

## Male *Heliothis virescens* Maintain Upwind Flight in Response to Experimentally Pulsed Filaments of Their Sex Pheromone (Lepidoptera: Noctuidae)

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Aspects of the intermittent fine structure of an odor plume were mimicked and experimentally modified in the wind tunnel using an air-pulsing device. Filaments of a behaviorally active blend of six sex-pheromone components created by the device in a temporally regular fashion elicited sustained upwind flight and source location in male *Heliothis virescens*. Males did not exhibit sustained upwind flight in significant numbers until a frequency of 4 filaments/s was delivered, at a loading of 1  $\mu$ g of the major component, Z11-16:Ald, with the other components loaded at their appropriate ratios. A loading of 10  $\mu$ g Z11-16:Ald was found to be optimal at this filament delivery rate. Electroantennogram recordings to different filament delivery rates of the complete blend indicated that a stationary male antenna can respond to the pulse rates used in this study. Importantly, when a main component necessary for upwind flight, Z9-14:Ald, was isolated into its own filaments and pulsed alternately against filaments of the five other components (including the other component essential for upwind flight, Z11-16:Ald), upwind flight to the source was significantly reduced (9%) compared to upwind flight and source location in response to filaments composed of the entire blend (30%), indicating that the complete pheromone blend must arrive on the antenna simultaneously for optimal evocation of sustained upwind progress. Neurophysiological evidence from other studies suggests that higher-order interneurons whose phasic response is enhanced when the entire blend is presented simultaneously may be of importance in explaining this behavioral

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*difference stemming from synchronous vs asynchronous arrival of the components.*

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### INTRODUCTION

The fine structure of an odor plume emanating from a point source is not a smooth gradient of homogeneous odor (Wright, 1958; Murlis and Jones, 1981; Murlis, 1986; Murlis *et al.*, 1990). Instead, due to eddies and small-scale turbulence at the source, the plume is heterogeneous in structure, with gaps of relatively clean air interspersing packets of odor-laden air. These packets, referred to as odor filaments, are formed close to the source and are borne away from it by the prevailing wind conditions. The intermittent fine structure of the pheromone plume is well maintained downwind of the source because it is mainly the wind, and not diffusion, that accounts for the displacement of the odor-bearing filaments. Indeed, the peaks and troughs in odor concentration can be detected by stationary moth antennae well downwind of the source both in the wind tunnel and in the field (Baker and Haynes, 1989). The same is true of plumes formed of ions as detected by stationary ion detectors (Murlis and Jones, 1981; Murlis, 1986; Murlis *et al.*, 1990).

Not surprisingly, the behavioral responses of a male moth to a conspecific female pheromone odor are reliant upon the intermittent nature of the signal. Several studies (Kennedy *et al.*, 1981; Willis and Baker, 1984; Baker *et al.*, 1985) have confirmed Wright's (1958) original notion that the intermittency of pheromone stimulation provided by the filaments is somehow important for orientation, a hypothesis that Wright's colleagues were unable to confirm with *Anagasta kühniella* in "homogeneous" pheromone clouds (Traynier, 1968). However, later studies with other species showed that the plume from a point source of odor within a homogeneous cloud creates enough of a rise and fall in concentration to sustain upwind flight, whereas a cloud alone fails to do so (Kennedy *et al.*, 1981; Willis and Baker, 1984; Baker *et al.*, 1985). Also, when the cloud itself is pulsed, swaths of clean air being interspersed with pheromone-bearing air, the moths are able to resume their upwind progress (Baker *et al.*, 1985). Moths also create their own intermittent stimulation by flying in and out of a homogeneous strip of pheromone-laden air bound down one side of a wind tunnel (referred to as a "side corridor") alongside a strip of clean air in the other half of the tunnel (Kennedy *et al.*, 1981; Willis and Baker, 1984). During their experiments on intermittent stimulation using two different species, both Kennedy *et al.* (1981) and Willis and Baker (1984) documented that initial

contact with pheromone, by a moth previously casting in clean air, was followed with an upwind surge by the moth.

Kramer (1986) performed a behavioral study with walking *Bombyx mori* males that showed that they responded better when pheromone was delivered at 3 pulses/s compared to continuously. This work was later expanded to show that modulation of long-lasting neuronal excitation, caused by hexadecadiene, an analogue of bombykol (Kaissling *et al.*, 1989), with pulses of a neuronal inhibitor compound (linalool) elicited upwind walking in *B. mori* males (Kramer, 1992). Cardé *et al.* (1984) determined the effect of an interrupted plume upon flying moths by mimicking the pulsing of the female pheromone gland but not the temporal fine structure of the plume.

