Male and Female Antennal Responses in *Heliothis virescens* and *H. subflexa* to Conspecific and Heterospecific Sex Pheromone Compounds

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ABSTRACT To determine whether *Heliothis virescens* and *H. subflexa*, two closely related sympatrically occurring species, differ in their antennal responses to conspecific and heterospecific pheromone compounds, we recorded electroantennogram (EAG) responses of male and female antennae of both species to eight different compounds loaded on filter paper dispensers. If antennal responses were found to differ in the two species, EAG-recordings from F_1 hybrids and backcrosses between these species could be used in developing an understanding of the genetic architecture of variation in olfactory signal perception. However, all compounds elicited EAG responses in all male antennae tested, and no quantitative differences in response were found between the two species, except for the response to 1 mg (Z)-11-hexadecenol (Z11–16:OH), which elicited larger EAG responses in *H. subflexa* than in *H. virescens* males. This difference is consistent with the idea that this pheromone component is less important in the biology of *H. virescens*. Female antennae of both species were less responsive to the major sex pheromone compound, (Z)-11-hexadecenal (Z11–16:Ald), than male antennae; 10 μg Z11–16:Ald, which elicited strong EAG responses in males, produced female EAGs similar to control puffs of air. However, higher doses of Z11–16:Ald elicited significant EAG responses in female antennae of both species. Female antennae of both species also responded to most other pheromone compounds, except Z11–16:OH. These results support the hypothesis that autodetection of sex pheromones occurs in females of both *H. virescens* and *H. subflexa*. Whether females behaviorally respond to any, or to combinations, of these compounds remains to be elucidated.

KEY WORDS *Heliothis virescens*, *Heliothis subflexa*, electroantennogram, species specificity, female autodetection

IN THE UNITED STATES, three heliothine species cooccur—*Helicoverpa zea* (Boddie, 1850) (*Hz*), *Heliothis virescens* (Fabricius, 1777) (*Hv*), and *Heliothis subflexa* (Guenée, 1852) (*Hs*)—of which the latter two are closely related (Cho et al. 1995, Fang et al. 1997). The three species have sexual communication systems that differ from each other through a combination of differences in the secondary pheromone components and kairomonal inhibitory compounds. In short, the main sex pheromone component for all three species is (Z)-11-hexadecenal (Z11–16:Ald). The main secondary pheromone component of both *Hz* and *Hs* is Z9–16:Ald (Klun et al. 1979, 1980b, 1982, Teal et al. 1981, Tumlinson et al. 1982, Vetter and Baker 1984, Vickers 2002), whereas for *Hv*, the main secondary component is Z9–14:Ald (Roelofs et al. 1974, Tumlinson et al. 1975, Klun et al. 1979, 1980a, Pope et al. 1982). In addition, (Z)-11-hexadecenol (Z11–16:OH) significantly increases attraction of *Hs* (Heath et al. 1990, Vickers 2002), whereas this compound inhibits attraction of *Hz* males (Teal et al. 1984, Quero and Baker 1999, Quero et al. 2001) and *Hv* males (when in concentrations >3% of the total blend; Vetter and Baker 1983). Another compound that inhibits attraction of *Hz* and *Hv* males is (Z)-11-hexadecenyl acetate (Z11–16:OAc) (Vickers and Baker 1997, Quero and Baker 1999, Quero et al. 2001). This compound, as well as Z7–16:OAc and Z9–16:OAc, is found in the glands and in volatile collections of *Hs* females (Klun et al. 1982, Heath et al. 1991), but does not seem to increase attraction of conspecific *Hs* males (Vickers 2002). Whether other compounds that are present in the pheromone glands of these heliothine females are behaviorally important in sexual communication or merely by-products of the biosynthetic pathways (Teal and Tumlinson 1986, Jurenka and Roelofs 1993) remains to be elucidated. The sympatric occurrence of

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these three species requires appropriate mate recognition, which in turn requires detection and perception of both conspecific and heterospecific pheromone compounds (Roelofs 1977).

