.9 ± 0.21 attempts per 5 min trial) and antennectomized (3.0 ± 0.13 attempts per 5 min trial) treatments.

Mating was also significantly reduced (5 ± 4.7%) in females presented with a hairpencil-ablated male ($n = 25$) versus those presented with a sham-operated male [$n = 35$; 75 ± 6.9%; $F(1,59) = 47.4$, $P < 0.001$]. For females presented with ablated-hairpencil males, frequencies of ovipositor extension, abdominal extension, abdominal curling and clasping were reduced, whereas moving away and flying away were significantly higher relative to sham-operated males (Figure 4B). Overall mating attempts by males were similar between sham-operated (3.0 ± 0.19 attempts per 5 min trial) and hairpencil ablation (2.9 ± 0.18 attempts per 5 min trial) treatments.

Successful mating was significantly affected by treatment in odor replacement trials (Figure 5). *Heliothis subflexa* and *H. zea* treatments did not improve mating success of hairpencil-ablated males more (5 ± 3.5%) than hexane. Presentation of the *H. virescens* hairpencil extract increased mating significantly, to 44 ± 12.8% (or 54% recovery relative to the

Table 2 Female *Heliothis virescens* behavioral responses to either filter papers loaded with compounds at concentrations found within one male equivalent (ME) of male *H. virescens* hairpencil extract (Teal and Tumlinson, 1989), 1 ME of a synthetic hairpencil blend, or 1 ME of hairpencil extracted in hexane

Treatment	Frequency of observed behaviors (±SEM)					
	Move away	Antennal flicking	Wing fanning	Ovipositor extension	Abdominal extension	Dragging
F-values	$F(9,265) = 2.6$, $P < 0.05$	$F(9, 265) = 14.7$, $P < 0.001$	$F(9,265) = 0.90$, $P > 0.05$	$F(9,265) = 4.0$, $P < 0.001$	$F(9,265) = 4.4$, $P < 0.001$	$F(9,265) = 1.0$, $P > 0.05$
10 µl Hexane	0.14 (0.049)BC	0.22 (0.059)D	1.00 (0.000)A	1.00 (0.000)A	0.06 (0.034)D	0.00 (0.00)A
5 ng 14:OOH	0.04 (0.040)B	0.76 (0.087)B	1.00 (0.000)A	1.00 (0.000)A	0.20 (0.082)CD	0.00 (0.00)A
200 ng 16:OAc	0.08 (0.055)BC	0.92 (0.055)AB	0.96 (0.040)A	1.00 (0.000)A	0.12 (0.066)D	0.00 (0.00)A
20 ng 16:OH	0.44 (0.101)A	0.96 (0.040)A	1.00 (0.000)A	0.96 (0.040)A	0.28 (0.092)ABC	0.04 (0.04)A
20 ng 16:OOH	0.16 (0.075)BC	0.40 (0.100)C	1.00 (0.000)A	1.00 (0.000)A	0.12 (0.067)D	0.04 (0.040)A
15 ng 18:OAc	0.28 (0.092)C	0.80 (0.081)B	0.96 (0.040)A	1.00 (0.000)A	0.04 (0.040)D	0.00 (0.00)A
10 ng 18:OH	0.12 (0.066)BC	0.56 (0.101)C	1.00 (0.000)A	0.84 (0.074)B	0.28 (0.095)BC	0.04 (0.040)A
10 ng 18:OOH	0.20 (0.082)BC	0.44 (0.101)C	1.00 (0.000)A	1.00 (0.000)A	0.16 (0.075)CD	0.00 (0.00)A
Synthetic blend	0.08 (0.055)BC	0.96 (0.040)A	1.00 (0.000)A	1.00 (0.000)A	0.44 (0.101)AB	0.08 (0.055)A
1 ME hairpencil extract	0.16 (0.075)BC	0.88 (0.066)AB	0.96 (0.040)A	1.00 (0.000)A	0.48 (0.102)A	0.04 (0.040)A

$n = 5$ females tested per compound and concentration (each female was exposed to a single compound five times during a trial). Means which are followed by different letters are significantly different from other treatments for a given behavior.

