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Fine-scale resolution of closely spaced pheromone and antagonist filaments by flying male *Helicoverpa zea*

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Abstract The limits of a male moth's ability to resolve closely spaced odor filaments have been investigated. Male *Helicoverpa zea* normally respond to their conspecific sex pheromone blend by exhibiting an upwind flight, which culminates in source contact by at least 50% of the bioassayed individuals. When loaded onto the same filter paper source containing this hitherto attractive pheromone blend, or onto a separate filter paper and co-emitted from the same pipette source with pheromone, (*Z*)-11-hexadecenyl acetate severely reduced upwind flight and source contact by male *H. zea*. A similar level of upwind flight inhibition was recorded when the antagonist (*Z*)-11-hexadecenyl acetate was emitted from its own point source placed 1 mm upwind of the pheromone point source, both plumes being simultaneously emitted in a continuous mode to form a confluent strand. However, (*Z*)-11-hexadecenyl acetate was less effective in reducing upwind flight and source contact when it was isolated and pulsed from its own source, placed 1 mm either upwind, downwind or cross-wind of a pipette source from which pheromone was simultaneously being pulsed, such that both filaments were separated in time by 0.001–0.003 s. These results suggest that male *H. zea* are able to distinguish between odor sources separated by as little as 1 mm in space and 0.001 s in time.

Key words *Helicoverpa zea* · Noctuidae · Lepidoptera · Sex pheromone · Antagonist

Abbreviations *Z11-16:Ald* *Z*-11-hexadecenal · *Z9-16:Ald* (*Z*)-9-hexadecenal · *Z11-16:Ac* (*Z*)-11-hexadecenyl · *Z7-12:OH* (*Z*)-7-dodecenol · *Z5-10:OH* (*Z*)-5-decenol · *Z5-10:Ac* (*Z*)-5-decenyl acetate

Introduction

The successful location of a sex pheromone source by a male moth has been shown to be dependent on blend composition, as well as plume structure (Willis and Baker 1984; Mafra-Neto and Cardé 1994; Vickers and Baker 1994, 1997). A species' sex pheromone blend commonly triggers upwind orientation in conspecific males. However, research has shown that the addition of certain interspecific compounds to a species' sex pheromone blend can cause cessation of orientation behavior (attraction) in conspecific males (Rothschild 1974; Liu and Haynes 1992; Vickers and Baker 1997; Fadamiro and Baker 1997). These compounds, known as antagonists, are usually components of the sex pheromone blends of congeneric or sympatric species, and their role in preventing mating mistakes between individuals of sympatric, related species has been suggested (e.g., Witzgall and Priesner 1991; Liu and Haynes 1993; Fadamiro and Baker 1997; Baker et al. 1998). For instance, (*Z*)-11-hexadecenyl acetate) (Z11-16:Ac), an important sex pheromone component of female *Heliothis subflexa* (Teal et al. 1981; Klun et al. 1982) has been reported as a behavioral antagonist of males of some sympatric heliothine species, including *H. virescens* (Vickers and Baker 1997) and *Helicoverpa zea* (Fadamiro and Baker 1997).

As potent as the antagonist may be, several studies have shown that it must be emitted from the same point

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source as the pheromone blend for optimal reduction of upwind flight and source location (Rothschild 1974; Witzgall and Priesner 1991; Liu and Haynes 1992, 1993). In a study on the flight behavior of male *Coleophora laricella* to pheromone and antagonist plumes, Witzgall and Priesner (1991) recorded virtually no suppression of upwind flight when the antagonist, (Z)-5-decenol (Z5-10:OH) was placed 5 cm apart from the pheromone source (*Z*)-5-decenyl acetate (*Z5-10:Ac*). Also, Liu and Haynes (1992) reported that (*Z*)-7-dodecenol (Z7-12:OH), a behavioral antagonist of male *Trichopulsia ni* was less effective in disrupting upwind flight when released from a source 5 cm cross-wind, or 10 cm upwind, of the pheromone source. Although, the above-mentioned studies were conducted by using continuous point-source plumes, thus making it difficult to measure the actual distances between pheromone and antagonist strands, the various authors, nonetheless, inferred that both pheromone and antagonist filaments must be received simultaneously for a complete expression of inhibition. As informative as these studies (Rothschild 1974; Witzgall and Priesner 1991; Liu and Haynes 1992, 1993) may be, only in studies in which the distances between filaments of pheromone and antagonist could be measured, or experimentally controlled, could we truly discern the limits of the degree of odor resolution. In an experiment in which odor was presented as pulsed filaments mimicking the natural fine-scale structure of an odor plume (Murlis and Jones 1981), Fadamiro and Baker (1997) recorded poorer suppression of upwind flight of male *H. zea* when filaments of the antagonist, Z11-16:Ac, were staggered with pheromone filaments such that both filaments were temporally separated in arrival time on the antennae by ca. 0.1 s. An experiment in which the distance between pheromone and antagonist filaments would be reduced was, therefore, the next logical step in our investigation on the resolution of closely spaced odor filaments by male moths.

Several moth species are now known to respond behaviorally to experimentally pulsed pheromone filaments mimicking the fine-scale structure of an odor plume (Vickers and Baker 1992, 1996; Mafra-Neto and Cardé 1994, 1995; Fadamiro and Baker 1997). Mafra Neto and Cardé (1994) demonstrated the importance of the physical structure of a pheromone plume on orientation. They recorded greater upwind orientation for male *Cadra cautella* following turbulent or mechanically pulsed pheromone plume than for those males following continuous narrow plumes. It follows, therefore, that the physical plume structure may be an important factor determining the degree of suppression of upwind flight that can be mediated by a behavioral antagonist.

Using the corn earworm, *H. zea*, the current study was carried out to investigate the limits of a male moth's ability to distinguish between filaments of pheromone and a behavioral antagonist. Experiments were designed to separate out possible interactions between plume composition, plume structure and suppression of upwind flight.

Materials and methods

Moths

H. zea larvae were reared in the laboratory on a pinto bean diet (Shorey and Hale 1965). Sex determination was made at the pupal stage. Males were separated from females and held in a 30 cm × 30 cm × 30 cm cage, placed in an environmental chamber on a 14:10 h L:D cycle at 25 °C and 55 ± 5% relativel humidity. Emerging adult males were supplied with a 10% sugar solution. Males used in behavioral bioassays were aged 3–5 days. Approximately 1 h before a daily flight test, individual males were placed under red light in 6 cm × 6 cm wire screen cages, held on plastic trays. The trays containing the males in their cages were then transferred into the wind tunnel for acclimation. Flight bioassays were conducted between the 5th and 8th hours of scotophase (Vetter and Baker 1984), and a male was scored only once and then discarded.