Electroantennogram (EAG) responses provide a general measure of odorant reception at the peripheral level (e.g., Roelofs 1977, Smith and Menzel 1989, Van der Pers and Minks 1998, Park et al. 2002). Because the specificity of EAG responses of male moth antennae to conspecific pheromone components has been instrumental in pheromone identifications, EAG recordings could be a diagnostic tool to relate differences in pheromone detection to genetic differences between *Hv* and *Hs.* EAG recordings are much easier to conduct than behavioral assays or single sensillum recordings, which makes the EAG a potentially simpler tool for relating differences in males' ability to detect conspecific and heterospecific compounds to genotypic differences.

Differences in EAG responses have been recorded between races of moth species (Fescemeyer and Hanson 1990, El-Sayed et al. 2003). Fescemeyer and Hanson (1990) found greater EAG responses in *ZZ*-males of *Ostrinia nubilalis* (Hübner, 1796) to the *Z*-isomer than to the *E*-isomer of 11-tetradecenyl acetate. Similarly, El-Sayed et al. (2003) found a higher EAG sensitivity (measured as smaller intercepts in the EAG concentration-response relationship) to Z11–14:Ald in *Choristoneura rosaceana* (Harris) males from British Columbia compared with males from Michigan and New York, which coincided with a higher relative amount of this compound in the pheromone glands of females from British Columbia.

EAG responses have been recorded from male antennae of *Hz* (Christensen et al. 1990, Park et al. 2002) and *Hv* (Almaas and Mustaparta 1990, Park et al. 2002), but these studies were not specifically focused on the differences between their sex pheromone components. To our knowledge, no EAG recordings have been conducted on *Hs* antennae. If there are species-specific EAG responses in *Hs* and *Hv* antennae, *Hs* males may show higher EAG responses to their species-specific C_{16} acetates and Z9–16:Ald, whereas *Hv* males may show higher EAG responses to Z9–14:Ald. In this study, we tested whether *Hv* and *Hs* male antennae showed species-specific EAG responses to the conspecific and heterospecific pheromone components that have shown to be behaviorally important in the sexual communication of these species.

We also recorded EAG responses of female antennae to determine whether EAG responses to conspecific and heterospecific sex pheromone components were species- and/or sex-specific. In general, olfactory antennal sensilla in female moths are thought to function mostly to perceive plant compounds (Ljungberg et al. 1993, Callahan et al. 2000, Rostelien et al. 2000, Burguiere et al. 2001), because females need to find suitable oviposition sites. Receptor neurons that respond to plant compounds generally do not respond to pheromone compounds (Almaas and Mustaparta 1991, Anton and Hansson 1994). Nevertheless, females may also perceive their own or other species' sex pheromones. This has been shown for several lepidopteran species (reviewed in Schneider et al. 1998, Pearson and Schal 1999), including the noctuids *Spodoptera littoralis* (Boisduval, 1833) (Ljungberg et al. 1993, Ochieng et al. 1995), *Trichoplusia ni* (Hübner, 1803) (Seabrook et al. 1987), and *Hv* (Almaas and Mustaparta 1990). However, the response of *Hv* female antennae to Z11–16:Ald and Z9–14:Ald were 100–10000-fold lower than the response of *Hv* male antennae (Almaas and Mustaparta 1991). *Hz* females did not seem to respond to female pheromone components (Christensen et al. 1990). While female heliothine pheromones attract males at long range, male-produced sex pheromones may be important during courtship. The male hairpencils of *Hv* are important in courtship behavior and mate acceptance by female *Hv* (Teal and Tumlinson 1989, Hillier and Vickers 2004). Because we included female antennae in our studies, we added the major component that is released by the hairpencils of *Hv* males, 16:OAc, to the series of compounds that were tested.

Materials and Methods

Moths. *Hv* and *Hs* were from laboratory colonies reared on artificial diet as described in Sheck and Gould (1993, 1995). Neonate larvae were reared in individual cups, from which pupae were removed, separated by sex, and placed in a room with a reversed light cycle (14 L:10 D, lights off from 0400 to 1400 hours). Newly eclosed adult males and females were collected daily and placed in separate plastic containers (diameter 11 cm, height 8 cm) with sugar water. The antennae used were of 3- to 7-d-old *Hs* males (n = 11) and 1- to 10-d-old *Hv* males (n = 17), *Hs* females (n = 11), and *Hv* females (n = 10).