Table 3 Female *Heliothis virescens* behavioral responses to filter paper discs loaded with four concentrations (10–500 ng) of seven individual hairpencil compounds

Compound	Amount (ng)	Frequency of observed behaviors (±SEM)					
		Move away	Antennal flicking	Wing fanning	Ovipositor extension	Abdominal extension	Dragging
14:OOH	10	0.12 (0.066)A	0.44 (0.101)A	1.00 (0.000)A	1.00 (0.000)A	0.08 (0.055)A	0.00 (0.000)A
	100	0.20 (0.082)A	0.64 (0.098)A	0.88 (0.066)A	1.00 (0.000)A	0.12 (0.066)A	0.00 (0.000)A
	250	0.12 (0.066)A	0.68 (0.095)AB	1.00 (0.000)A	1.00 (0.000)A	0.12 (0.066)A	0.00 (0.000)A
	500	0.20 (0.082)A	0.84 (0.075)B	0.96 (0.040)A	1.00 (0.000)A	0.24 (0.087)A	0.00 (0.000)A
16:OAc	10	0.16 (0.075)A	0.20 (0.082)A	0.56 (0.101)A	0.80 (0.082)A	0.36 (0.098)A	0.12 (0.066)A
	100	0.12 (0.066)A	0.88 (0.066)B	0.88 (0.066)B	0.92 (0.055)AB	0.48 (0.102)AB	0.08 (0.055)A
	250	0.08 (0.055)A	0.92 (0.055)B	0.96 (0.040)B	1.00 (0.000)B	0.56 (0.101)AB	0.12 (0.066)A
	500	0.12 (0.066)A	1.00 (0.000)B	0.84 (0.074)B	0.92 (0.055)AB	0.68 (0.0/96)B	0.12 (0.066)A
16:OH	10	0.16 (0.075)A	0.92 (0.055)A	1.00 (0.000)A	0.76 (0.087)AB	0.36 (0.098)A	0.16 (0.075)A
	100	0.16 (0.075)A	0.00 (0.000)B	0.92 (0.055)A	0.80 (0.082)A	0.04 (0.040)B	0.00 (0.000)B
	250	0.20 (0.082)A	0.04 (0.040)B	0.92 (0.055)A	0.76 (0.087)AB	0.48 (0.102)A	0.24 (0.087)A
	500	0.08 (0.055)A	0.60 (0.100)C	0.96 (0.040)A	0.60 (0.100)B	0.44 (0.101)A	0.12 (0.067)A
16:OOH	10	0.04 (0.040)A	0.92 (0.055)A	0.92 (0.055)A	0.96 (0.040)A	0.24 (0.087)B	0.04 (0.040)A
	100	0.04 (0.040)A	0.32 (0.095)B	0.80 (0.082)AB	0.68 (0.095)B	0.36 (0.098)AB	0.20 (0.082)AB
	250	0.04 (0.040)A	0.00 (0.000)C	0.72 (0.092)B	0.80 (0.082)AB	0.56 (0.101)B	0.24 (0.087)B
	500	0.20 (0.082)A	0.88 (0.066)A	0.92 (0.055)A	0.80 (0.082)AB	0.52 (0.102)B	0.12 (0.066)AB
18:OAc	10	0.24 (0.087)A	0.92 (0.055)A	1.00 (0.000)A	1.00 (0.000)A	0.08 (0.055)A	0.00 (0.000)A
	100	0.32 (0.095)AB	0.68 (0.095)B	0.96 (0.040)A	1.00 (0.000)A	0.32 (0.095)B	0.08 (0.055)A
	250	0.44 (0.101)BC	0.84 (0.075)AB	1.00 (0.000)A	1.00 (0.000)A	0.12 (0.066)AB	0.04 (0.040)A
	500	0.52 (0.102)C	0.80 (0.082)AB	1.00 (0.000)A	1.00 (0.000)A	0.20 (0.082)AB	0.08 (0.055)A
18:OH	10	0.12 (0.066)A	0.92 (0.055)A	1.00 (0.000)A	0.84 (0.075)A	0.32 (0.095)A	0.04 (0.040)A
	100	0.04 (0.040)A	0.44 (0.101)B	0.76 (0.087)B	0.68 (0.095)AB	0.44 (0.101)AB	0.28 (0.092)B
	250	0.12 (0.066)A	0.04 (0.040)C	0.64 (0.098)B	0.80 (0.082)AB	0.36 (0.097)A	0.24 (0.087)B
	500	0.08 (0.055)A	0.88 (0.066)A	1.00 (0.000)A	0.64 (0.098)B	0.64 (0.097)B	0.16 (0.075)AB
18:OOH	10	0.00 (0.000)A	0.80 (0.082)A	0.76 (0.087)A	0.88 (0.067)A	0.12 (0.066)A	0.00 (0.000)A
	100	0.08 (0.055)A	0.00 (0.000)B	0.68 (0.095)A	0.76 (0.087)A	0.20 (0.082)A	0.00 (0.000)A
	250	0.04 (0.040)A	0.68 (0.095)A	0.80 (0.082)A	0.80 (0.082)A	0.52 (0.102)B	0.08 (0.055)A
	500	0.04 (0.040)A	0.84 (0.075)A	1.00 (0.000)B	0.88 (0.067)A	0.88 (0.066)C	0.36 (0.098)B
Hexane	–	0.11 (0.032)	0.11 (0.032)	0.79 (0.041)	0.84 (0.038)	0.09 (0.029)	0.04 (0.021)

