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Interspecific Pheromone Plume Interference Among Sympatric Heliothine Moths: A Wind Tunnel Test Using Live, Calling Females

Jonathan P. Lelito · Andrew J. Myrick · Thomas C. Baker

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Abstract Three species of North American heliothine moths were used to determine the level at which interspecific female interference of male attraction to conspecific females occurs. We used live calling females of *Heliothis virescens*, *H. subflexa*, and *Helicoverpa zea*, as lures for conspecific males in a wind tunnel, and then placed heterospecific females on either side of the original species such that the plumes of the three females overlapped downwind. In nearly all combinations, in the presence of heterospecific females, fewer males flew upwind and contacted or courted the source than when only conspecific females were used in the same spatial arrangement. Males did not initiate upwind flight to solely heterospecific female arrangements. Our results show that the naturally emitted pheromone plumes from heterospecific females of these three species can interfere with the ability of females to attract conspecific males when multiple females are in close proximity. However, the fact that some males still located their calling, conspecific females attests to the ability of these male moths to discriminate point source odors by processing the conflicting information from interleaved strands of attractive and antagonistic odor filaments on a split-second basis.

Keywords *Heliothis* · *Helicoverpa* · Pheromone blend · Behavioral antagonist · *Z*11-16:Ald · *Z*9-16:Ald · *Z*11-16:OH · Z9-14:Ald · Flight behavior · Olfactory orientation

J. P. Lelito (✉) · A. J. Myrick · T. C. Baker
104 Chemical Ecology Laboratory, Center for Chemical Ecology, Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA
e-mail: jpl207@psu.edu

Introduction

The heliothine moths are a well-studied group of crop pests in North America. The two most economically important species are the tobacco budworm, *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), and the corn earworm, *Helicoverpa zea* Boddie. A third species, which is a pest of tomatillo in Mexico, is *Heliothis subflexa* Guenée. All three of these moths share the same major pheromone blend component, (*Z*)-11-hexadecenal (*Z*11-16:Ald), but differ in the other components of their respective blends. In *H. virescens*, the secondary component is (*Z*)-9-tetradecenal (*Z*9-14:Ald; Roelofs et al. 1974; Tumlinson et al. 1975; Klun et al. 1980b; Pope et al. 1982; Vetter and Baker 1983; Teal et al. 1986), while for *H. zea*, the secondary component is (*Z*)-9-hexadecenal (*Z*9-16:Ald; Klun et al. 1979, 1980a; Vetter and Baker 1984; Pope et al. 1984). Finally, in *H. subflexa*, secondary components include *Z*9-16:Ald, (*Z*)-11-hexadecen-1-ol (*Z*11-16:OH), and (*Z*)-11-hexadecenyl acetate (Z11-16:Ac; Teal et al. 1981; Klun et al. 1982; Heath et al. 1990, 1991; Vickers 2002; Groot et al. 2006).

The secondary components of one species of moth can act as behavioral antagonists if they are added to the sex pheromone blend of another species (Cardé et al. 1977; Löfstedt 1990, 1993; Löfstedt et al. 1991; Gries et al. 1996; Potting et al. 1999; Quero and Baker 1999), often most strongly where the species in question are sympatric and synchronic (Cardé et al. 1977; Guerin et al. 1984; Gemeno et al. 2000; McElfresh and Millar 1999, 2001; Gries et al. 2001; El-Sayed et al. 2003; Groot et al. 2007). This is expected as a result of selection against mating mistakes by males in areas where the species co-occur.

These antagonistic effects are well known in wind tunnel studies for the heliothine moths, mainly from adding the

heterospecific secondary components onto the same point source as the synthetic conspecific pheromone blend (Vickers et al. 1991; Vickers and Baker 1997; Fadamiro and Baker 1997; Fadamiro et al. 1999). Both Z11-16:OH and Z11-16:Ac from the *H. subflexa* blend antagonize the attraction of male *H. virescens* (Vetter and Baker 1983, 1984; Vickers and Baker 1997) and *H. zea* (Teal et al. 1984; Quero and Baker 1999; Quero et al. 2001). However, the antagonistic effects caused by the coemission of a heterospecific compound from the same point source does not necessarily mean that a confluent plume from a nearby heterospecific female would interfere with the attraction of a male to a conspecific female whose plume was being overlapped. Plumes are comprised of individual strands of odor interspersed with pockets of clean air, the strands having been sheared off at the source and later shredded into finer substrands by microturbulence (Murlis 1986).