EAGs. The EAG-setup used here is the same as described by Gemeno et al. (2003), with slight modifications. Males and females were anesthetized with a brief pulse of CO_2 and one antenna was excised with fine forceps. The proximal end of the antenna was placed in the narrow end of a Pasteur pipette, while the distal end was placed in a second glass capillary. Ag–AgCl wires, 0.5 mm diameter, connected the saline-filled capillaries to a Grass P-16 amplifier (Astro-Med, West Warwick, RI) with coaxial wire and BNC connectors. The antenna was introduced into a 1-cm-diameter glass tube, which carried clean humidified air continuously over the antenna at 1.5 liters/min. The test sample was delivered through a rubber septum at the end of a lateral branch of the air delivery tube, 8 cm upwind from the antenna. The signal was acquired through an A/D board installed in a HP5890 GC and recorded and analyzed with ChemStation software (Agilent Technologies, Palo Alto, CA).

Stimuli. Pheromone components were obtained from *PHERO*BANK (Wageningen, The Netherlands), Shin-Etsu Chemical (Tokyo, Japan), and Bedoukian Research (Danbury, CT). The following synthetic compounds were tested (% purity by GC indicated): Z11–16:Ald (98.8%), Z9–14:Ald (95.5%), Z9–16:Ald (97.1%), 16:OAc (99.7%), Z7–16:OAc (98.0%), Z9–16:

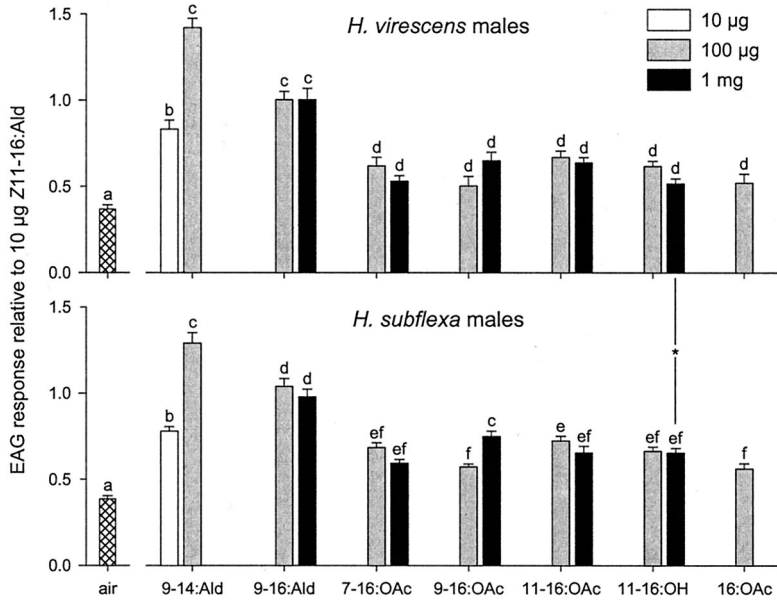

Fig. 1. Normalized mean ± SEM EAG responses of male antennae. The amounts of 10 µg, 100 µg, and 1 mg refer to the amount loaded on the filter paper, which was subsequently introduced in the Pasteur pipette. All male EAG responses were normalized relative to 10 µg Z11–16:Ald, i.e., the EAG response to each compound was divided by the response to 10 µg Z11–16:Ald. All components are the *z*-isomers. Within each species, means without a letter in common differ significantly ($P < 0.05$). Differences in EAG response between the two species are indicated between the graphs where significant differences ($P < 0.05$) were found; no indication means no significant differences. Significant differences within and between species were determined using least square means with a Tukey adjustment for multiple comparisons.