Control trials were conducted using hexane. $n = 5$ females tested per compound and concentration (each female was exposed to a single compound five times during a trial = 25 trials per concentration, 100 trials per odorant). Means which are followed by different letters are significantly different ($P < 0.05$) from other concentrations for a specific compound.

sham-operated males). For behavioral responses, moving away was significantly increased for the hexane control, and reduced for *H. virescens* extract, relative to pairing between sham-operated males and females (Figure 6). Females did not fly away in *H. virescens* or sham-operated male treatments. Abdominal curling and clasping were significantly reduced in hexane, *H. subflexa* and *H. zea* treatments, relative to the sham-operated pairs and the *H. virescens* extract treatment (Figure 6). Abdominal extension was reduced in the hexane and *H. subflexa* treatments and dragging behavior was greatest to the hexane, followed by the pairing between sham-operated males and females, *H. zea* and

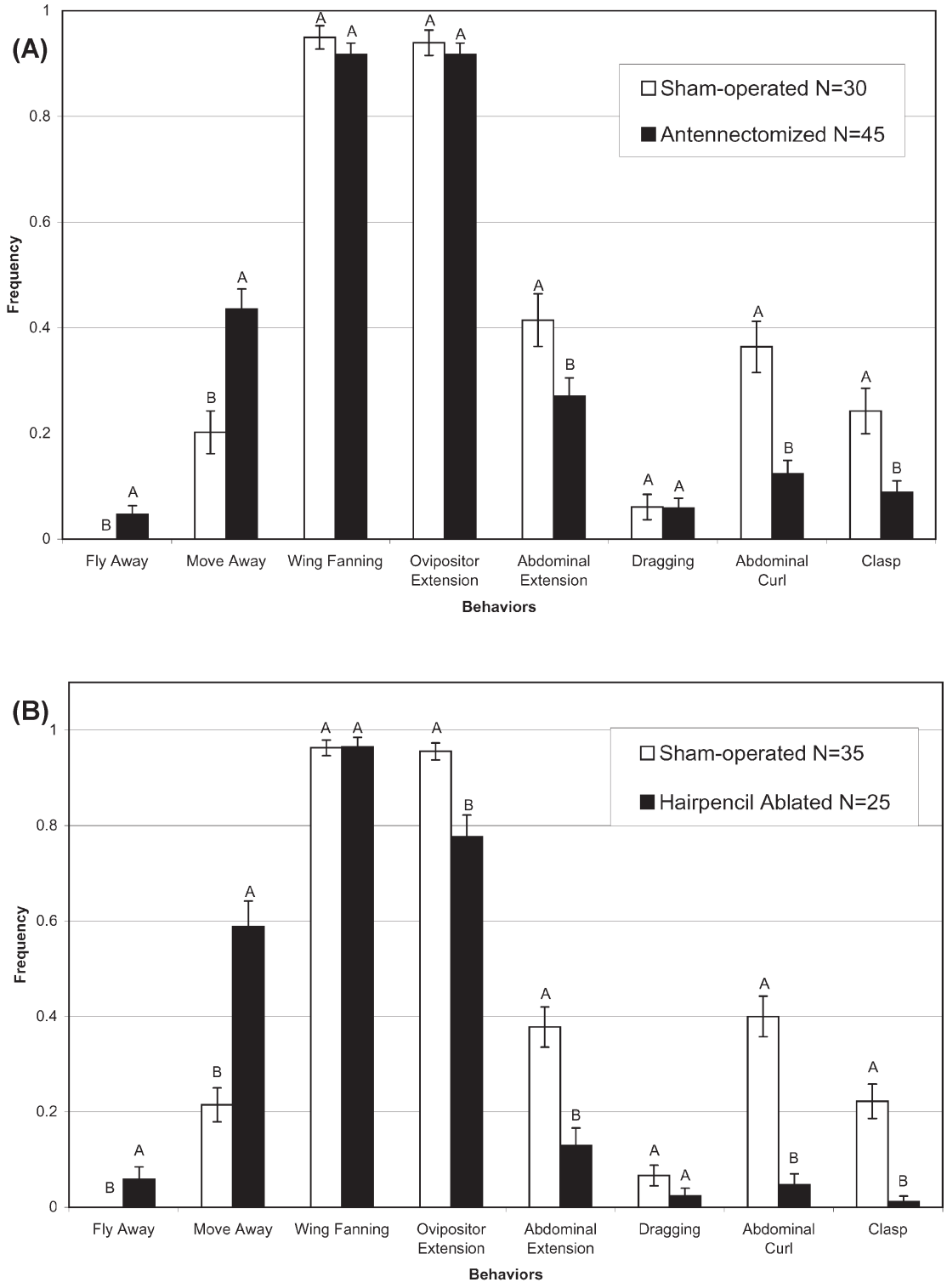