Wind tunnel

The wind tunnel was of dimension 2.4 m × 1 m × 1 m and modified after Miller and Roelofs (1978). Males were released individually at a height of about 23 cm above the floor 170 cm downwind of the odor source. Wind speed and temperature in the wind tunnel measured 40 cm s^{-1} and 25 °C, respectively. Lighting was achieved by using a mixture of red and white light, measuring about 0.5 lx. Each male was held in the plume for 30 s before release and was allowed 2 min to take-off from its cage. Calibration of the apparatus and visualization of filaments was done by using smoke plumes of TiCl4.

Odor strands generation

Odor sources consisted of either a sex pheromone binary mixture of (*Z*)-11-hexedecenal (Z11-16:Ald; 10 µg) and (*Z*)-9-hexedecenal (Z9-16:Ald; 0.5 µg), a three-component blend made of the above binary mixture plus the antagonist, Z11–16:Ac (1, 2.5 or 5 µg, depending upon experiment) placed on the same filter paper, or the antagonist at these same loadings placed on a separate filter paper and, depending on the experiment, the filter paper was placed in the same pipette as the pheromone or in a separate one. The filter papers were placed in 14.7-cm-long pasteur pipettes and the treatments were made by using the binary pheromone blend with or without antagonist such that 10 µl of each solution was measured over the surface of square-ending filter papers (3 cm × 0.5 cm, Whitman no. 1), by using a micropipette. All chemical compounds were made from neat materials maintained in our laboratory and each was found to be >98% pure by gas chromatoghraphy (GC). Where necessary, filter papers containing 10 µl of hexane were used as the blank control.

Odor filaments were generated by using the stimulus-flow controller (Syntech, The Netherlands), an air-pulsing device described in Vickers and Baker (1992). The air pulser was either set to produce multiple pulses over a long period of time at the rate of 5 pulses s^{-1} (Fig. 1B), or to generate continuous 'turbulent' plumes (Fig 1A). Flow rate and pulse duration were held constant at 5 m s^{-1} and 0.02 s, respectively. Pipettes containing filter paper wicks loaded with odorants were held in a holding device within the tunnel with the tip of each pipette pointing upward. The holding device could hold one or more pipettes at the same time and the distance between two pipettes could be adjusted.

Experimental protocol

Males exposed to the different odorants were assessed for their ability to exhibit various behavioral parameters, including upwind flight and progress greater than mid-way to the source (reaching a distance of 15 or 40 cm to the source, respectively). The number of moths contacting the source was also recorded. Flight tracks were

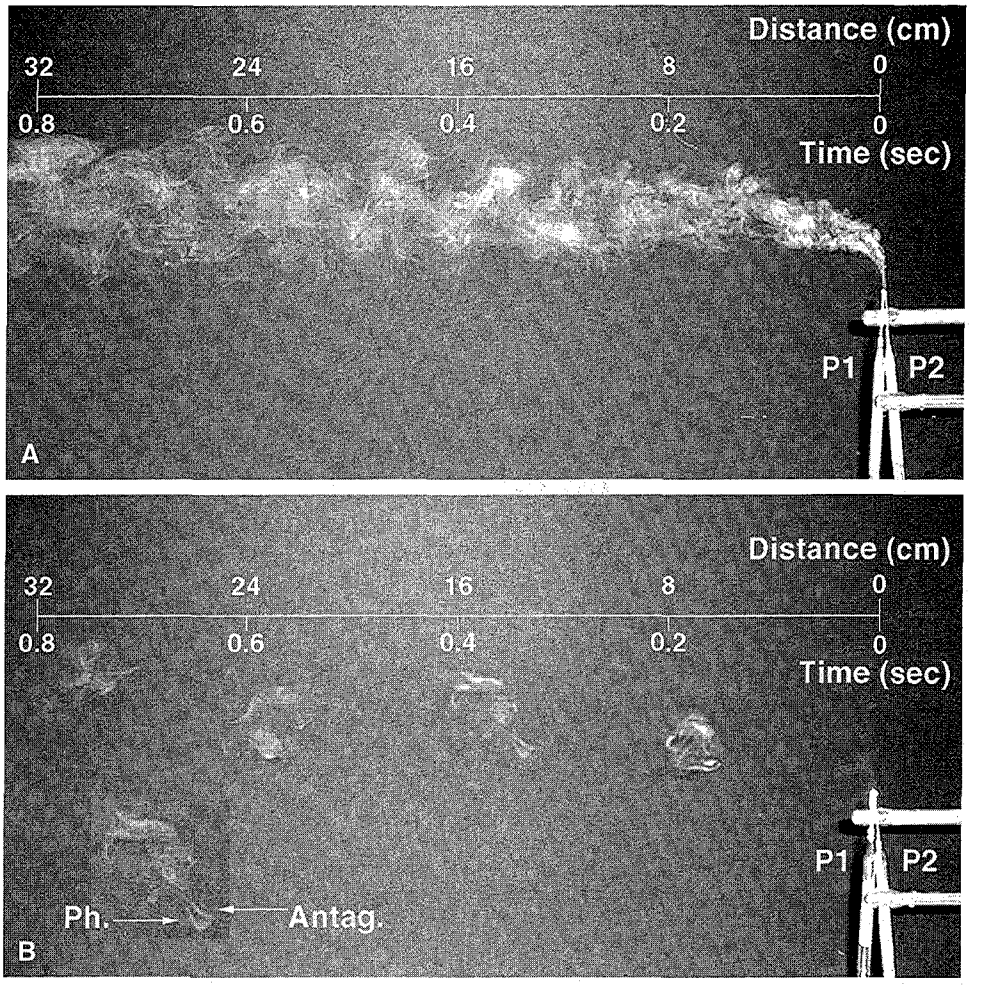

Fig. 1 Relative differences in the structures of the pheromone plumes used in this study as visualized by $TiCl^4$-generated smoke plumes: **A** continuous plumes of pheromone (*Ph.*) and antagonist (*Antag.*) simultaneously generated at a flow rate of 5 ml s^{-1} from two pipette sources (*P1* and *P2*) whose tips are separated by 1 mm; **B** pulsed strands of pheromone (*Ph.*) and antagonist (*Antag.*) simultaneously generated at the rate of 5 filaments s^{-1} with a 0.002-s duration and 5 ml s^{-1} flow rate from two pipette sources (*P1* and *P2*) whose tips are separated by 1 mm

obtained by video-recording male response from above the wind tunnel using a Sony RSC 1050 rotary shutter camera. The camera's field of view encompassed 1 m of the length of the wind tunnel and 0.75 m of its width. Video tapes were played back frame-by-frame on a Toshiba video tape deck and relayed to a Panasonic monitor. The male's position every 0.03 s was transcribed onto a sheet of acetate. Tracks were later digitized on an Hitachi digitizing pad (Puma Plus), and analyzed by using a track analysis computer program. A total of five experiments were conducted. Experiments were randomized and significant differences within behavioral categories were established by using a χ^2 2 × 2 test of independence with Yates correction of continuity (Parker 1979). Statistical analysis of the triangle of velocity data was by two-way analysis of variance (ANOVA) and means were compared using the LSD test (SAS Institute 1989).