Moths have an exceptionally good ability to fly upwind to and locate sources of their pheromone, even when sources of behaviorally antagonistic compounds are placed 10 cm away to create confluent plumes (Witzgall and Priesner 1985; Liu and Haynes 1994; Baker et al. 1998; Fadamiro et al. 1999). For instance, *H. virescens* males were able to discriminate strands of their two-component pheromone from strands of the antagonist Z11-16:Ac when the strands were experimentally generated to be separated by only 1 mm (Baker et al. 1998; Fadamiro et al. 1999). When the antagonist was coemitted in every strand along with the pheromone, however, attraction was negligible, and the suppression of attraction was apparent on a strand-by-strand basis in the behavior of flying males in both *H. virescens* (Vickers and Baker 1997) and *H. zea* (Quero et al. 2001).

Our goal in the current study was to examine the behavior of males in a wind tunnel in response to live, calling conspecific females in the close presence of live, calling heterospecific females. The experiments examine the effects of heterospecific blends that arrived downwind on flying males' antennae in naturally mixed or staggered strands. Using each of the six species pairs possible, we tested the hypothesis that the sex pheromone plumes from heterospecific live heliothine moths may interfere with the attraction of males to their conspecific females when the time-averaged plumes are known to overlap. However, when sufficient proportions of heterospecific strands might arrive asynchronously on the antenna with conspecific strands, we hypothesized that low levels of attraction to conspecific females should still occur.

Methods and Materials

Insects Moths of the three species used in this experiment were maintained over many generations on a modified pinto bean diet (*H. zea* and *H. virescens*; Shorey and Hale 1965) and a corn–soy diet (*H. subflexa*; Vickers 2002). Single larvae were placed into individual diet-filled cups, removed at pupation, and sexed under a dissecting scope. Sexed pupae of each species were placed into screen cages lined with paper toweling and provided with a 10% sucrose solution for adults to feed on upon eclosion. Each day, any live, properly formed adults of either sex were removed and placed into a separate cage, sorted by species. Male and female moths of the three species were kept in separate Percival environmental chambers (Percival Scientific, Boone, IA, USA) at 29–30°C and 50% or higher relative humidity, under a reversed light cycle, 16:8-h light/dark, with the scotophase starting at 9:30 A.M. Dead and malformed moths were discarded daily, and the sucrose–water dispenser was refilled. The entire screen cage was sprayed twice daily (just before lights-off and just after lights-on) with distilled water. All male moths were between 3 and 6 d of age, and all female moths were between 2 and 6 d of age when utilized for these experiments.

Behavioral Assays The wind tunnel, with a width of 1.2 m, a length of 2.8 m, and a height of 1 m at its peak (a bowed shape based on Miller and Roelofs 1978), was set to a wind speed of 60 cm s^{-1}, the room darkened to 2.96 lx (2.20 lx of red light and 0.76 lx of white light) emanating from eight overhead incandescent light sources (four red, four white) connected to a variable transformer. Just before the onset of the scotophase, 15 male moths of each of the three species, in individual 6 by 6-cm metal screen cages, were placed into the wind tunnel itself. At the same time, at least 10 female moths of each species were placed into individual metal screen cages, in an adjacent fume hood in the darkened wind tunnel room to prevent the males from being exposed to pheromone prior to flight. All flights were conducted in the last 4 hr of the scotophase, giving the moths several hours to adjust to the conditions in the wind tunnel room.

Control flights were performed each day to judge the response level of males of each species to conspecific females under our wind tunnel conditions. Three calling females of the same species were placed in individual metal screen cylinders (4 cm diameter×6 cm height) on a metal screen pedestal 35 cm above the wind tunnel floor in a triangular arrangement with the apex of the three-female triangle facing upwind (Fig. 1). The separation between the two downwind cages was only 2–3 cm, and thus the maximum distance that plumes from the calling females (depending on where they were situated when calling) would be separated by was 4 cm. Females were placed into the wind stream and allowed to acclimate for up to 10 min, by which time they had resumed calling. Once all three