Figure 4 Female *Heliothis virescens* behavioral responses during mating trials with male *H. virescens*: **(a)** comparison between sham-operated ($n = 30$) and antennectomized females ($n = 45$) and **(b)** comparison between females exposed to males with either sham-operated ($n = 35$) or ablated ($n = 25$) hairpencils. For antennectomized females, frequency of both abdominal extension [$F(1,74) = 6.4$, $P < 0.001$] and abdominal curl [$F(1,74) = 16.9$, $P < 0.001$] were significantly reduced, while moving away [$F(1,74) = 10.5$, $P < 0.001$] and flying away [$F(1,74) = 2.5$, $P < 0.01$] significantly increased. Frequencies of ovipositor extension [$F(1,59) = 17.8$, $P < 0.001$], abdominal extension [$F(1,59) = 17.0$, $P < 0.001$], abdominal curling [$F(1,59) = 39.1$, $P < 0.001$] and clasping [$F(1,59) = 20.7$, $P < 0.001$] were reduced, whereas moving away [$F(1,59) = 36.6$, $P < 0.001$] and flying away [$F(1,59) = 8.4$, $P < 0.01$] were significantly higher for females paired with hairpencil-ablated males relative to sham-operated males. Means with no letters in common are significantly different from other extracts for a specific behavior.