Experiment 1

An initial experiment was conducted to investigate the effect of separating out pheromone (10 μg Z11-16:Ald + 0.5 μg Z9-16:Ald) and antagonist (2.5 μg Z11-16:Ac) filaments and pulsing them simultaneously at 5 filaments s^{-1}. Four treatments were compared: A) pipette (P1) containing pheromone blend plus a second pipette (P2) containing hexane (blank), both separated along the wind line (P2 upwind of P1) by 1 mm; B) pipette (P1) containing pheromone blend plus a second pipette (P2) containing antagonist, both separated along the wind line by 5 cm; C) pipette (P1) containing pheromone blend plus a second pipette (P2) containing antagonist, both separated along the wind line by 1 mm; and D) pipette (P1) containing both pheromone blend and antagonist loaded onto the same filter paper plus a second pipette (P2) containing hexane, both separated along the wind line by 1 mm. In all treatments, the pipette (P1) containing the pheromone blend was always placed in front (downwind) of pipette P2. In this, and later experiments, the distance between a pair of pipettes was measured tip-to-tip. Thirty-six males were released to each treatment.

Experiment 2

A second experiment was conducted to check for possible effects of the order of presentation of partitioned filaments. This experiment was similar in set-up to Experiment 1, except that a lower level of the antagonist (1 μg or 10% of Z11-16:Ac) was used in order to pick up any subtle differences in behavior. In all eight treatments tested, the two pipettes holding odor sources were separated along the wind line. In four of these (A, C, E, G), the pipette (P1) holding the pheromone blend was always placed in front (downwind) of pipette P2, as in Experiment 1 above. This order of presentation was reversed in the remaining four treatments, such that the P1 pipette holding the pheromone blend was placed behind the P2. Thirty-two males were tested for each treatment.

Experiment 3

Having established in the first two experiments that males were able to resolve closely spaced filaments generated by placing one pipette upwind of the other (separation along the wind line), a third experiment was conducted to test if a similar odor resolution will occur when two pipettes were separated cross-wind (across the wind line separation). Three treatments were compared: A) pipette (P2) containing antagonist placed 1 mm upwind of pipette (P1)

containing pheromone blend; B) pipette (P2) containing antagonist placed 1 mm cross-wind of pipette (P1) containing pheromone blend; and C) pipette (P2) containing hexane placed 1 mm cross-wind of pipette (P1) containing both pheromone blend and antagonist. The last treatment served as a negative control. Antagonist in this experiment was a 2.5 µg dose of Z11-16:Ac. Twenty males were tested for each treatment.

Experiment 4

A fourth experiment involved the use of a higher dose of the antagonist (5 µg of Z11-16:Ac or 50% Z11-16:Ac in relation to the major pheromone component, 10 µg Z11-16:Ald). In addition to serving as a confirmatory test for the earlier experiments, this experiment was designed to test the effect of plume structure (continuous versus pulsed plumes) on inhibition of upwind flight. Using smoke plumes of $TiCl^4$, it was observed that continuous plumes generated from two closely spaced pipette sources tended to appear more completely mixed than pulsed plumes from two closely-spaced sources (Fig. 1). We therefore hypothesized that more inhibition of upwind flight by filaments of pheromone and antagonist originating from separate pipettes might result from continuous plumes than from pulsed plumes. To test this hypothesis, odor was presented to males either as continuous plumes or as pulsed filaments. Six treatments were tested: A) pulsed strands simultaneously generated from a pipette (P1) containing a filter paper loaded with pheromone plus a second pipette (P2) containing a filter paper loaded with hexane (blank); B) continuous plumes simultaneously generated from a pipette (P1) containing a filter paper loaded with pheromone plus a second pipette (P2) containing a filter paper loaded with hexane; C) pulsed strands simultaneously generated from a pipette (P1) containing a filter paper loaded with pheromone plus a second pipette (P2) containing a filter paper loaded with antagonist; D) continuous plumes simultaneously generated from a pipette (P1) containing a filter paper loaded with pheromone plus a second pipette (P2) containing a filter paper loaded with antagonist; E) pulsed strands simultaneously generated from a pipette (P1) containing a filter paper loaded with both pheromone and antagonist plus a second pipette (P2) containing a filter paper loaded with hexane; F) continuous plumes simultaneously generated from a pipette (P1) containing a filter paper loaded with both pheromone and antagonist plus a second pipette (P2) containing a filter paper loaded with hexane. The tips of the pair of pipettes in all treatments were separated along the wind line by 1 mm, with pipette P2 always placed upwind of pipette P1. Pulsed filaments were generated at 5 pulses s^{-1}. Under this protocol, filaments of pheromone and antagonist originating from separate pipettes, would at maximum be separated by 1 mm in space and 0.003 s in time if the male moth were stationary in the 40 cm s^{-1} wind. Thirty-six males were released to each treatment.

Experiment 5

As a follow-up to Experiment 4, a final experiment was conducted in which the antagonist was added to the pheromone pipette on a second filter paper rather than on the same paper, as in Experiments 1–4 above. This was done to test if loading the antagonist on a separate paper, but generated from the same pipette with pheromone would result in the same level of suppression of response, as when both pheromone and antagonist compounds were loaded onto the same filter paper. Pheromone (10 µg Z11-16:Ald + 0.5 µg Z9-16:Ald) and antagonist (5 µg Z11-16:Ac) plumes were either generated continuously, or pulsed in eight treatments: A) pulsed strands simultaneously generated from a pipette (P1) containing a filter paper loaded with pheromone blend plus a second pipette (P2) containing a filter paper loaded with hexane (blank); B) continuous plumes simultaneously generated from a pipette (P1) containing a filter paper loaded with pheromone blend plus a second pipette (P2) containing a filter paper loaded with hexane; C) pulsed strands simultaneously generated from a pipette (P1) containing two filter papers, one loaded with pheromone blend and the other loaded with hexane plus a second pipette (P2) containing a filter paper loaded with hexane; D) continuous plumes simultaneously generated from a pipette (P1) containing two filter papers, one loaded with pheromone blend and the other loaded with hexane plus a second pipette (P2) containing a filter paper loaded with hexane; E) pulsed strands simultaneously generated from a pipette (P1) containing a filter paper loaded with pheromone blend plus a second pipette (P2) containing a filter paper loaded with antagonist; F) continuous plumes simultaneously generated from a pipette (P1) containing a filter paper loaded with pheromone blend plus a second pipette containing a filter paper loaded with antagonist (P2); G) pulsed strands simultaneously generated from a pipette (P1) containing two filter papers, one loaded with pheromone blend and the other loaded with antagonist plus a second pipette (P2) containing a filter paper loaded with hexane; H) continuous plumes simultaneously generated from a pipette (P1) containing two filter papers, one loaded with pheromone blend and the other loaded with antagonist plus a second pipette (P2) containing a filter paper loaded with hexane. In all treatments, pipette P2 was always placed upwind of pipette P1, with both pipette tips separated by 1 mm. Pulsed filaments were generated at 5 pulses s^{-1}. The blank (hexane-added) pieces of filter papers in two of the treatments were added to control for possible reductions in pheromone emission rates when the second (antagonist-containing) filter paper was added to the pheromone-emitting pipettes (P1). Thirty-four males were released to each treatment.