In locating the source of a sex pheromone male moths are thought to integrate two behavioral mechanisms, optomotor anemotaxis (Kennedy, 1940) and counterturning (Kennedy and Marsh, 1974; Marsh *et al.*, 1978; Baker *et al.*, 1984). First, in optomotor anemotaxis, males visually monitor the wind-induced drift resulting from the discrepancy between their heading (course) and their actual track over the ground. The moth makes appropriate compensatory movements to steer in the upwind direction when stimulated by pheromone and across the wind upon losing pheromone (Kennedy and Marsh, 1974; Marsh *et al.*, 1978). The amount of wind-induced drift is revealed by the triangle of velocities (Kennedy and Marsh, 1974; Marsh *et al.*, 1978). Second, the counterturning aspect of odor-mediated responses by flying insects appears to be internally driven and is manifested by a series of temporally regular reversals back and forth across the windline during both upwind flight and casting following odor loss (Kennedy and Marsh, 1974; Marsh *et al.*, 1978; Kennedy *et al.*, 1980; Kennedy, 1983, 1986; Baker *et al.*, 1984; Baker, 1985; Baker and Haynes, 1987; Baker, 1990). Counterturning is integrated with the anemotactic component during casting to result in the track legs between reversals switching from being oriented moderately off the windline, but upwind, to being oriented at 90° or more across the windline, so that no upwind progress is made.

An alternative hypothesis (Preiss and Kramer, 1986a,b) suggests that counterturning is a reflection of internal "noise" within an upwind steering mechanism set at 0° (due upwind). However, moths reverse their tracks back and forth across the windline even in zero wind (provided they have been flying upwind in pheromone prior to wind off) (Baker *et al.*, 1984; Baker, 1985; David and Kennedy, 1987; Willis and Cardé, 1990). In this instance the track matches the course; thus the moths actively appear to steer across the former windline.

Receptor cells for pheromone components are housed in sensilla on the antenna. Each cell is known to have the lowest threshold to only one compound (not necessarily released by conspecific females), and axons from these cells converge in the macroglomerular complex of the deutocerebrum (Christensen

and Hildebrand, 1987a). Several neurophysiological studies have now replicated the intermittent stimulation that a male moth would receive during upwind flight in a sex-pheromone plume at both the antennal neuronal level (Kaissling, 1986; Baker *et al.*, 1988; Grant *et al.*, 1989) and the deutocerebral/higher brain center level (Christensen and Hildebrand, 1988; Christensen *et al.*, 1989b). Neurophysiological studies of projection interneurons within the deutocerebrum as well as protocerebral and descending interneurons have revealed phasic and tonic response profiles to pheromone blends (Christensen *et al.*, 1989a,b; 1991; Kanzaki *et al.*, 1991). Furthermore, specialist projection neurons that have a definitive response profile to the complete blend of pheromone components have been located in some species of moths. These cells may be especially relevant in mediating upwind flight response to the appropriate blend (Christensen *et al.*, 1989a,b) because males of one species, have been found to have the lowest behavioral threshold to the complete blend of components released by the female compared to partial blends or to single components (Linn *et al.*, 1985; Linn and Roelofs, 1989).

The nature of the theoretical behavioral mechanisms and the observed responses of male moths to intermittent stimulation coupled with the existence of blend-sensitive interneurons led to the recent proposal of a new model for sustained upwind flight (Baker, 1990), whereby a long-lasting tonic neural response at the onset of pheromone stimulation is suggested to maintain the counterturning aspect of the behavior, causing prolonged casting flight after pheromone loss. A phasic neural response to each individual filament would promote reiterative upwind surges, accounting for upwind progress. Thus each odor filament would cause a surge upwind, temporarily suppressing counterturning, while each packet of clean air would allow the waning of the phasically driven surge and the expression of the tonically driven counterturning oscillator. Other models to date (Kennedy and Marsh, 1974; Marsh *et al.*, 1978; Preiss and Kramer, 1986a,b) have centered largely on debating the existence and functioning of the orientation mechanisms (namely, optomotor anemotaxis and counterturning) using behavioral evidence. This latest model (Baker, 1990) attempts to go farther and link observed behaviors with known results from neurophysiological experiments. The strength of the model is that it is supported by both behavioral and neurophysiological evidence and thereby places the behavioral mechanisms onto a sound neuroethological foundation, especially with respect to counterturning.

We report here the results of experimentally regulating the intermittent fine structure of a pheromone plume and measuring the success of males in sustaining their upwind flight to these modifications in a wind tunnel. Using our technique we show, among other findings, that the complete blend of sex-pheromone components of *Heliothis virescens* needs to arrive on the antenna simultaneously in order to elicit significant levels of sustained upwind progress.

## MATERIALS AND METHODS

### Moths

*H. virescens* larvae were reared from eggs on a modified pinto bean diet (Shorey and Hale, 1965). Males were separated from females at the pupal stage and were maintained in an environmental chamber on a 14:10 L:D cycle at 25°C. Males were flown in the wind tunnel when they were between 3 and 8 days old. Prior to scotophase on the day that the moths were to be used, males were placed in individual screen cages 6 cm in diameter × 6 cm high (Vetter and Baker, 1983). These cages were placed on plastic trays (20 individual cages per tray) and the trays were returned to the environmental chamber. Males were flown between the fifth and the eighth hours of scotophase (Vetter and Baker, 1983). At least 1 h prior to the flight period the trays were removed from the environmental chambers and were placed inside the wind tunnel to afford the moths a period of acclimation to the conditions therein.