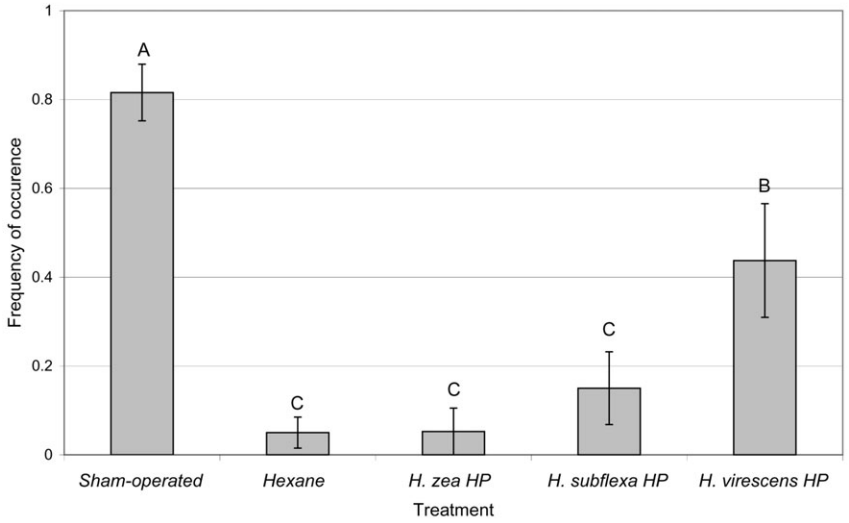

Figure 5 Frequency of mate acceptance by female *Heliothis virescens* exposed to males with either sham-operated hairpencils ($n = 38$) or ablated hairpencils plus a filter paper odor source loaded with 1 ME hairpencil extracts of either *Helicoverpa zea* ($n = 19$), *Heliothis subflexa* ($n = 20$) or *H. virescens* ($n = 20$). Control trials were conducted using hexane only [$n = 42$; $F(4,128) = 89.1$, $P < 0.001$]. Means with no letters in common are significantly different from other extracts for a specific behavior.

H. subflexa treatments (Figure 6). Male mating attempts were significantly increased in the *H. zea* treatment, and the *H. virescens* treatment showed significantly fewer attempts than the sham-operated males, *H. zea* and *H. subflexa* treatments (Figure 7).

Discussion

Female *H. virescens* use species-specific male hairpencil odors as cues for mate acceptance. No single compound alone appears essential in producing behavioral responses, however blend specificity was important, as females discriminated *H. virescens* hairpencil extracts from *H. zea* and *H. subflexa*.

Hairpencil extracts and inter-specific discrimination

Assays of female behavioral responses to conspecific and inter-specific hairpencil extracts indicated that females can discriminate between hairpencil extracts. While all odorants elicited an increase in antennal flicking, *H. virescens* extract increased abdominal extension (preliminary trials showed that females will often extend the abdomen to the substrate when being courted by conspecific males, possibly scent marking with their own pheromone, indicating sexual excitement or readiness to mate; Teal *et al.*, 1981) and both *H. zea* and *H. subflexa* extracts caused females to move or fly away from the odor source. This provides evidence that female *H. virescens* can discriminate between hairpencil extracts of other related heliothines, and that hairpencil odors may act to: (i) produce quiescence or make females receptive to mating; and (ii) distinguish appropriate suitors (conspecifics) from other species. Comparisons between blends (synthetic or extracts) and single compounds support the fact that the odorant blend significantly affects female behavior. Odor quality may therefore be used as a means of mate choice, with females accepting males with a highly specific blend of hairpencil compounds.

Analysis of the effects of single compounds indicated that 18:OAc frequently caused females to move away. This effect was concentration-dependent, increasing in occurrence to 53% of females at the 500 ng concentration. This compound occurs in the *H. virescens* hairpencil blend in a low concentration (6.5 ng) and females may use this compound as a means of distinguishing mates—either good quality males, or simply conspecifics (Teal and Tumlinson, 1989). Antennal flicking was increased in most treatments, relative to hexane controls, likely indicating that females detected a given odorant. Antennal movement has been quantified as a reliable behavioral response to female sex pheromone in *Periplaneta americana* L. (Okada *et al.*, 1990) and also in assays of female *H. virescens* responses to oviposition stimuli (Ramaswamy, 1990). Wing fanning and ovipositor extension were monitored as indications of female 'calling' or pheromone release. Overall, no treatments reduced either wing fanning or ovipositor extension, indicating that none of the single hairpencil compounds tested inhibited female calling at the concentrations tested in this study, as reported by Hendricks and Shaver (1975). Abdominal extension was increased in response to all single compounds, except 18:OAc. This effect was concentration-dependent, increasing in frequency with increasing concentrations of 16:OOH, 16:OAc, 18:OH and 18:OOH. Dragging was also increased in most treatments, relative to hexane, perhaps indicating increased scent-marking, due to the apparent presence of a male. Several compounds elicited abdominal extension and it remains unclear which single compounds are most important to female *H. virescens*. Since trials on hairpencil extracts