OAc (99.2%), Z11–16:OAc (97.1%), and Z11–16:OH (97.8%). Each compound was dissolved in CH_2Cl_2 to 1, 10, and 100 µg/µl. Ten microliters of each solution was loaded on a piece of folded filter paper (Whatman #1, 1.5 cm^2), and the filter paper was air dried to evaporate the solvent and placed into a Pasteur pipette. Two milliliters of room air was delivered to the antenna as a rapid puff from a calibrated glass syringe (Perfektum, Fisher) and through the pipette containing the test compound. Each sample was puffed three times, and the average EAG amplitude constituted the experimental unit. All samples were tested in random order on each antenna. Air was used as a negative control (puffed in the same way as samples) at the start, half way, and at the end of each test period with each antenna. To control for variation in response among antennae, all male responses were normalized relative to 10 µg Z11–16:Ald, the major pheromone component of both species. Female antennae of both species were less sensitive to Z11–16:Ald; therefore, female EAG responses were normalized relative to 100 µg Z11–16:Ald. Because each stimulus was preceded or followed by the standard, the average amplitude of each set of three EAG responses was divided by the average EAG amplitude in response to the nearest set of three puffs of the respective standard. In this way, the response to the standard is set to 1.

Statistical Analysis. Differences in EAG responses were analyzed using a mixed linear model, fitted with the procedure MIXED of the computer program SAS, version 8.02 (SAS Institute 2000). Data were square-root transformed to normalize the variance. Statistical differences between the sexes could not be determined because the standard differed in concentration, and thus, the normalized unit differed as well. Hence, separate analyses were performed for males and females. After fitting the model with fixed main effects and interaction for different moths and chemicals and a random effect for the antenna, we compared (1) within each sex of each species, which compounds differed from the negative control (air) and which compounds differed from each other; (2) virgin *Hv* with virgin *Hs* males; and (3) virgin *Hv* with virgin *Hs* females. All comparisons were made using least square means, with a Tukey adjustment for multiple comparisons.

Results

In males, EAG responses to all compounds at all doses tested were significantly different from responses to air puffs (Fig. 1). In all antennae tested, the largest EAG responses were recorded for Z9–14:Ald when using 100 µg; therefore, we tested 10 and 100 µg instead of 100 µg and 1 mg, as was used for most other compounds. The three unsaturated C_{16} acetates elicited significantly lower responses than the aldehydes tested, whereas they elicited similar responses as Z11–16:OH and 16:OAc. In *Hv* male antennae, no differences in EAGs were found between the three unsat-

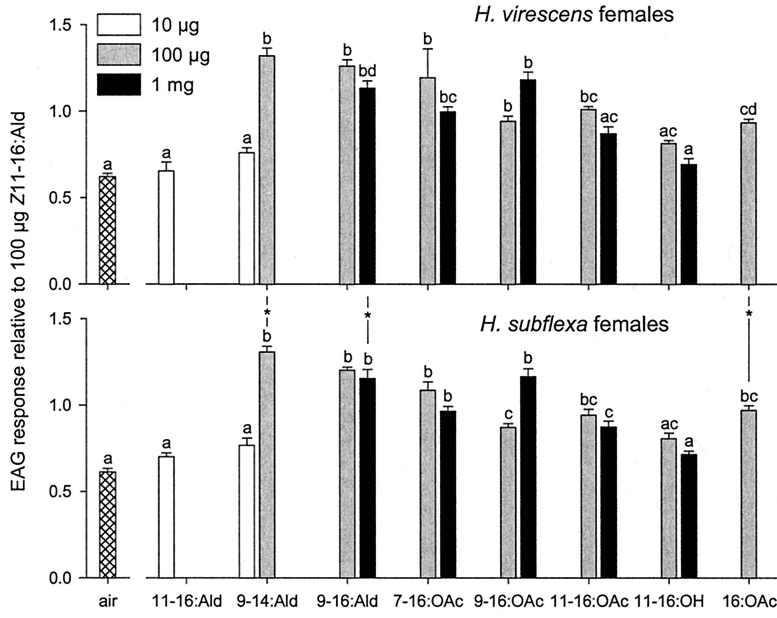

Fig. 2. Normalized mean ± SEM EAG responses of female antennae. All female EAG responses were normalized relative to 100 µg Z11–16:Ald, i.e., the EAG response to each compound was divided by the response to 100 µg Z11–16:Ald. All compounds are the *Z*-isomers. Within each species, means without a letter in common differ significantly ($P < 0.05$). Differences in EAG response between the two species are indicated between the graphs where significant differences ($P < 0.05$) were found; no indication means no significant differences. Significant differences within and between species were determined using least square means with a Tukey adjustment for multiple comparisons.