### Wind Tunnel

The wind tunnel utilized in this study is based on a design modified from Miller and Roelofs (1978), measuring 1 m (width at the floor level) × 0.9 m (high) × 3.65 m (length). Wind is generated by a 0.25-hp fan and pheromone-laden air is removed to the outside using a vacuum unit. The conditions within the wind-tunnel room were maintained as follows: 25°C, 0.5 lux (incandescent and red light mixture), and 60% RH. The wind speed was measured at 40 cm/s. Higher wind speeds made the puffs produced by the stimulus flow controller lean closer to the horizontal.

### Stimulus-Flow Controller

The stimulus-flow controller (=puffer) was custom-made by Murphy Developments Inc., The Netherlands (Model SFC-2). It consisted of two independent channels, each having a stimulus outlet port and an exhaust port. Pressurized air from a compressed air cylinder passed through the exhaust port of each channel at a controlled flow rate (1 to 50 ml/s). The air was filtered clean and dried before entering the intake valve of the puffer to prevent dust or water from entering the machine. A solenoid-activated switch within the machine channeled air from the exhaust port to the stimulus port of each channel according to a controlled frequency (ranging from 10/s to 1 pulse/10 s) and duration (0.02 to 10 s). In addition, each channel could be pulsed simultaneously or alternately with the other channel. Each stimulus port was connected to a stainless-steel pipette retainer with a rubber O-ring seal via 1.5 m of plastic tubing (4-mm o.d. × 2-mm i.d.). Glass Pasteur pipettes (Fisher Scientific, Catalog

No. 13-678-6A) were inserted into the retainers and air was pulsed through them by the puffer. Pipettes connected to both outlet ports were placed in a holding device within the wind tunnel, the barrel of each pipette pointing upward (Fig. 1). The holding device did not, in this position, affect the airflow over the pipette tips. In this series of experiments the flow rate and pulse duration were held constant at 5 ml/s and 0.02 s, respectively. Thus each pulse of air issuing from the pipettes had a volume of 0.1 ml<sup>3</sup>. In all experiments the puffer was employed in the alternating mode. Throughout this paper we refer to the following three terms noninterchangeably: a *filament* is the air extruded from the pipette delivered in each *puff*; each *puff* is produced by the mechanical *pulsing* of the stimulus-flow controller.

#### Pheromone Loading Procedure

Circular filter papers (Whatman No. 1) were cut into square-ending wicks 3.5 cm in length and 0.5 cm wide. The wicks were narrow enough to allow them to be pushed into the Pasteur pipette but also wide enough to prevent their falling out when the pipette was inverted. The filter-paper wicks were loaded with various complements of the known behaviorally active components from the female sex-pheromone gland: Z11-16:Ald, Z9-14:Ald, 16:Ald, 14:Ald, Z9-16:Ald, and Z7-16:Ald (Vetter and Baker, 1983; Teal *et al.*, 1986). These compounds were obtained from highly concentrated stock solutions maintained in the laboratory of T.C.B. Purity of the starting compounds was found to be greater than 99% by capillary gas chromatography (GC) on a Varian Model 3740 GC using a 30-m DB-225 column. A dosage series was prepared in hexane and the dosage loaded corresponded to the major component present, Z11-16:Ald (100), the other components being loaded at their appropriate ratios of 2.5:50:5:1:1, respectively, to match known female emission ratios (Pope *et al.*, 1982; Vetter and Baker, 1983). Ten microliters of solution were distributed evenly over the surface of each filter-paper wick using a micropipette. The hexane solvent was allowed to evaporate and the wick was placed inside the glass pipette.

#### Visualization of the Filaments

In order to visualize that the filaments generated by the puffer remained as separate entities during their passage through the wind tunnel, two pipettes had their barrels loaded with a small amount of cotton wool. A few drops of TiCl<sub>4</sub> were then placed on the cotton wool, the pipettes were fitted into their holder, and the puffer was operated at a variety of frequencies (ranging from 1 to 10 pulses/s). The smoke filaments created were observed throughout the 3-m length of the wind tunnel (Fig. 1).