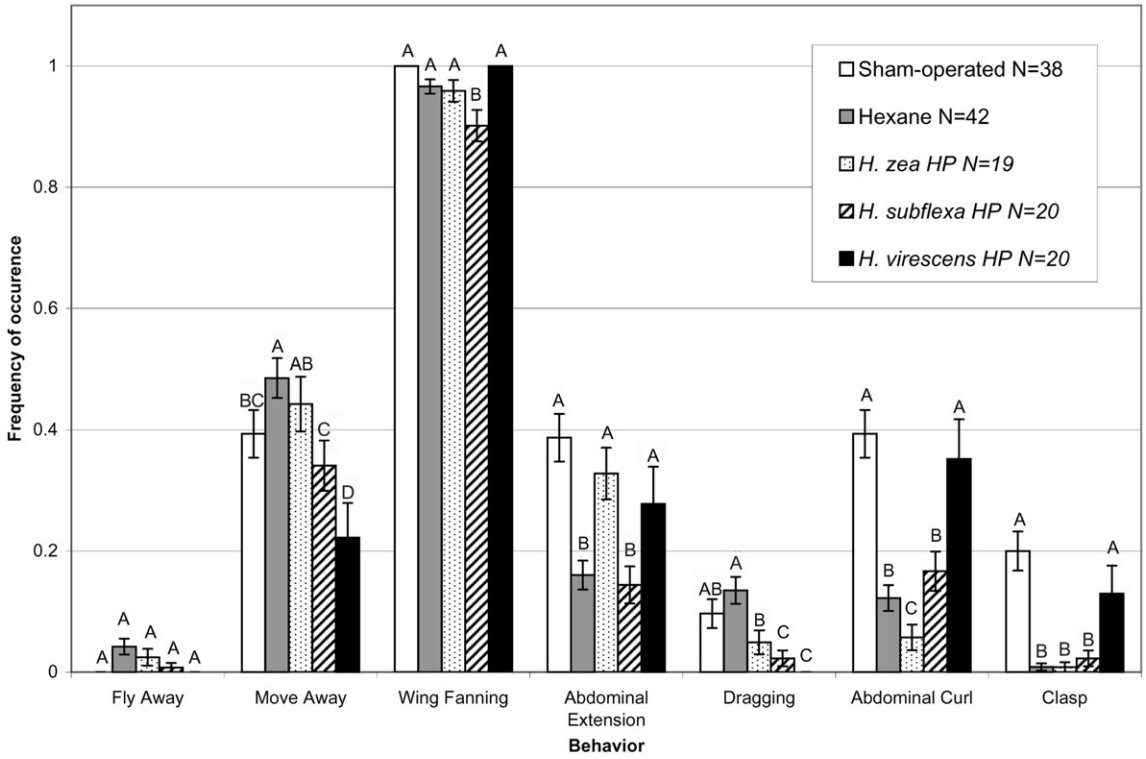

Figure 6 Behavioral responses of female *Heliothis virescens* exposed to males with either sham-operated hairpencils ($n = 38$) or ablated hairpencils plus a filter paper odor source loaded with 1 ME hairpencil extracts of *Heliothis subflexa* ($n = 20$), *Helicoverpa zea* ($n = 19$) or *H. virescens* ($n = 20$). Control trials were conducted using hexane only ($n = 42$). Moving away was increased with the hexane blank, and reduced for *H. virescens* extract [$F(4,695) = 4.3$, $P < 0.01$]. Wing fanning was reduced in *H. subflexa* treatments [$F(4,695) = 5.6$, $P < 0.001$] and abdominal curling [$F(4,695) = 19.4$, $P < 0.001$] and clasping [$F(4,695) = 20.9$, $P < 0.001$] were significantly reduced in hexane, *H. subflexa* and *H. zea* treatments. Abdominal extension was reduced in the hexane and *H. subflexa* treatments [$F(4,695) = 10.0$, $P < 0.001$] and dragging was greatest to the hexane, followed by the pairings with sham-operated males, *H. zea* and *H. subflexa* treatments [$F(4,695) = 5.7$, $P < 0.001$]. Means with no letters in common are significantly different from other extracts for a specific behavior.

and blends have already demonstrated that the presence of these single compounds is insufficient to cause a significant increase abdominal extension, it appears that odor quality is again important in female behavioral responses. While individual odorants (at concentrations of 10–500 ng) may elicit a high frequency of abdominal extension, the simultaneous presentation of odorants in a blend may reduce this. Concentrations of individual compounds were considerably lower in extract and blend trials, perhaps also reducing the effect of single compounds within blends tested. One of several single compounds are sufficient to produce abdominal extension in females, however the specificity of female behavior between species extracts must depend on the ratio of compounds in that species' blend.