Odor collections

Compounds emitted from the pipettes that had been used in the experiments were collected as they issued from the tip in 25-cm-long glass collection tubes (3 mm ID). The tip of a pipette, containing a filter-paper strip loaded with an odorant, was inserted into a collection tube, and the connection was sealed with Teflon tape. The collection tube was placed in a container (20 cm long × 3 cm ID) filled with dry ice. The odor pipette was then connected to the flow controller, and the released compound was collected using 3072 20-ms pulses with the air flow set at 15 ml s^{-1}. Collection tubes were washed with 50 µl of HPLC-grade hexane containing (*Z*)-10-pentadecenyl acetate (30 pg μl^{-1}) as an internal standard. Collections were analyzed using GC-MS in selective ion mode. Collected amounts were calculated as mean (3 replicates/treatment) picograms/pulse (0.3 ml) and corrected for differences in relative abundance of the selected ions relative to the internal standard. Trap breakthrough was checked and confirmed negative for all odor pipettes by analyzing collected material in a second, in-series-connected, glass tube. All GC-MS analyses were performed by using a Hewlett-Packard 5890 GC with a direct interface to a Hewlett-Packard 5972 mass selective detector (30-m DB-225 capillary column, electron impact, 70 eV).

Results

Experiment 1

Ninety-two percent of males released to pheromone (10 µg Z11-16:Ald + 0.5 µg Z9-16:Ald) filaments alone flew upwind and 47% of the released males contacted the source. (Fig. 2A). The proportions of males exhibiting these same behaviors for this treatment were not significantly different from those of males tested to the treatments in which pheromone and antagonist (2.5 µg Z11-16:Ac) filaments were generated from different sources, separated along the wind line either by 5 cm (upwind flight = 92%, source contact = 47%; Fig. 2B), or by 1 mm (upwind flight = 94%, source

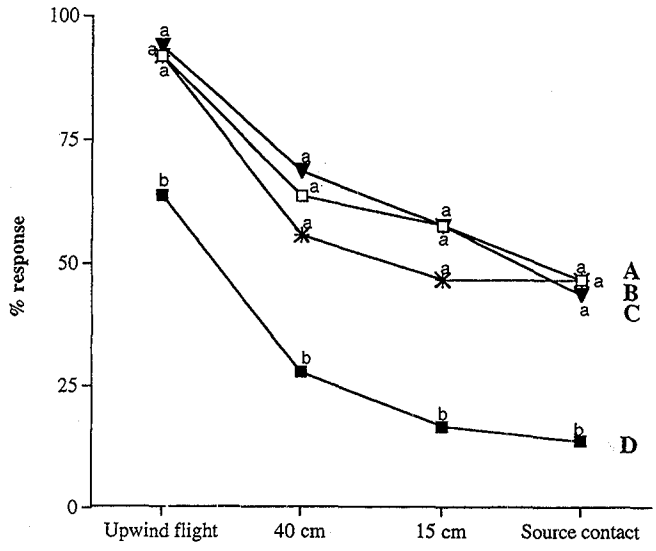

Fig. 2 Summary of results in experiment 1: percentage of male *H. zea* responding to pulsed strands of pheromone alone, or to pulsed strands of pheromone containing the antagonist, either as a separate or co-emitted filaments. Four treatments were compared, with P1 and P2 indicating pipette 1 and pipette 2, respectively: *A* P1 pheromone, P2 blank, both separated by 1 mm; *B* P1 pheromone, P2 antagonist, both separated by 5 cm; *C* P1 pheromone, P2 antagonist, both separated by 1 mm; *D* P1 pheromone/antagonist co-emitted, P2 blank, both separated by 1 mm. In all treatments, pipettes P1 and P2 were separated along the wind line, with P2 always placed upwind of P1. Pheromone was 10 μg Z11-16:Ald [(*Z*)-11-hexadecenal] + 0.5 μg Z9-16:Ald [(*Z*)-9-hexadecenal], while the antagonist was a 2.5-μg dose of Z11-16:Ac [(*Z*)-11-hexadecenyl acetate]. In this and later figures, percentage responses in the same behavioral category having no letters in common are significantly different at $P < 0.05$

contact = 44%; Fig. 2C). However, a significantly lower proportion of males released to the treatment in which pheromone and antagonist filaments were co-emitted from the same source exhibited upwind flight (64%), and contacted source (14%), compared with the other three treatments (Fig. 2D).

Experiment 2

The order of presentation of partitioned filaments did not significantly affect male response to the different odor treatments (Table 1). For the two positive control treatments in which males were exposed to odor from a pair of pipettes, one containing pheromone and the other blank, percentages of males exhibiting upwind flight and source contact were similar, regardless of whether the pheromone-containing pipette was placed downwind (100% upwind flight; 50% source contact), or upwind (100% upwind flight; 47% source contact) of the blank (hexane-loaded) pipette (Table 1). Similarly, no significant effect of order of odor presentation was recorded for the four treatments (C, D, E, F) in which pheromone and antagonists were presented to males from different pipettes. Percentages of source contact were 44% and 56%, when the pheromone-containing pipette was placed 1 mm downwind or upwind of the antagonist, respectively (Table 1, treatments C and D, respectively). Also, when the pheromone-containing pipette was placed 5 cm downwind or upwind of the antagonist, percentages of males contacting source were 44% or 41%, respectively (Table 1, treatments E and F, respectively).

Experiment 3

Placing the antagonist-containing pipette 1 mm cross-wind of the pheromone-containing pipette resulted in similar levels of response, as when the antagonist-containing pipette was placed 1 mm upwind of the pheromone-containing pipette (Table 2). When both pipettes were separated 1 mm cross-wind, 100% of the males tested took flight upwind with 55% arriving at the source (Table 2B). These proportions were comparable to the percentages of males that exhibited upwind flight (100%) and source contact (50%), when the antagonist-containing pipette was placed 1 mm upwind of the

Table 1 Summary of results in experiment 2: percentage of male *H. zea* responding to partitioned, pulsed pheromone and antagonist plumes, in which the antagonist source was either placed upwind, or downwind of the pheromone source. In all treatments, pipette P2 was always placed upwind of pipette P1, both pipettes being separated by 1 mm or 5 cm, depending upon the treatment. Thirty two males were tested for each treatment. Pheromone was 10 μg Z11-16:Ald [(*Z*)-11-hexadecenal] + 0.5 μg Z9-16:Ald [(*Z*)-9-hexadecenal]. Antagonist was 1 μg Z11-16:Ac [(*Z*)-11-hexadecenyl acetate] (10% of Z11-16:Ald). Odor filaments were generated at 5 pulses s^{-1}