urated acetates, Z11–16:OH, and 16:OAc, whereas in *Hs* male antennae, 100 µg Z9–16:OAc and 16:OAc elicited significantly lower EAG responses than all other compounds tested. Comparisons of male responses showed no differences in EAG response between virgin *Hv* and *Hs* males (Fig. 1), except to 1 mg Z11–16:OH, which elicited significantly stronger responses in *Hs* than in *Hv* male antennae.

In females of both species, two compounds elicited EAG responses that did not differ from responses to air: 10 µg Z9–14:Ald and both doses of Z11–16:OH (Fig. 2). In addition, in *Hv* female antennae, 1 mg of Z11–16:OAc did not elicit an EAG response different from air. Most other compounds elicited similar EAG responses, which were all significantly different from air. When responses were compared between females of the two species, three compounds elicited significantly higher EAG responses in *Hs* females: 100 µg Z9–14:Ald, 1 mg Z9–16:Ald, and 100 µg 16:OAc (Fig. 2).

Discussion

There is ample evidence that the pattern of EAG responses to odorants can be species-specific (e.g., Smith and Menzel 1989, Visser and Yan 1995, Visser et al. 1997, Park et al. 2002) or even race-specific (Fescemeyer and Hanson 1990, Linn et al. 1999, El-Sayed et al. 2003). However, although *Hv* and *Hs* use different, but overlapping, blends of pheromone components, our study found no differences between *Hv* and *Hs* male EAG responses for most pheromone components of these two species.

Several comments are warranted on the methods we used in our EAG assays. Fist, we stimulated each antenna three times with the same stimulus, averaged the three resulting amplitudes, and normalized them relative to the average of three EAG responses to standards (10 µg Z11–16:Ald for male antennae and 100 µg Z11–16:Ald for female antennae). Despite the constraints of manual puffing, this procedure insured a high degree of repeatability, as evidenced by extremely low variance of the EAG amplitudes (Figs. 1 and 2). Second, the compounds we tested differ widely in their vapor pressures, and filter papers may emit different amounts of equally loaded compounds. Third, as is typical in EAG experiments, filter papers were loaded with high doses to obtain EAG responses, especially from female antennae. An important consideration is that, at high doses, the antenna might respond to minor contaminants, which may be pheromonal or kairomonal. However, even at high doses, the flux of each compound over the antennal preparation with each puff is only a small fraction of the amount on the filter paper. This procedure was designed to serve as a diagnostic assay to differentiate two species, and it clearly does not reflect the sensitivity of moths in behavioral assays.

Nevertheless, we found a significant difference between *Hv* and *Hs* males in EAG responses to Z11–16:OH. There is an ongoing debate on the role of this compound in *Hs* and *Hv* (reviewed by Vickers 2002).

Females of both species produce Z11–16:OH, but only *Hs* females emit it, and in this species, Z11–16:OH has been found to be an essential pheromone component (Heath et al. 1990, Vickers 2002). Interestingly, Teal et al. (1981) found an antagonistic effect of Z11–16:OH on *Hs* males, but Heath et al. (1990) found this to be true only when Z11–16:OH exceeded 3% of the total blend. Contributing to uncertainty of the role of Z11–16:OH are field trapping studies (e.g., Ramaswamy et al. 1985), showing that small amounts of Z11–16:OH increase trap catches of *Hv* males. However, the observation that the antennae of *Hv* males are significantly less sensitive to this compound than *Hs* supports the idea that Z11–16:OH is less important in the biology of *Hv*.