Discriminatory responses observed in extracts also appear blend-enhanced, producing significant frequencies of females moving or flying away from the odor source. Thus the potential for mate acceptance and reproductive isolation (by escape from incorrect 'smelling' males) for female *H. virescens* is likely found within the mixture of compounds in male hairpencils, and cannot be attributed to single compounds producing specific behaviors. The importance of blend ratios in hairpencil activity has not been demonstrated previously in heliothine moths. In studies on *Acrolepiopsis assectella* Zeller, Thibout *et al.* (1994) found that four hairpencil compounds were important in eliciting female acceptance posture, and that synergism was not produced by blending compounds. Whereas it is not clear in this study if hairpencil compounds act synergistically on *H. virescens* females, the presence of one or more compounds in a blend appears to be important. Future research will investigate the role of blends in female behavioral and electrophysiological responses.

Hairpencil and antennal ablation assays

Experiments on hairpencil extracts and single compounds with individual females provide evidence that female *H. virescens* can detect and behaviorally respond to hairpencil odors, and discriminate between closely related species, apparently based on blend characteristics. Is the detection of these odors important in mating behavior? Removal of either the female antennae or male hairpencils resulted in a significant decrease in successful mating, despite the number of mating attempts between pairings with sham-operated males and experimental treatments being similar. Females which were unable to detect hair-