Treatment	Percentage response			
	Upwind flight	Flight reaching 40 cm to source	Flight reaching 15 cm to source	Source contact
A Pheromone (P1) + blank (P2) 1 mm separation	100[a]	75[a]	66[a]	50[a]
B Blank (P1) + pheromone (P2) 1 mm separation	100[a]	69[a]	50[a]	47[a]
C Pheromone (P1) + antagonist (P2) 1 mm separation	100[a]	75[a]	56[a]	44[a]
D Antagonist (P1) + Pheromone (P2) 1 mm separation	100[a]	69[a]	63[a]	56[a]
E Pheromone (P1) + antagonist (P2) 5 cm separation	100[a]	78[a]	59[a]	44[a]
F Antagonist (P1) + pheromone (P2) 5 cm separation	94[a]	66[a]	56[a]	41[a]
G Pheromone/antagonist (P1) + blank (P2) 1 mm separation	75[b]	16[b]	6[b]	6[b]
H Blank (P1) + pheromone/antagonist (P2) 1 mm separation	72[b]	13[b]	9[b]	9[b]

[a, b] Percentages in the same column having no letters in common are significantly different at $P < 0.05$

Table 2 Summary of results in experiment 3: percentage of male *H. zea* responding to partitioned, pulsed pheromone and antagonist plumes, in which the antagonist source was placed 1 mm upwind, or 1 mm cross-wind of the pheromone source. Pipettes P1 and P2 were separated by 1 mm either along the wind line (P2 upwind of P1), or across the wind. Twenty males were tested for each treatment. Pheromone was 10 μg Z11-16: Ald + 0.5 μg Z9-16:Ald. Antagonist was 2.5 μg Z11-16:Ac (25% of Z11-16:Ald). Odor filaments were generated at 5 pulses s^{-1}

Treatment	Percentage response			
	Upwind flight	Flight reaching 40 cm to source	Flight reaching 15 cm to source	Source contact
A Pheromone (P1) + antagonist (P2) 1 mm separation along the wind line	100[a]	70[a]	65[a]	50[a]
B Pheromone (P1) + antagonist (P2) 1 mm separation cross-wind	100[a]	85[a]	70[a]	55[a]
C Pheromone/antagonist (P1) + blank (P2) 1 mm separation cross-wind	70[b]	15[b]	5[b]	5[b]

[a,b] Percentages in the same column having no letters in common are significantly different at $P < 0.05$

pheromone-containing pipette (Table 2A). As expected, a significant suppression of response (70% upwind flight; 5% source contact) was recorded when both pheromone and antagonist were placed in the same pipette, which was now placed 1 mm cross-wind of the hexane-containing pipette (Table 2C).

Experiment 4

As in the first three experiments, significant numbers of males exposed to pheromone alone, either as a point-source continuous plume (upwind flight = 86%, source contact = 63%), or as pulsed filaments (upwind flight = 89%, source contact = 69%) exhibited upwind response (Figs. 3B and 3A, respectively). When pheromone and antagonist (5 μg Z11-16:Ac) filaments were pulsed from two different pipettes separated along the wind line by 1 mm, males flew upwind in great numbers (83%), but fewer (31%) located source (Fig. 3C). Yet, these males responded better than those released to continuous plumes of pheromone and antagonist generated from two pipette sources separated along the wind line by 1 mm: 74% and 14% for upwind flight and source contact, respectively (Fig. 3D). However, a near-complete suppression of response was recorded for males released to pulsed filaments (upwind flight = 37%, source contact = 0%), or continuous plumes (upwind flight = 50%, source contact = 10%) containing both pheromone and antagonist filaments co-emitted from the same source (Figs. 3E and 3F, respectively).

Experiment 5

Results similar to those in experiment 4 were obtained in this final experiment in which odorants were loaded on separate filter papers and generated from the same pipette source either as continuous, or as pulsed plumes (Fig. 4). Significantly higher proportions of upwind flight and source contact were recorded for males tested to the four treatments in which pheromone alone was pulsed or continuously emitted, either from a pipette containing a single filter paper loaded with pheromone alone, or from a pipette containing two filter papers, one loaded with pheromone and the other with hexane (blank). For these four treatments, upwind flight averaged 100% while source contact ranged between 68% and 82% (Fig. 4A–D). Similarly, males receiving pulsed filaments of pheromone and antagonist (5 μg Z11-16:Ac) generated from two pipettes separated along the

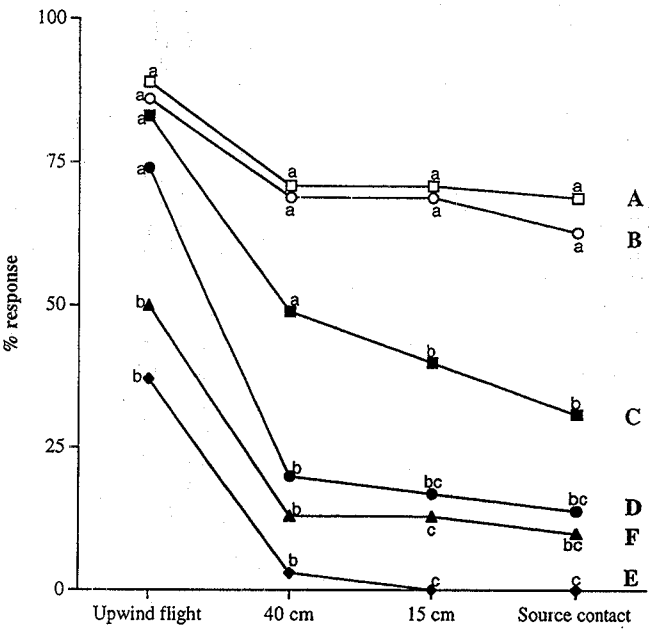

Fig. 3 Summary of results in experiment 4: percentage of *H. zea* males responding to pulsed or continuous plumes of pheromone alone or to plumes of pheromone containing the antagonist, either as a separate, or co-emitted plumes. Six treatments were compared, with P1 and P2 indicating pipette 1 and pipette 2, respectively: *A* pulsed, P1 pheromone, P2 blank; *B* continuous, P1 pheromone, P2 blank; *C* pulsed, P1 pheromone, P2 antagonist; *D* continuous, P1 pheromone, P2 antagonist; *E* pulsed, P1 pheromone/antagonist co-emitted, P2 blank; and *F* continuous, P1 pheromone/antagonist co-emitted, P2 blank. In all treatments, pipette P2 was placed 1 mm upwind of pipette P1. Pheromone was 10 μg Z11-16:Ald + 0.5 μg Z9-16:Ald, while the antagonist was a 5-μg dose of Z11-16:Ac. Pulsed filaments were generated at the rate of 5 pulses s^{-1}, while continuous plumes were at a flow rate of 5 ml s^{-1}