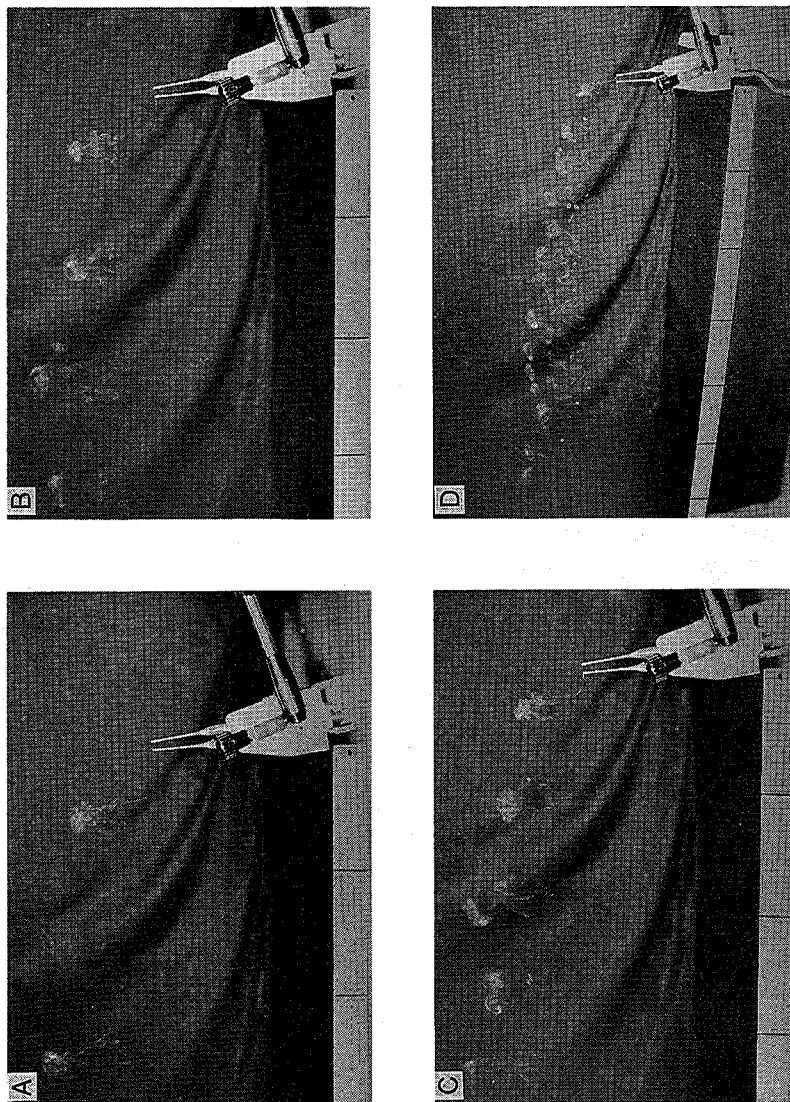


Fig. 1. Four frequencies of pulse delivery using  $TiCl_4$  smoke illustrate the mimicked intermittent structure of the experimental plume: (A) 1 pulse/s, (B) 4 pulses/s, (C) 5 pulses/s, and (D) 10 pulses/s. The filaments remained visually distinct throughout the length of the wind tunnel at all but the highest delivery rate (D). The meter rule along the floor of the wind tunnel is marked every 10 cm. In some photographs a small trail of smoke can be seen emanating from the pipette tip. This trail is caused by the accumulation of  $TiCl_4$  on the outer surface of the pipette during extended pulsing and is not known to occur in pheromone-laden pipettes.

### Electroantennogram Recording of the Filaments

A more biologically relevant way to confirm the integrity of the filaments over a range of delivery rates was provided by the use of the electroantennogram (EAG). A single, male *H. virescens* antenna was placed between two electrodes at a distance of 1.5 m from the pipette-tip source and EAG recordings were made according to the technique used by Baker and Haynes (1989). Pipettes contained filter-paper wicks loaded with 10  $\mu\text{g}$  of Z11-16:Ald plus the other five components at their appropriate ratios (Pope *et al.*, 1982; Vetter and Baker, 1983). Filaments were generated at a range of frequencies by the puffer (from 1 to 10 filaments/s) and the DC response of the antenna was recorded, after amplification on a Gould 2200 brush recorder.

### Behavioral Experiments

#### *Frequency of Filaments*

The frequency of filament production required to sustain upwind flight was examined by systematically varying the number of filaments of the complete sex-pheromone blend delivered per second. The loading of Z11-16:Ald was held constant at 1  $\mu\text{g}$ , with the other components present at their appropriate ratios (Pope *et al.*, 1982; Vetter and Baker, 1983).

Baker and Haynes (1989) found that a stationary antenna in the wind tunnel was stimulated by a plume from a rubber-septum point source between one and three times per second at a wind velocity of 50 cm/s. The following delivery rates were thus chosen to encompass the stimulatory rates previously found: 2 filaments/3 s, 1 filament/s, 2 filaments/s, 4 filaments/s, and 10 filaments/s. The frequency of pulses generated did not affect the amount of pheromone produced in each puff as checked by collecting the puffs in a dry-ice-cooled glass tube and quantifying the amount on a gas chromatograph. Two controls were also included: two pipettes containing filter-paper wicks loaded with 10  $\mu\text{l}$  hexane were pulsed in order to control for responses to sounds or other cues generated by the puffer, and also, pipettes laden with pheromone-bearing filter-paper wicks (1  $\mu\text{g}$  Z11-16:Ald plus the full complement of minor components) were attached to the puffer but were not pulsed. This second control was to check for possible leakage of pheromone from the pipette tips. The behavior of the moths was noted as follows: taking flight, upwind flight in response to pheromone, and successful location of the source of pheromone. As the males could not actually land on the source due to the filaments being produced by two glass pipette tips, approach to within 5 cm of the source and hovering was scored as "location." Observations were aided by the use of night-vision goggles.