EAG responses to Z9–14:Ald were high in both *Hs* and *Hv* male antennae, relative to the other compounds tested. Recently, Baker et al. (2004) found specific olfactory receptor neurons in both *Hv* and *Hs* that are sensitive to Z9–14:Ald, which likely explains the high EAG responses in both species. High EAG responses to Z9–14:Ald were also found in *Hv* by Almaas and Mustaparta (1991) and in *Hz* (Christensen et al. 1991). In *Hv*, a high response to this compound would be expected, because it is the main secondary pheromone component, abundantly present in pheromone gland extracts (Roelofs et al. 1974, Tumlinson et al. 1975, Klun et al. 1980a, Teal et al. 1986), and makes up to 18% of the emitted volatiles (Teal et al. 1986). In *Hz*, Z9–14:Ald has been found to function as an antagonist (Klun et al. 1979). However, Vickers et al. (1991) found that small amounts of Z9–14:Ald can substitute for Z9–16:Ald without significantly impacting the levels of upwind flight and source location of *Hz* males in a wind tunnel. In *Hs*, the function of Z9–14:Ald is unclear. Klun et al. (1982) reported its presence in small amounts in *Hs* female glands, which we recently confirmed with gas chromatography-mass spectrometry (GC-MS) (Groot et al. 2005). So far, however, neither attraction nor repellence to this compound has been found in *Hs* males (Vickers 2002).

Z9–16:Ald, at stimulus doses of 100 μg and 1 mg, elicited male EAG responses in both *Hv* and *Hs* male antennae that were similar to the main component, Z11–16:Ald (the standard). In comparison, Baker et al. (2004) did not find a response to Z9–16:Ald in any of the sampled olfactory receptor neurons in *Hv*, so that our finding of similar EAG responses in both *Hv* and *Hs* males to this compound is somewhat surprising. Z9–16:Ald is the main secondary pheromone compound of *Hs* (Teal et al. 1981, Tumlinson et al. 1982, Heath et al. 1991, Vickers 2002), as well as of *Hz* (Klun et al. 1979, 1980b, Vetter and Baker 1984, Vickers et al. 1991). Its function in *Hv* is dubious. Z9–16:Ald has been found in small amounts in *Hv* pheromone gland extracts (Klun et al. 1980a, Tumlinson et al. 1982, Teal et al. 1986, Groot et al. 2005) and female volatiles (Teal et al. 1986), whereas trap catches of *Hv* males increased when Z9–16:Ald and Z11–16:OH were omitted from the blend (Tumlinson et al. 1982). However, in wind-tunnel assays, deletion of Z9–16:Ald from the blend led to a reduction in all close-range behaviors, especially in hovering and copulation attempts by *Hv* males (Teal et al. 1986).

The three unsaturated C_{16} acetates are unique to the pheromone of *Hs* females—in *Hv* and *Hz*, these compounds are most likely immediately converted to the corresponding aldehydes (Teal and Tumlinson 1986, 1987, Jurenka and Roelofs 1993), if produced at all. We found no differences between *Hv* and *Hs* males in their EAG responses to these acetates. The interspecific function of Z11–16:OAc is clear: it is the main inhibitor for *Hv* males (Vickers and Baker 1997), as well as for *Hz* males (Fadamiro and Baker 1997, Fadamiro et al. 1999, Quero et al. 2001). Paradoxically, Z11–16:OAc emitted by *Hs* females does not seem to be essential for attracting conspecific males in a wind tunnel (Vickers 2002), although in field assays, Teal et al. (1981) and Tumlinson et al. (1982) found a decrease in trap catches when the acetates were omitted from the blends. Unfortunately, when the acetates were deleted in those studies, Z9–16:OH and Z11–16:OH were added to the synthetic blends (Teal et al. 1981, Tumlinson et al. 1982), which might have inhibited attraction (Heath et al. 1990).