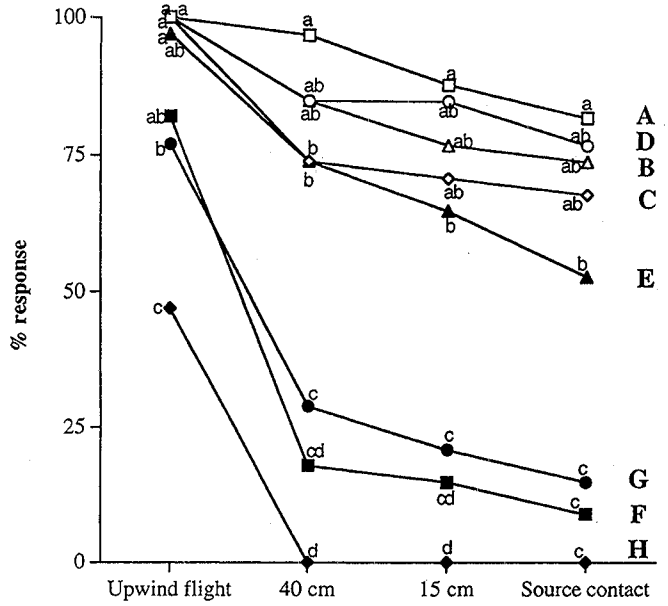

Fig. 4 Summary of results in experiment 5: percentage of *H. zea* males responding to pulsed or continuous plumes of pheromone alone or to plumes of pheromone containing the antagonist, either as a separate, or co-emitted plumes. In this experiment, the antagonist was added to the pheromone-containing pipette on a separate piece of filter paper, and the corresponding control treatments had a blank piece of filter paper added to the pheromone-containing pipette. Eight treatments were compared, with P1 and P2 indicating pipette 1 and pipette 2, respectively: *A* pulsed, P1 pheromone, P2 blank; *B* continuous, P1 pheromone, P2 blank; *C* pulsed, P1 pheromone/blank, P2 blank; *D* continuous, P1 pheromone/blank, P2 blank; *E* pulsed, P1 pheromone, P2 antagonist; *F* continuous, P1 pheromone, P2 antagonist; *G* pulsed, P1 pheromone/antagonist co-emitted, P2 blank; and *H* continuous, P1 pheromone/antagonist co-emitted, P2 blank. In all treatments, pipette P2 was placed 1 mm upwind of pipette P1. Pheromone was 10 μg Z11-16:Ald + 0.5 μg Z9-16:Ald, while the antagonist was a 5-μg dose of Z11-16:Ac. Pulsed filaments were generated at the rate of 5 pulses s^{-1}, while continuous plumes were at a flow rate of 5 ml s^{-1}

wind line by 1 mm flew upwind (97%) and located the source (53%) in great numbers (Fig. 4E). Although up to 82% of males exposed to continuous plumes of pheromone and antagonist simultaneously emitted from two separate pipettes exhibited upwind flight, only (9%) located source (Fig. 4F). These lower proportions were comparable to those of males receiving both pheromone and antagonist molecules co-emitted from the same pipette either as pulsed filaments (77% upwind flight and 15% source contact; Fig. 4G), or as continuous plumes (47% upwind flight and 0% source contact; Fig. 4H).

Flight tracks

Flight tracks were analyzed for three groups of males in experiment 4: males responding to pulsed pheromone filaments alone; males responding to simultaneously pulsed separate strands of pheromone and antagonist (5 μg Z11–16:Ac) with their pipette tips separated along the wind line by 1 mm; and males responding to pheromone and antagonist strands co-emitted from the same pipette. Compared to the first two treatments, only three of the males in the last category released to co-emitted pheromone and antagonist filaments flew in the field of view of the recording camera, due to the significant suppression of upwind flight by this treatment.

Males responding to antagonist molecules added to the atmosphere either as partitioned or co-emitted filaments in the last two treatments flew with significantly slower airspeeds and groundspeeds than males responding to pheromone filaments alone (Table 3). Males flying to co-emitted pheromone and antagonist filaments exhibited significantly greater course angle than males responding to pheromone filaments alone, or than males responding to partitioned pheromone and antagonist filaments. Furthermore, males flying in response to pheromone filaments alone, or to separate strands of pheromone and antagonist flew straighter upwind, with a trend toward smaller track angles than males responding to co-emitted pheromone and antagonist filaments (Table 3, Fig. 5). Frequency histogram distribution of the track angles of males responding to the three treatments obtained by classifying track angles into 10° bins from −180° to +180° showed that while the distribution of flight track angles of males in the first two categories was preponderantly unimodal, with a cluster around 0 degrees (upwind), the distribution of

Table 3 Analysis of anemotactic and counterturning behaviors of male *H. zea* responding to three odor treatments. Odor filaments were generated at 5 pulses s^{-1} with a 0.02 s duration and 5 ml s^{-1} flow rate. Males responding to pheromone filaments alone (n = 8) flew faster than males exposed to males responding to partitioned (1 mm separation along the wind line) pheromone (10 μg Z11-16:Ald + 0.5 μg Z9-16:Ald) and antagonist (5 μg Z11-16:Ac) filaments (n = 12), or males responding to co-emitted pheromone and antagonist filaments (n = 3). Males orienting to co-emitted pheromone and antagonist filaments flew with greater course angle than those males flying to the first two treatments

Odor filaments	Air speed (cm s^{-1})	Ground speed (cm s^{-1})	Track angle (deg)	Course angle (deg)	Counterturning frequency (reversals/s)
Pheromone alone	105.65 ± 12.88[a]	75.21 ± 11.84[a]	41.34 ± 16.63[a]	25.77 ± 10.73[b]	3.67 ± 0.62[a]
Pheromone + antagonist isolated	83.19 ± 16.48[b]	52.96 ± 12.89[b]	45.27 ± 17.37[a]	22.32 ± 7.26[b]	3.50 ± 0.49[a]
Pheromone/antagonist co-emitted	76.45 ± 14.07[b]	54.12 ± 14.86[b]	63.70 ± 17.48[a]	36.59 ± 10.55[a]	3.04 ± 0.58[a]
P	0.03*	0.01*	0.12	0.004*	0.36

[a,b] Values (means ± SD) in the same column having no letters in common are significantly different at $P < 0.05$*