#### *Effect of Pheromone Loading*

From the previous experiment it was demonstrated that there was a significant increase in upwind flight activity at the 4 filaments/s delivery rate (Table I). The dosage of Z11-16:Ald, with other components present at appropriate



**Table I.** The Effect of the Number of Filaments Produced per Second on the Ability of Moths to Sustain Upwind Progress to the Source<sup>a</sup>

Number of filaments produced	N	Percentage*		
		Take flight	Upwind flight	Locate source
2 per 3 s	57	82 cd	11 bc	4 bc
1 per s	59	86 bcd	8 c	3 c
2 per s	59	95 abc	13 bc	3 c
4 per s	57	100 a	25 ab	16 ab
10 per s	51	98 ab	35 a	29 a
Hexane-loaded pipettes, pulsed	48	71 de	2 c	0 c
Pheromone-loaded pipettes, no filaments produced	53	64 e	2 c	2 c

<sup>a</sup>The loading of Z11-16:Ald in all treatments was 1  $\mu\text{g}$ , with the other components present at appropriate ratios (see text for details).

\*Percentages in the same column having no letters in common are significantly different according to a  $\chi^2$  2  $\times$  2 test of independence with Yates' correction ( $P < 0.05$ ).

ratios, loaded onto the filter-paper wicks was next varied while holding the delivery rate constant at 4 filaments/s. The following loadings were examined: 100 ng, 1  $\mu\text{g}$ , 10  $\mu\text{g}$ , and 100  $\mu\text{g}$ . Again, to control for leakage, response to the 100- $\mu\text{g}$  pipettes, with no air pulses, was recorded. Behavioral observations made on the males were as in Frequency of Filaments (above).

#### *Effect of Temporal Partitioning of Blend Quality*

A binary mixture of Z11-16:Ald and Z9-14:Ald at an optimal ratio [40:1 loading on rubber septa (Vetter and Baker, 1983)] will elicit upwind flight in *H. virescens* males (Roelofs *et al.*, 1974; Tumlinson *et al.*, 1975; Teal *et al.*, 1986). The other four components improve the complete repertoire of flight behaviors but are not essential for upwind flight (Vetter and Baker, 1983; Teal *et al.*, 1986). Having established the optimal loading of Z11-16:Ald on the filter-paper wick to be 10  $\mu\text{g}$  (Table II), we proceeded to examine the effects of separating out these two main components of the female sex pheromone of *H. virescens* and pulsing them alternately at various delivery rates.

For *component* filaments, 10  $\mu\text{g}$  of Z11-16:Ald plus the four other minor components (excluding Z9-14:Ald) were loaded onto one filter-paper wick and placed into a single pipette. The other pipette was loaded with 0.25  $\mu\text{g}$  of Z9-14:Ald, the other component essential for upwind flight behavior. For *blend*-containing filaments, the Z11-16:Ald mixture and Z9-14:Ald were combined on the same filter-paper wick and one wick was loaded into each pipette. All attempts to measure the amount of Z11-16:Ald and Z9-14:Ald in each puff

Table II. The Effect of Filament Concentration on Upwind Flight<sup>a</sup>

Concentration loaded (Z11-16:Ald)	N	Percentage*		
		Take flight	Upwind flight	Source location
100 ng	78	76 b	8 bc	4 b
1 $\mu$ g	77	93 a	16 b	7 b
10 $\mu$ g	79	97 a	44 a	33 a
100 $\mu$ g	79	99 a	42 a	32 a
100 $\mu$ g, no filaments produced	75	75 b	3 c	2 b

<sup>a</sup>The rate of filament production was held constant at 4 filaments/s. The concentration loaded represents the amount of the major component, Z11-16:Ald, the other components being present at their respective ratios (see text for details).

\*Percentages in the same column having no letters in common are significantly different according to a  $\chi^2 2 \times 2$  test of independence with Yates' correction ( $P < 0.05$ ).

from 10- $\mu$ g pipettes failed to detect pheromone. However, at a loading of 100  $\mu$ g Z11-16:Ald, the emission rates of Z11-16:Ald and Z9-14:Ald were found to be  $4.1 \times 10^{-3}$  and  $5.8 \times 10^{-4}$  ng/puff, respectively, when the loading ratio was 40:1. This emitted ratio of 7:1 is close to the optimal ratio produced by a female and lies between previously reported female-release ratios (Pope *et al.*, 1982; Teal *et al.*, 1986). Also, using the pulsing device on a continual air-flow regimen through a 100- $\mu$ g blend pipette (100  $\mu$ g Z11-16:Ald and 2.5  $\mu$ g Z9-14:Ald on the same filter-paper wick), we collected amounts of these two main components giving a 15:1 ratio (Vickers and Baker, unpublished results).