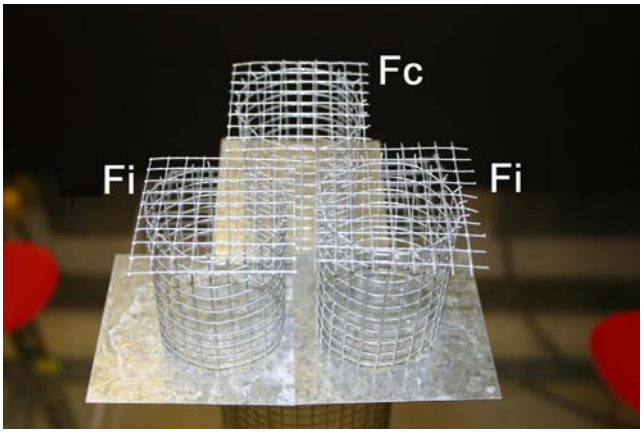

Fig. 1 Arrangement of female cages in the control and experimental wind tunnel experiments conditions. *Fc* indicates the conspecific female position. *Fi* indicates the interference female positions for the experimental treatments; during control flights for each species, all three positions are occupied by conspecific females. The direction of air movement in the wind tunnel is from *top* to *bottom* in the image

females were visibly calling, males were released in the center of the wind tunnel by hanging their cage, open end upwind, on a pedestal 35 cm high, 1 m directly downwind from the females. Each male was given up to 2 min to respond, after which nonresponders' cages were removed, while responding males were captured and removed with a net. Between each male test, the females were observed, and in the few instances when they were no longer visibly calling, they were replaced with another individual from the fume hood; the new females were given time to resume calling if the move from the hood to the wind tunnel had disturbed them. Males of a given species were also tested against three females of each of the other two species. Each individual male was exposed only to one of these treatments and then discarded.

For each male, we scored his progress toward completion of the entire flight sequence: "no flight," "wing fanning," "flight," "casting," "locked on plume," "half the distance to source," "10 cm from source," "contact source," and "hair pencils and courtship." The temperature at the beginning and end of the flight period was averaged to yield the temperature recorded for that day.

After the control flights were complete, experimental flights were performed. These were conducted in a manner similar to the control flights, except that the apical female in the triangular arrangement was a conspecific of the male being tested, and the other two cages held individual heterospecific females of the same species (Fig. 1). The same protocol of observing the females in a calling posture and replacement of noncalling ones was used. Males again were given 2 min to respond. Experimental flights were conducted such that individual females of both "interfering" species were assessed; that is, male *H. subflexa* were tested to *H. subflexa* females under interference from two *H. virescens* and subsequently from two *H. zea* females. As before, an individual male of a given species only was exposed to one experimental treatment and not used again.

For simplicity, treatments will hereafter be referred to by three-letter combinations. For example, the array of three *H. subflexa* females would be abbreviated as "SSS," while an array comprised of one *H. subflexa* female in the presence of two *H. zea* females would be abbreviated "ZSZ," because this annotation most closely reflects the spatial arrangement of the species of females on the platform.

Plume Strand Analyses The degree to which plume strands of females were or were not coincident in their arrival downwind was measured by using cages of calling females placed in the positions on the platform as described above. We used calling females of *Trichoplusia ni* and *H. virescens* and the Quadroprobe four-antenna biosensor (Park et al. 2002) on which two *T. ni* male antennae and two *H. virescens* antennae were placed on the four-channel simultaneous electroantennogram (EAG) probe. The probe was situated in the wind tunnel 1 m downwind of the calling females where males had been released earlier in the behavioral experiments. The system for odor classification, using a computer algorithm (Myrick et al. 2005; Hetling et al. 2006), was first trained to classify the plume strands from calling *T. ni* and *H. virescens* females, as well as a synthetic mixture of 10 μg of each of the two major components of both species emitted from a filter paper with greater than 95% accuracy for all training odors. *T. ni* and *H. virescens* females were used due to the need to discriminate the major pheromone components of females so closely placed together in upwind cages. Threshold for detection "events" (EAGs) from plume strands was set at 50 μV. Readings from the Quadroprobe were then taken in the confluent plumes from calling *T. ni* and *H. virescens* females from 1 m downwind using the caged female configurations described above (Fig. 1). EAGs from the calling females of the three heliothine moth species used in behavioral experiments could not be placed in different classes due to the predominant EAG responses resulting from the major component of all three species, Z11-16:Ald. A total of 83s (195 strands), 33s (85 strands), and 33s (135 strands) was used for the Quadroprobe algorithm training sessions in the plumes from *H. virescens* females, *T. ni* females, and the synthetic mixture, respectively.