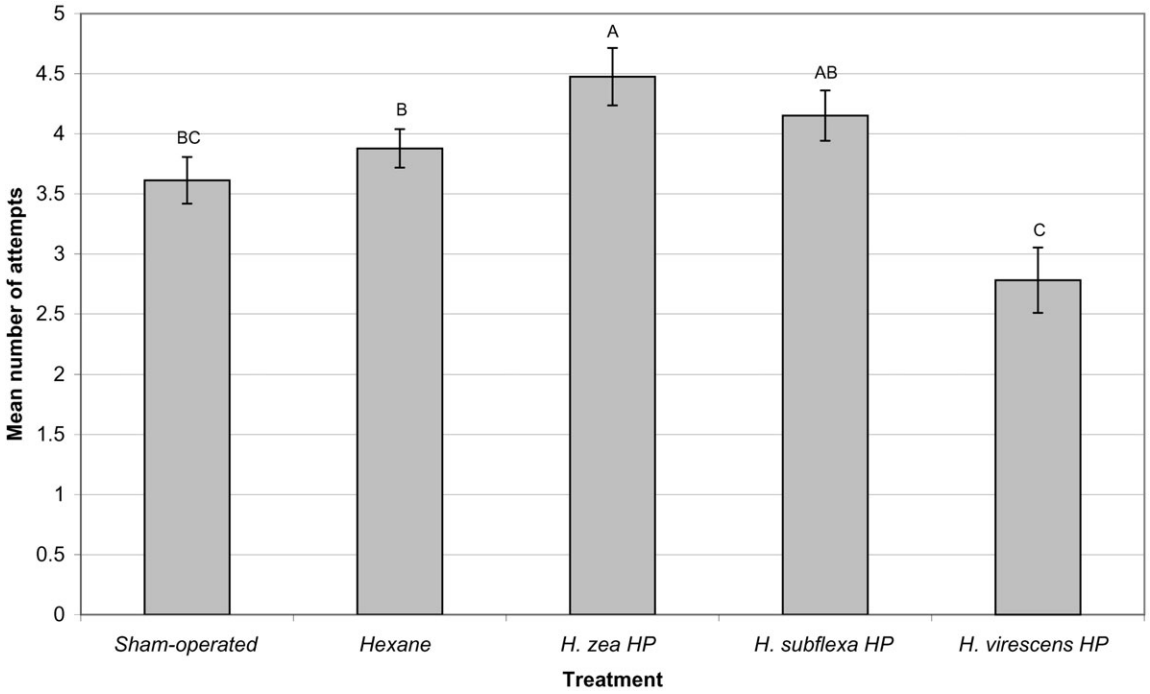

Figure 7 Mean number of mating attempts between *Heliothis virescens* females and males with either sham-operated hairpencils ($n = 38$) or ablated hairpencils plus a filter paper odor source loaded with 1 ME hairpencil extract of either *Heliothis subflexa* ($n = 20$), *Helicoverpa zea* ($n = 19$) or *H. virescens* ($n = 20$). Control trials were conducted using hexane only ($n = 42$). Significantly fewer attempts were made in the *H. virescens* treatment than the sham-operated males, *H. zea* or *H. subflexa* treatments [$F(4,695) = 4.2$, $P < 0.001$]. Means which are followed by different letters are significantly different.

pencil emissions from courting males were much less likely to present their genitalia by curling or extending the abdomen, and much more likely to move and fly away from the male. This suggests that male *H. virescens* hairpencil odors prevent females from moving away (arrestment), and also act to make females more receptive to mating (aphrodisiac).

To further confirm the effect of hairpencil odors on female mate acceptance, trials were conducted using hairpencil-ablated males, with odors introduced on filter papers loaded with either conspecific or inter-specific hairpencil extracts. As in the hairpencil extract trials, females responded specifically to the presence of the *H. virescens* hairpencil extract which restored mating between ablated males and normal females. Hairpencil extracts of *H. subflexa* and *H. zea* did not restore mating between pairs of *H. virescens*. Particular behavioral responses to *H. virescens* extract which were increased (to the same level as sham-operated pairs) were abdominal extension, abdominal curling and genital clasping (successful mating). Moving and flying away were reduced relative to the hexane control and other species' extracts. The exhibition of these female behaviors in response to either the presence or absence of *H. virescens* hairpencil odor agrees with results from extract and ablation experiments, indicating that: (i) hairpencil odors produce specific behavioral responses in female *H. virescens*; (ii) in the absence of the conspecific male blend, or in the presence of an inter-specific hairpencil extract, females will move away from a courting male (or odor source); (iii) female behavioral responses to hairpencil odors are important in mating, with females mating in the presence of the conspecific hairpencil blend, and rejecting males in the absence of the conspecific male blend, or in the presence of an inter-specific hairpencil extract.

Also evident from odor replacement trials is that male courting is significantly lower in the presence of 1 ME of *H. virescens* hairpencil extract than with *H. zea*, *H. subflexa*, or a hexane control. It is therefore possible that *H. virescens* males are inhibited from courting or approach from a distance by conspecific hairpencil odors. Future studies using multiple, competing males, or female mate choice assays with *H. virescens* will test this effect.

Hairpencil odors produce several effects in *H. virescens*. Reproductive isolation may be achieved by female escape in the presence of an incorrect species' blend. In the presence of a conspecific hairpencil extract, females become more quiescent, engage in mate acceptance behavior and are more likely to mate with courting males. Finally, hairpencil secretions may be used by males as a means of repelling other competing conspecifics during courtship.

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