For the *component* treatment, each filament contained alternately the Z11-16:Ald mixture and then Z9-14:Ald, while for the *blend* treatment each filament contained both the Z11-16:Ald mixture and Z9-14:Ald (Fig. 2). Frequencies of filament delivery were selected to ensure comparability between the component and the blend treatments. For instance, 2 filament/s blend is comparable to 4 filament/s component (Fig. 2). A moth flying directly upwind, intersecting every filament, would receive two stimulations with the Z11-16:Ald mixture and two stimulations with Z9-14:Ald every second, *simultaneously* with the *blend* filaments and *staggered* with the *component* filaments. Likewise other treatments in this experiment, 1 filament/s blend vs 2 filament/s component, and 5 filament/s blend vs 10 filament/s component, are comparable. Again, the behavior of the males in response to these different stimulatory regimens was noted as in Frequency of Filaments (above).

#### Statistical Analysis

In all three behavioral experiments, treatments were presented on the basis of a randomized complete block design. Differences within behavioral categories for each experiment were compared using a  $\chi^2 2 \times 2$  test of independence with Yates' correction (Steel and Torrie, 1961).

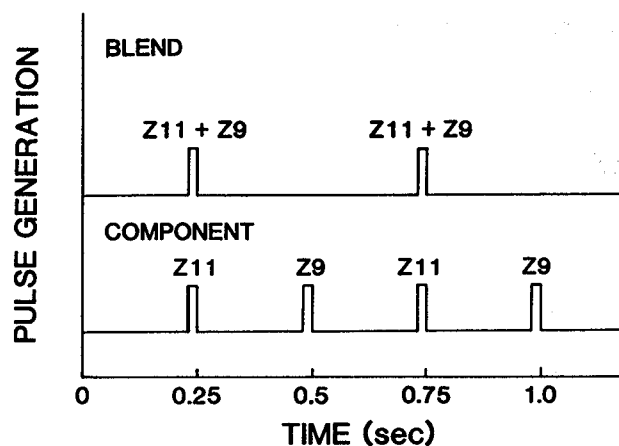


Fig. 2. A schematic representation of the filaments created by a comparable *blend* and *component* stimulus regimen. A moth flying directly upwind to a 2 pulse/s blend will encounter Z11-16:Ald (Z11) and Z9-14:Ald (Z9), plus the four other minor components, twice in a second. The 4/s component delivery rate is comparable because the moth receives the same stimuli per second (*i.e.*, two Z11-16:Ald and two Z9-14:Ald) but these main components are in alternate filaments, as opposed to being blended in the same filament.

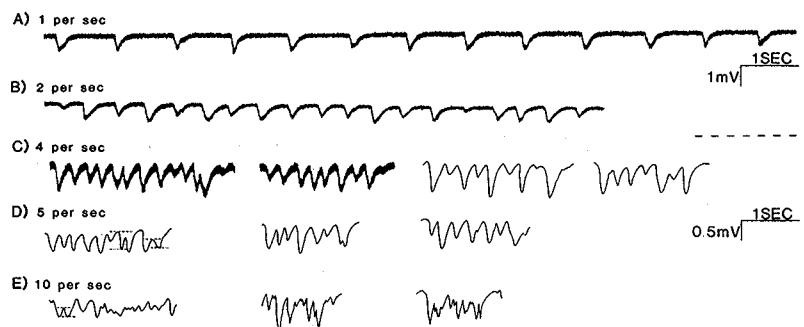
## RESULTS

### Visualization of the Filaments

Each packet of smoke issued from the pipette barrel as a thin "pencil" of molecules that then swelled into a blob. These smoke filaments did appear to remain as separate entities during passage through the length of our wind tunnel (Figs. 1A-C) at all but the highest filament delivery rate, 10 filaments/s in this study. Here, by halfway along the wind tunnel, filaments of smoke were mingling together and could not be easily separated visually (Fig. 1D).

### Electroantennogram Recording of the Filaments

EAG recordings confirmed, using the moth's own detector, that filaments of actual pheromone were in fact distinctly spaced in time (Figs. 3A-E). Trains of EAG depolarizations in response to a delivery frequency of 1 filament/s of the complete sex-pheromone blend could be recorded for extended periods (Fig. 3A). As the frequency of filament delivery increased, the ability of the antenna to respond to the arrival of each filament diminished. At 10 filaments/s, the fastest delivery rate possible (without actually moving the antenna up the wind tunnel), the antenna did not recover to baseline between filaments although there



**Fig. 3.** Electroantennogram recordings of different filament delivery rates indicate that a stationary male antenna is capable of responding to the pulse rates used in these experiments: (A) 1 filament/s; (B) 2 filaments/s; (C) 4 filaments/s; (D) 5 filaments/s; (E) 10 filaments/s. As the pulse rate was increased, the antenna appeared less capable of responding to the arrival of each filament but, even at 5 filaments/s (D), still recovered to near-baseline between filaments. At 10 filaments/s the peak-to-trough EAG amplitudes (between dashed lines) were nearly always reduced, and the antenna quite often failed to respond significantly to each filament. However, there were 1-s periods where 10 distinct "blips" occurred on the trace (E). Note that the vertical millivolt scale bar for A and B is different from that for C-E.

were periods, illustrated in Fig. 3E, where the EAG recording had 10 distinct DC "blips" in a 1-s period.