Statistical Analyses A Fisher's exact test was used to compare each category of male response among female treatments within each species of male assessed (Fisher 1922). Chi-square contingency tests (χ^2) were used to examine the difference in response rates between contacting

the source and extending hair pencils for each species of male, under each treatment (Steel and Torrie 1960).

Results

Males of all three species exhibited a significant reduction in at least one of their upwind flight attraction behaviors when two heterospecific females were positioned next to their own female, compared to when three conspecific females were present (Figs. 2, 3, 4, 5, 6, and 7). No males of any species responded with attraction-related behaviors to an array of three heterospecific females; it is important to note that none even "locked on" to the plumes of heterospecific females (Figs. 2, 3, 4, 5, 6, and 7).

The presence of either *H. subflexa* or *H. zea* females on either side of a *H. virescens* female significantly reduced the upwind flight behavior of the *H. virescens* males, starting with the "locking on" stage (Figs. 2 and 3). At subsequent stages of upwind flight, reductions caused by the heterospecific females were not as severe, and several of the *H. virescens* males were able to land at the cage of the centrally located, conspecific female (Figs. 2 and 3).

It is interesting to note that the effects of heterospecific females seemed to carry over to the courtship stage after males had landed, even though the pheromone components they were exposed to near the cage were more likely to be only conspecific. Seventy-one percent of *H. virescens* males that contacted the conspecific female array also exhibited the hair pencil courtship display, whereas in the ZVZ and SVS treatments, the courtship responses were significantly lower, 44.4% and 37.5%, respectively ($p<0.05$; Chi-square 2×2 test of independence).

Similarly, the presence of either *H. virescens* or *H. zea* females significantly reduced the upwind flight behavior of *H. subflexa* males to a conspecific, starting downwind at the "locking on" to the plume stage (Figs. 4 and 5). Several of the *H. subflexa* males were able to land at the cage of the centrally located conspecific female (Figs. 4 and 5).

The effects of heterospecific females around *H. subflexa* females seemed to carry over to the courtship stage. For *H. subflexa* males flying to the SSS treatment, 41.2% of those that contacted the source also exhibited the hair pencil courtship display. However, in response to the VSV and ZSZ treatments, the percentages of males that had contacted the source that also exhibited courtship was significantly lower, 0% and 20%, respectively ($p<0.05$; Chi-square 2×2 test of independence).

H. zea males did not respond as well to conspecific females as did *H. virescens* and *H. subflexa*, but their responses to either of the heterospecific female controls (SSS and ZZZ) were significantly lower (Figs. 6 and 7). The presence of heterospecific females on either side of a *H. zea* female significantly reduced the upwind flight behavior of the *H. zea* males, with the interference from *H. subflexa* females (Fig. 6) being greater than that of *H. virescens* females (Fig. 7), in that, in the latter case, only "locking on" to the plume stage was affected. *H. subflexa* females interfered with male *H. zea* attraction to *H. zea* females at every stage. For *H. zea*, such a low percentage of males landed on their conspecific female cages that no analysis of the subsequent hair pencil displays of these landed males would be meaningful.