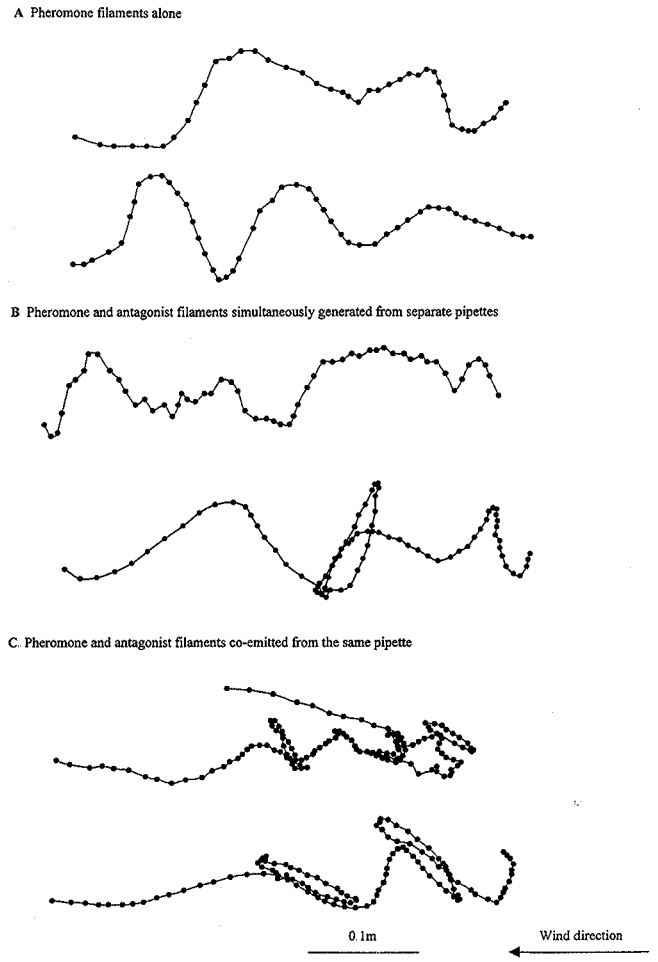

Fig. 5 Typical flight tracks exhibited by *H. zea* males in response to three different odor filaments generated at the rate of 5 pulses s^{-1}: pheromone filaments alone (**A**); pheromone and antagonist filaments simultaneously generated from different pipettes separated along the wind line by 1 mm (**B**); and pheromone and antagonist filaments co-emitted from a single pipette (**C**). Pheromone was 10 μg Z11-16:Ald + 5% Z9-16:Ald, while the antagonist was 5 μg of Z11-16:Ac. Males responding to the two strands containing antagonist filaments, whether partitioned (**B**), or in the same source as pheromone (**C**) exhibited significant reduction in air and ground speeds. Flight tracks of males in the third category exposed to co-emitted pheromone and antagonist filament (**C**) were characterized by several loops and a trend for comparatively greater track and course angles

track angles of the three males in the third category that responded to co-emitted pheromone and antagonist filament was not unimodal (Fig. 6).

Odor collections

The amount of the major pheromone component, Z11-16:Ald, emitted from the pipette containing two filter papers, one loaded with pheromone and the second with antagonist (2.480 ± 0.540 pg per pulse; mean ± SD), was similar to that emitted from the pipette containing the pheromone-loaded filter paper plus blank filter paper (2.620 ± 0.890 pg per pulse). Although higher amounts of pheromone (8.130 ± 0.026 pg per pulse) and antagonist (0.041 ± 0.02 pg per pulse) were emitted when generated simultaneously from different pipettes than when both were co-emitted from the same pipette (2.480 ± 0.540 pg per pulse and 0.011 ± 0.001 pg per pulse, for pheromone and antagonist, respectively), the emission ratio of pheromone to antagonist issuing from separate pipettes (198:1) was similar to when issuing from the same pipette (225:1).

Discussion

When loaded onto the same filter paper source containing pheromone blend, or placed on separate filter paper and co-emitted from the same pipette source, Z11-16:Ac significantly reduced the amount of upwind flight and source contact by male *H. zea*. A similar level of upwind flight antagonism was recorded when the antagonist was emitted from its own pipette source, placed 1 mm upwind of the pheromone pipette, both plumes being simultaneously emitted in a continuous mode to form a confluent strand. However, Z11-16:Ac was less effective in reducing upwind flight and source contact when it was pulsed from its own source placed 1 mm upwind, downwind, or cross-wind of a simultaneously pulsed pheromone source, such that both filaments were separated in time by 0.001–0.003 s. Measurements of the ratios of pheromone to antagonist emitted showed that this differential odor resolution was not due to differences in emission from partitioned versus co-emitted pheromone and antagonist filaments.

Analysis of flight tracks showed that males responding to these pulsed, partitioned pheromone and antagonist filaments exhibited flight tracks similar in shape and angular deviations to those flying to pheromone filaments alone, although the former males were significantly slower. Whereas the three males that responded to the treatment in which pheromone and antagonist filaments were co-emitted from the same source exhibited stunted tracks characterized by several loops and comparatively greater track and course angles, males tested to pheromone filaments alone, or to partitioned pheromone and antagonist pulsed filaments flew straighter upwind with more or less unimodal track angle distributions. These results suggest that males released to pulsed filaments from partitioned pheromone and antagonist sources showed similar response to those males released to pheromone filaments alone, and that both groups of males flew more directly upwind than males released to co-emitted pheromone and antagonist filaments.

Both the quality and structure of pheromone plumes have been shown to influence track shape and track angle distributions of flying male moths. For instance, male *Ephestia cautella* responding to incomplete or off-ratio pheromone blends reportedly exhibited slower and more meandering upwind flight, compared with males