## Behavioral Experiments

### *Frequency of Filaments*

There was no significant sustained upwind flight until a delivery rate of 4 filaments/s was reached (Table I). At 2 filaments/s many moths kept station by counterturning in place above the takeoff platform, as often happens before locking onto the plume, but they were largely unsuccessful in locking on. At the highest filament delivery rate of 10 filaments/s, the greatest number of moths commenced upwind flight and reached the source, although the difference was not significantly greater than to 4 filaments/s. There was no significant response to the two controls, indicating minimal leakage from the pipette tips and also a lack of response to any other cues emanating from the puffer (Table I).

### *Effect of Pheromone Loading*

With a delivery rate of 4 filaments/s, the most effective loading of Z11-16:Ald, with the five other components present at their respective ratios, was found to be 10  $\mu\text{g}$  (Table II). Upwind flight reached a level of 44%, with many males continuing to the source (33% of the total). A 10-fold increase in the loading to 100  $\mu\text{g}$  did not result in a commensurate increase in the level of

**Table III.** The Effects on Sustained Upwind Progress of Pulsing *Blend* Filaments Composed of the Six Known Active Sex-Pheromone Components of *Heliothis virescens* or of Pulsing *Component* Filaments Alternating Z11-16:Ald (+ Four Minor Components) Against Z9-14:Ald<sup>a</sup>

Filament treatment	N	Percentage*		
		Take flight & lock	Upwind flight	Source location
1/s blend	64	41 bc	11 bc	0 c
2/s component	61	34 c	3 c	0 c
2/s blend	59	46 bc	17 b	2 bc
4/s component	59	44 bc	17 b	2 bc
5/s blend	56	84 a	48 a	30 a
10/s component	54	57 b	35 a	9 b

<sup>a</sup>The loading of the major component (Z11-16:Ald) was 10  $\mu$ g. Moths taking flight and attempting to lock onto the plume were noted in this experiment.

\*Percentages in the same column having no letters in common are significantly different according to  $\chi^2$  2  $\times$  2 test of independence with Yates' correction ( $P < 0.05$ )

sustained upwind flight, there being no statistical difference between the two treatments. Loadings of 100 ng and 1  $\mu$ g produced some upwind progress but the levels were significantly less than to the 10- and 100- $\mu$ g loadings. Again, the control indicated that there was negligible chance of males responding to leakage of pheromone from the pipettes (Table II).

#### *Effect of Temporal Partitioning of Blend Quality*

Of the comparable treatments in this experiment, only two caused upwind flight to be initiated at high levels: 5 filament/s blend and 10 filament/s component (Table III). Although similar numbers of males commenced upwind flight to both treatments (48 vs 35%, respectively), significantly fewer males sustained their upwind progress all the way to the source in response to the component filaments (30% blend vs 9% component). This important difference in response levels is not reflected at lower delivery frequencies, where few males were able to remain locked onto the plume and locate the source origin (Table III). This outcome was anticipated from the lack of response to blend filaments at a delivery rate of 2 filaments/s in experiment I (Table I).

## DISCUSSION

The results of the final *blend vs component* filament experiment clearly indicate that the blend of components must arrive on the antenna simultaneously for optimal levels of sustained upwind flight and source location to occur. Staggering the arrival of the Z11-16:Ald mixture and Z9-14:Ald significantly lowers source location in *H. virescens* males compared to that when all components

are blended in the same filament (Table III). The asynchronous arrival of the two main components of *H. virescens* sex pheromone, even by such a short time interval (less than 0.1 s if the moth is flying upwind), may translate into a suboptimal ability at the antennal lobe level to transmit the correct information about repeated filament arrival. Evidence exists in other species of moth that interneurons selectively responsive to blends are present in the deutocerebrum, higher brain centers, and descending pathways (Christensen *et al.*, 1989a,b; Kanzaki *et al.*, 1991). Some of these deutocerebral projection interneurons in *Manduca sexta* (Christensen and Hildebrand, 1987b; Christensen *et al.*, 1989a,b) have a very phasic response profile to each odor pulse, with a short inhibitory onset caused by one component, the trienal (or its mimic, C-15) (Tumlinson *et al.*, 1989), followed by a burst of spiking caused by the other component, bombykal. There is a sharp offset to the spiking burst due to continuing inhibition from the trienal; in some cases, however, the trienal is excitatory and bombykal is inhibitory (Christensen and Hildebrand, 1987b; Christensen *et al.*, 1989b).