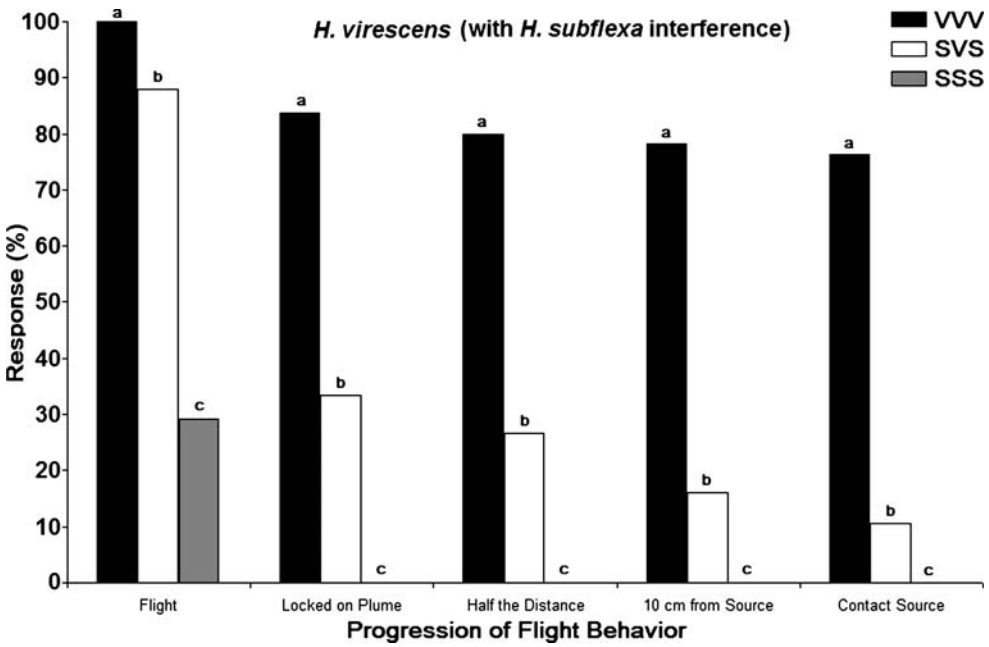

Fig. 2 Responses of male *H. virescens* to conspecific (*VVV*) and heterospecific (*VSV* and *SSS*) treatments involving *H. subflexa* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

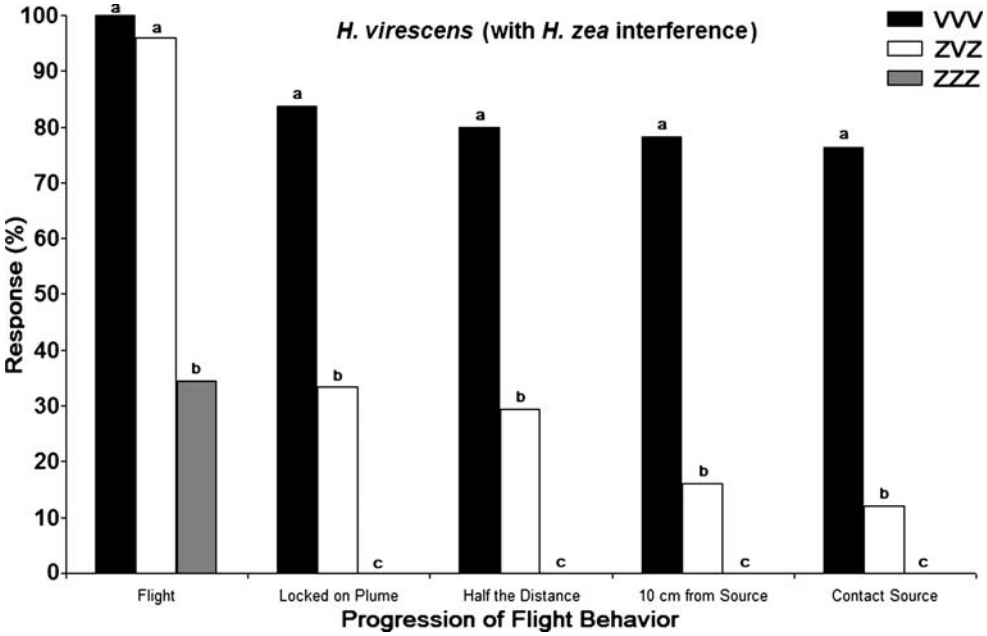

Fig. 3 Responses of male *H. virescens* to conspecific (*VVV*) and heterospecific (*VZV* and *ZZZ*) treatments involving *H. zea* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

The degree of coincident arrival of plume strands, as measured by the Quadroprobe four-antenna system using two heterospecific, calling females, was substantial. The fact that females of the two species were placed so closely together in their cages on the platform perhaps makes this not so surprising. Strands classified as "mixture" comprised 670 out of the 1,525 strands arriving on the probe (43.9%) for the VTV (*H. virescens*/*T. ni*/*H. virescens*) placement. Strands classified as *T. ni* comprised 600 out of 1,525 strands (39.3%), and those identified as *H. virescens* were 255 out of 1,525 (16.7%). For the TVT arrangement, 285 out of 625 strands (45.6%) were classified as "mixture," 270 (43.2%) were classified as *T. ni*, and 70 (11.2%) were classified by the algorithm as *H. virescens*.