16:OAc is the major pheromone component emitted by *Hv* males during courtship (Teal and Tumlinson 1989, Hillier and Vickers 2004); male *Hs* produce 733-fold less 16:OAc than male *Hv* (286.1 versus 0.39 ng) (Teal and Oostendorp 1995a). 16:OAc was detected by antennae of both males and females of both species, although the response of *Hs* male antennae to this compound was marginal (Fig. 1). Autodetection of this male pheromone component by males of both species suggests that 16:OAc encodes information for males of both *Hs* and *Hv*. Female antennae of both species responded to 16:OAc, but surprisingly, EAG responses of *Hs* females to this compound were significantly higher than those of *Hv* females. This may suggest an antagonistic function in *Hs* females during courtship.

Our finding of an overall lower EAG response to pheromone components in females of both species reflects a general trend in female moths (Christensen et al. 1990, Schneider et al. 1998). Nevertheless, all pheromone compounds, except the alcohol, elicited significant EAG responses that were different from the air control in at least one of the concentrations tested, and it can thus be stated that, in *Hs* and *Hv* females, autodetection occurs, i.e., detection of their conspecific pheromone compounds. Generally, the antennal lobe in females lacks the male-specific macroglomerular complex (MGC), although in *Manduca sexta* (Linnaeus, 1763) females (Rössler et al. 1998, King et al. 2000, Rospars and Hildebrand 2000), as well as in *Hv* females (Berg et al. 2002), two enlarged compartments were found in a position corresponding to the MGC. The function of these large female glomeruli (LFG) is not clear (Berg et al. 2002), but given the anatomical relatedness between LFG and MGC, it is possible that the LFG may be involved in detection of a pheromone released by courting males (King et al. 2000) or in detection of conspecific and/or heterospecific female pheromone components. However, Gali-

zia et al. (2000) showed that, while plant odors elicited activity in the ordinary glomeruli of both *Hv* males and females, pheromone components failed to elicit any activity in the antennal lobe of *Hv* females.

Several functions have been discussed for autodetection of pheromone components (McNeil 1991, Schneider et al. 1998): autodetection may establish social contacts among females, such as in lek formation, joint calling, or spacing on food plants (Den Otter et al. 1978, 1996); it may be adaptive for females to locate local population centers, i.e., males, and thus increase local chances of mating (Birch 1977); it may be a way to control the timing of pheromone release (Palaniswamy and Seabrook 1985); and autodetection may be used for spacing to avoid interference among pheromone plumes (Schneider et al. 1998). However, only few studies have been conducted to determine if and how female calling behavior is affected by conspecific or heterospecific pheromone plumes. Saad and Scott (1981) conducted repellency tests of virgin and mated females of *H. armigera* (Hübner, 1808) and *H. zea.* They found that virgins of both species were repelled by conspecific virgins and by mated females as well as by the heterospecific pheromone extract, whereas mated females were repelled by conspecific virgins; mated females were not repelled by each other (Saad and Scott 1981). In other species, the presence of conspecific pheromone caused virgin females to call at an earlier (Palaniswamy et al. 1978) or later times (Noguchi and Tamaki 1985).

Because EAG responses only indicate reception at the peripheral level, it is too early to speculate on the behavioral significance of autodetection by *Hv* and *Hs* females. For the three sympatrically co-ocurring heliothines—*Hz*, *Hv*, and *Hs*—it would be interesting to determine whether and how the presence of conspecifics and heterospecifics affects their calling behavior, and perhaps even their pheromone composition, especially because there is so much overlap in their pheromone blends, and females modulate pheromone emissions during the scotophase (Teal and Oostendorp 1995b).

In conclusion, EAG responses in *Hv* and *Hs* are very similar, and the variation between the two species is too small to serve as a quantitative trait to which genetic differences could be correlated in a similar way as variation in pheromone production (Groot et al. 2004, Sheck et al. 2005). Behavioral responses and central nervous system activity show higher differentiation between the two species (Vickers and Baker 1997, Vickers 2002). When variation in these responses can be correlated to genetic differences, we may gain a better understanding of the genetic architecture of variation in signal perception and response in *Hv* and *Hs.*

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