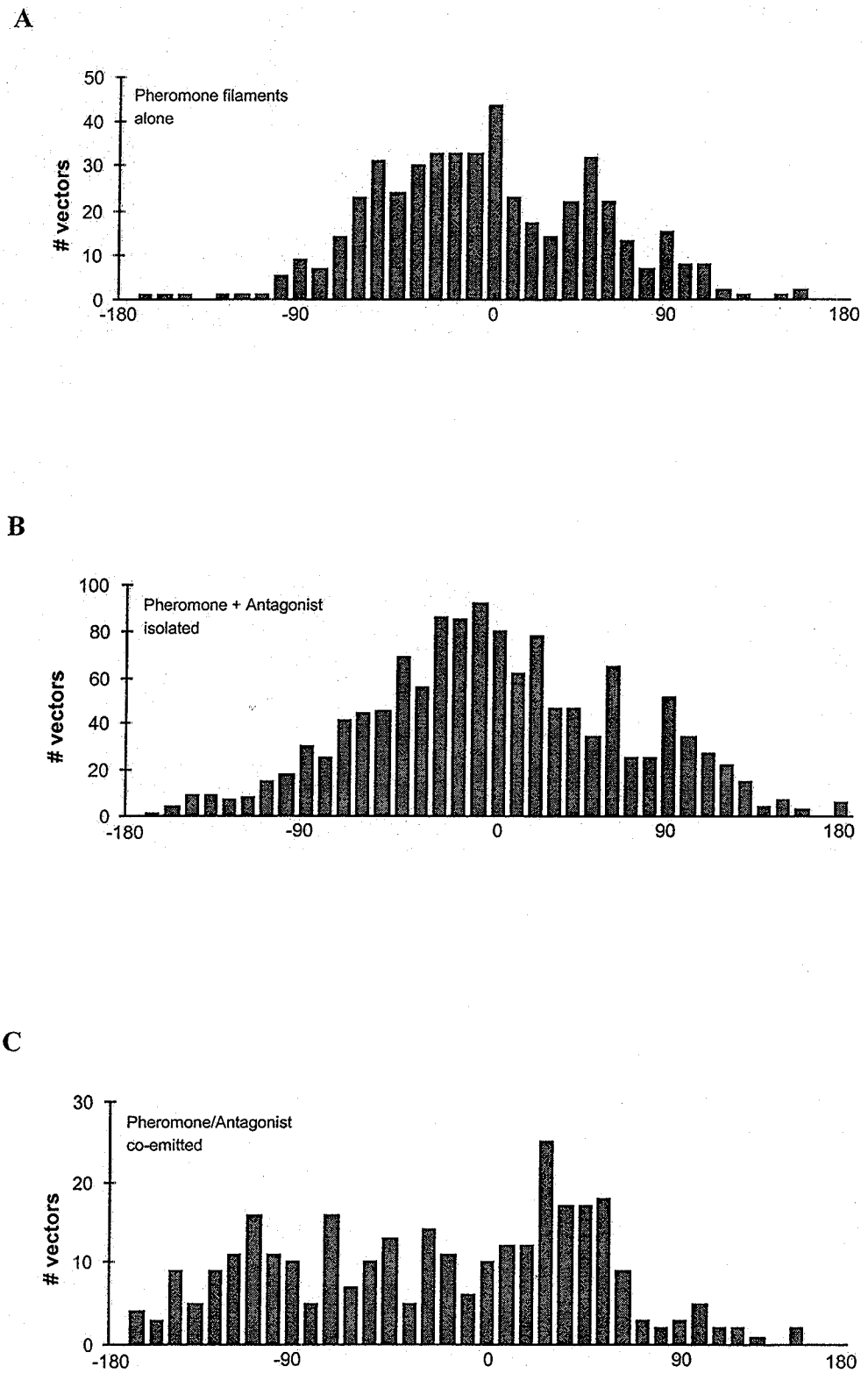

Fig. 6 Frequency distribution of the track angles steered by *H. zea* males in response to three different odor filaments generated at the rate of 5 pulses s^{-1}: pheromone filaments alone (**A**, $n=8$); pheromone and antagonist filaments simultaneously generated from different pipettes separated along the wind line by 1 mm (**B**, $n=12$); and pheromone and antagonist filaments co-emitted from a single pipette (**C**, $n=3$). Pheromone was 10 µg Z11-16:Ald + 5% Z9-16:Ald, while the antagonist was 5 µg of Z11-16:Ac. Track angles were classified into 10° bins from −180° to +180°. Males responding to pheromone filaments alone (**A**), or to partitioned pheromone and antagonist filaments (**B**) exhibited more direct upwind flight as evident by the unimodal distribution of their track angles, compared to the non-unimodal distribution of the track angles of males exposed to pheromone and antagonist filaments co-emitted from the same source (**C**)

responding to a complete pheromone blend (Quartey and Coaker 1993). Similar results were recorded for *Grapholita molesta* males released to off-ratio pheromone plumes (Willis and Baker 1988). Liu and Haynes (1993) also recorded a significant reduction in airspeed for male *T. ni* responding to an antagonist-tainted pheromone blend. The underlying mechanisms involved in the suppression of attraction and slower net upwind progress commonly observed for moths exposed to antagonist-tainted pheromone plumes have been elucidated by Vickers and Baker (1997). They attributed the stunted surges recorded for male *H. virescens* responding

to antagonist-tainted single filaments to the inability of these males to make significant changes in their airspeeds, course angles, and in their tempo of counterturning (Vickers and Baker 1997).

Mafra Neto and Cardé (1994, 1995) had demonstrated with *Cadra cautella* males the significant effect of plume structure on orientation. They recorded straighter, faster upwind flights with a unimodal distribution of track angles to fast-pulsed plumes than to slow-pulsed or continuous narrow plumes. In the current study, plume structure was also shown to have a profound effect on upwind flight, or its suppression. We recorded poorer suppression of upwind flight when the pheromone and antagonist sources were partitioned and simultaneously pulsed, compared to when both sources were generated as continuous plumes. As observed with smoke plumes of $TiCl^4$ (Fig. 1), the poorer suppression of upwind flight recorded for pulsed plumes may be explained by the incomplete mixing of pulsed filaments, compared to the more completely mixed strands produced downwind by turbulence and mixing following the continuous emission of the strands. However, the incomplete mixing of pulsed partitioned pheromone and antagonist plumes in the current study would only separate the filaments by, at maximum, 1 mm in space and by 0.003 s in time if the moth were stationary in the 40 cm s^{-1} wind.

That male *H. zea* were able to distinguish between filaments separated by 1 mm and in time by 0.001–0.003 s is intriguing. We propose that this remarkably high degree of resolution of closely spaced odor filaments, begins with the co-compartmentalization of two receptor neurons, one tuned to the antagonist and the other to a pheromone component, within the same antennal sensilla (Baker et al. 1998). Further resolution could also occur due to integration by interneurons in the antennal lobe. In the majority of moths studied thus far, receptor neurons tuned to antagonists are co-compartmentalized within the same sensilla as pheromone-component-tuned neurons (O'Connell et al. 1983; Van der Pers et al. 1986; Akers and O'Connell 1988; Hansson 1988). Such co-compartmentalization is found in heliothine moths (Berg et al. 1995a, b), including *H. zea* (Cossé et al. 1998). Two differentially tuned neurons cannot optimally report the synchronous arrival of the two components to which they are tuned unless they are located at the same point in space (Baker et al. 1998). Co-compartmentalization, such as is found in the Z11-16:Ac and Z9-16:Ald receptor neurons of *H. zea* (Cossé et al. 1998) entails that two different neurons are being housed in the same cuticular walls and bathed in the same aqueous solution of binding proteins, optimizing the ability of both neurons to sample the air at the same point in space and time. This mechanism of co-compartmentalization of neurons might also be involved in the accurate reporting and discrimination of pheromone component blend ratios, when such ratios are critical to male mating success, as in the Tortricidae and Crambidae (Akers and O'Connell 1988; Cossé et al. 1995).

Clearly, there should be an advantage conferred by the ability of a male to detect in the air non-conspecific compounds that will prevent erroneous upwind flight and mating mistakes. This is of particular importance when congeneric or sympatric species occupy the same ecological niche, such as occurs in heliothine moths. In this group which consists of at least four sympatric North American species that share Z11-16:Ald as a major sex pheromone component, Z11-16:Ac is only produced as a sex pheromone component by female *H. subflexa* and *H. phloxiphaga* (Teal et al. 1981; Klun et al. 1982). It therefore makes sense that this compound is a behavioral antagonist to upwind flight of *H. virescens* males (Vickers and Baker 1997) and *H. zea* males (Fadamiro and Baker 1997) when it is present in every filament of pheromone indicating that it originates from a single, non-conspecific female source. Males that can continue to fly upwind when they detect strands of pure conspecific pheromone, however, even in the presence of incompletely admixed antagonist filaments should be favored. Males that can discriminate strands of pure pheromone amongst those containing antagonist are in effect discriminating two separate female emitters upwind, one being a conspecific female.

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