Discussion

Despite years of studies on the behavioral antagonism caused by the addition of synthetic heterospecific heliothine pheromone components to the synthetic sex pheromone blends of other heliothine species, there has been a relative lack of information about the degree of antagonism caused by actual overlapping plumes emitted by heterospecific

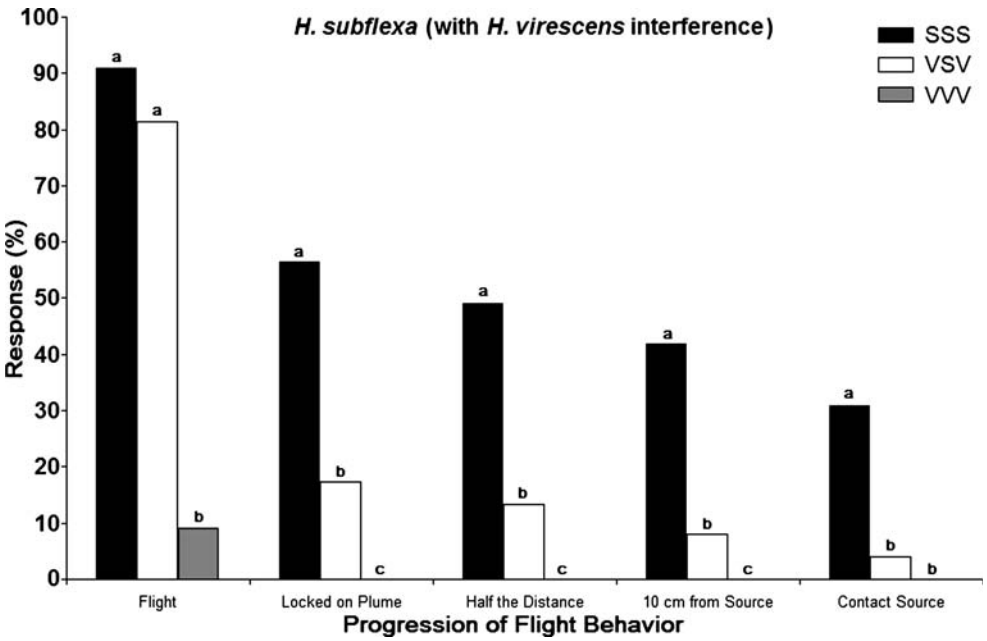

Fig. 4 Responses of male *H. subflexa* to conspecific (*SSS*) and heterospecific (*VSV* and *VVV*) treatments involving *H. virescens* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

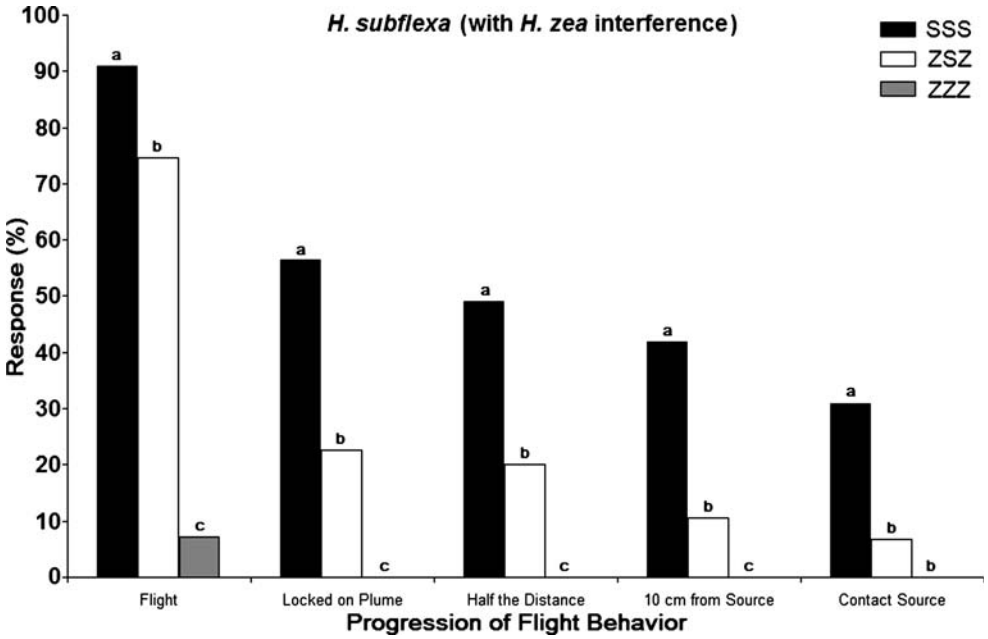

Fig. 5 Responses of male *H. subflexa* to conspecific (*SSS*) and heterospecific (*ZSZ* and *ZZZ*) treatments involving *H. zea* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

heliothine females. The question of naturally emitted female–female sex pheromone plume interactions relates directly to the possible selection pressures that may have occurred over evolutionary time. Practical issues of sex pheromone monitoring trap specificity, not the evolutionary forces that determine species-specific communication channels, were the initial driving force behind the identification of these field-trapping heliothine blends.

Our results using live, calling females of these three heliothine species show that there is no cross-attraction whatsoever between males of one species and females of the other two species. This is in agreement with field-trapping studies that used synthetic blends, as well as those that used live females, as lures in field-trapping tests (Haile et al. 1973; Sparks et al. 1979a, b; Klun et al. 1979, 1982; Carpenter et al. 1984; Lopez and Witz 1988). The interfering effects of a confluence of plumes in previous field-trapping tests using cages of calling females could not be verified due to the uncertainties produced by the ever-shifting wind fields under these conditions. In the current experiments, these uncertainties were eliminated.

It had been inferred from studies that used synthetic lures that male *H. subflexa* will not fly upwind to females of either *H. virescens* or *H. zea* because *H. virescens*

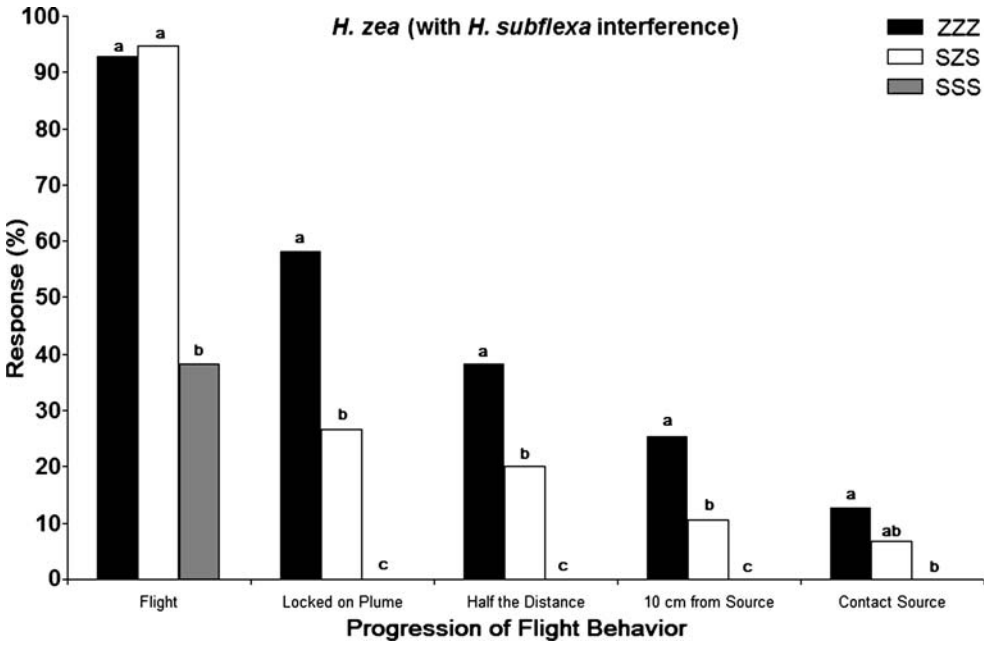

Fig. 6 Responses of male *H. zea* to conspecific (*ZZZ*) and heterospecific (*SZS* and *SSS*) treatments involving *H. subflexa* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

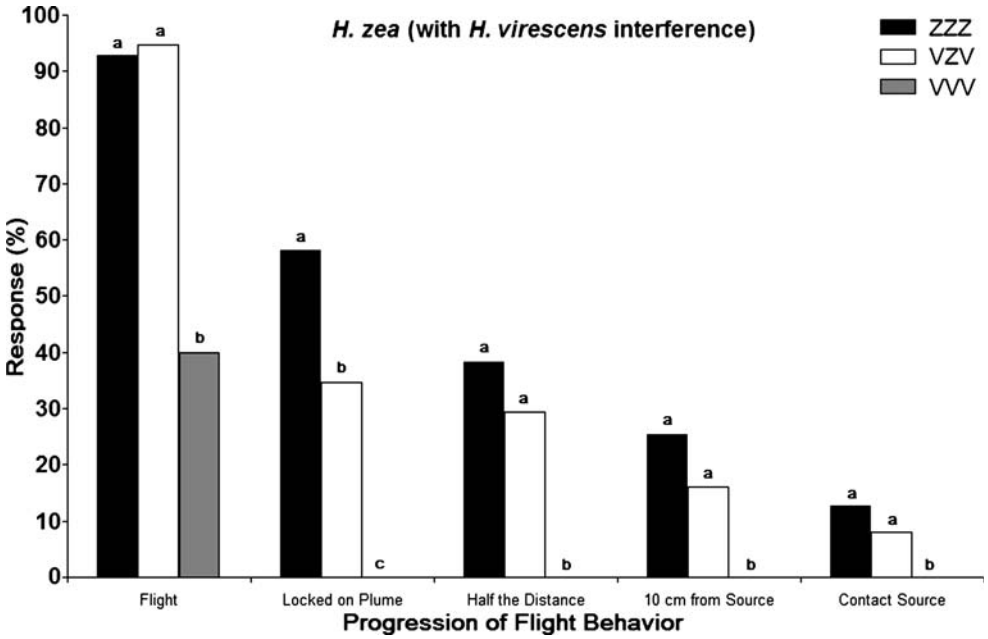

Fig. 7 Responses of male *H. zea* to conspecific (*ZZZ*) and heterospecific (*VZV* and *VVV*) treatments involving *H. virescens* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

females emit trace amounts, at best, of Z9-16:Ald and Z11-16:OH, and *H. zea* females do not emit Z11-16:OH (Klun et al. 1979; Pope et al. 1984; Heath et al. 1990; Vickers et al. 1991). Our results support these suppositions, showing no cross-attraction of *H. subflexa* males to females of the other two species.

It had been assumed that neither *H. virescens* nor *H. zea* males will fly upwind to *H. subflexa* females because they emit Z11-16:OH, which is antagonistic to males of both these species (Klun et al. 1982; Teal et al. 1984; Vickers and Baker 1997; Quero and Baker 1999). It had also been assumed that *H. virescens* males would not be attracted to *H. zea* females because they do not emit the essential component Z9-14:Ald, and *H. zea* males would not be attracted to *H. virescens* females because the amount of Z9-14:Ald they emit relative to Z11-16:Ald acts as a behavioral antagonist. Field-trapping tests that used live, calling females (Haile et al. 1973; Sparks et al. 1979a, b) had supported these ideas, and our results confirm them.

We have further shown that the plume strands from heterospecific heliothine females contain sufficient amounts of the above-mentioned antagonistic compounds to interfere with upwind flight and source location of conspecific females by males when the plume strands are intertwined. This had been conjectured in past studies (Vickers et al. 1991; Vickers 2002), based on the potential antagonism imposed by the known heterospecific antagonist compounds applied to single sources, but it had not been rigorously tested before by using live, calling females.

To determine the precise effects when heterospecific females are in close proximity, we measured the percentages of plume strands that might be registered on heliothine male antennae downwind from the release point as "mixture" (coincident strands from two females) or else as the "pure" blend of either species. We found that nearly 50% of the strands in two configurations, VTV and TVT, were classified as "mixture" from the plumes of calling females placed in such close proximity. We suggest that this tendency toward "mixture" strands is responsible for the interspecific interference in male attraction we document here. This, in combination with the presence of antagonists in the noncoincident odor strands, reduces attraction of a given species of male moth to conspecifics. Nevertheless, sufficient numbers of strands of pure pheromone reach the males' olfactory systems to allow a significant percentage to locate their conspecific females in this closely spaced array.

Two unexpected instances of interference occurred, and they were unexpected because there are no known antagonists involved that can explain the reduced levels of upwind flight. First, *H. virescens* male attraction was reduced by the presence of *H. zea* plumes, the only explanation based on known behavior-modifying compounds for these species being that the ratio of Z9-14:Ald to Z11-16:Ald would be diluted due to the lack of emission of Z9-14:Ald by *H. zea* females. Second, the reduction in upwind flight by *H. subflexa* males in the presence of either *H. virescens* or *H. zea* females was also unexpected due to the lack of any known antagonism in *H. subflexa* males to any compounds emitted by the females of these two species. Barring the possibility of antagonism from other coemitted compounds from the females of either of these species, the only other explanation for reduced attraction of *H. subflexa* males would be that a ratio shift caused by overemission of some compounds by the other species would register suboptimally in the *H. subflexa* central

nervous system (Vickers et al. 1991; Vickers 2002), as suggested previously by Klun et al. (1982).

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