Such interneurons that respond with an enhanced phasic pattern only to the blend may be important in sustained upwind flight to the pheromone according to the model of Baker (1990); they may shorten the response latency of both the anemotactic upwind surge with the onset of the filament and the quick extinction of the surge when clean air is contacted and casting flight commences in the moth (Baker, 1990). Christensen and Hildebrand (1988) found such blend-responsive  $-/+/-$  deutocerebral interneurons in *M. sexta* that could "follow" a pulse delivery rate of 10 filaments/s. However, these phasic interneurons specifically responsive to blends have not yet been found in *H. virescens*, although their presence appears possible (Christensen *et al.*, 1989b). Deutocerebral neurons in *H. virescens* were capable of following single-component stimulus pulses at 4 filaments/s (Christensen *et al.*, 1989b).

Our results show that a pulse rate of 10 filaments/s is not suboptimal to sustained upwind flight; therefore, these results are consonant with the proposition that *H. virescens* males have deutocerebral interneurons capable of responding to very high frequencies of filament interception. Indeed, males, as they thrust upwind, will effectively increase the number of filaments that the antennae cut through. That is not to say that each and every filament is intersected or processed by a freely flying moth, but it is almost certain that, at least during short intervals, the frequency of filaments encountered by a moth in our experiments will be greater than the output of the puffer. The somewhat overall low level of source location was not surprising given the fact that we should not anticipate our pulsing device, lacking interpulse strands of pheromone, to replicate flight levels observed with point source plumes that continuously emit filaments.

Interestingly, with our stationary EAG preparation the amplitudes of the peak-to-trough DC potentials were somewhat attenuated at the highest delivery

rate (Fig. 3E). The attenuation may well be counteracted somewhat by a higher airspeed of the moth or by higher windspeeds. Baker and Haynes (1989) demonstrated in *Grapholita molesta* that a higher windspeed created a sharper onset and a quicker return to baseline of the EAG than did a slower windspeed.

Olfactory receptor neurons in the antennae of male moths have been challenged with different pulsed odor regimes (Baker *et al.*, 1988; Grant *et al.*, 1989) up to a rate of 4 pulses/s. In some circumstances, especially high concentrations or cool temperatures, antennal neurons in some species cannot adapt fast enough to respond to repeated pulses (Baker *et al.*, 1988, 1989), possibly explaining behavioral arrestment of upwind flight before the source is reached under suboptimal conditions (Linn *et al.*, 1988; Löfstedt *et al.*, 1985). When *H. virescens* males were presented with high concentrations of pheromone, even under cool temperatures, no increased arrestment was observed, and likewise no significant adaptation of antennal neurons was recorded (Baker *et al.*, 1989). The sensory processing system of this species thus seems robust at handling rapid arrival of filaments and high concentrations, at least during synchronous arrival of components. Males did not seem capable of sustaining their upwind flight when fewer than 4 filaments/s were pulsed (Table I) at a loading of 1  $\mu\text{g}$  Z11-16:Ald. At a delivery rate of 4 filaments/s, it is possible that a single surge and suppression of counterturning lasts long enough for a moth to intersect the next filament (Baker, 1990), thereby repeating the phasic stimulation cycle and extending upwind progress without significant periods of casting. This possibility needs to be experimentally examined, however, with single pulses. If males are capable of responding, behaviorally, to a single pulse of pheromone, then the whole process of upwind flight may be entirely reiterative as proposed by Baker (1990). That is, each response to a filament with an upwind movement (a single iteration) might be the basic building block repeated again and again during the upwind flight process.

Given the decline in upwind flight response at less than 4 filaments/s (Table I), we might predict that *H. virescens* males respond to the loss of pheromonal odor by beginning to cast about 0.25 s after last pheromone contact. Other species of moths have been shown to have reaction times to the loss of pheromone in this range: *G. molesta*, 0.15 s (Baker and Haynes, 1987), and *Antheraea polyphemus*, 0.5 s (Baker and Vogt, 1989). More concentrated filaments may produce a more pronounced upwind surge and thus a more sustainable upwind flight pattern. A 10- $\mu\text{g}$  loading of Z11-16:Ald in the mixture produced a significantly greater number of upwind flights than either 100-ng or 1- $\mu\text{g}$  loadings (Table II). Increasing the loading 10-fold, to 100  $\mu\text{g}$ , did not result in any increase in upwind flight activity at a delivery rate of 4 filaments/s. A possibility not precluded by these experiments is that the 100- $\mu\text{g}$  loading would have elicited more flights than a 10- $\mu\text{g}$  loading at a delivery rate of 2 filaments/s. Although we know the amount of pheromone in each puff from 100- $\mu\text{g}$  loadings to be 4.1

$\times 10^{-3}$  ng of Z11-16:Ald and  $5.8 \times 10^{-4}$  ng of Z9-14:Ald, when the loading ratio was 40:1, we have been unable to collect measurable amounts at loadings of 10  $\mu$ g or less, even when hundreds of puffs were collected (Vickers and Baker, unpublished results). A 10-fold increase in loading may not result in a linear increase in the concentration of each pheromone puff.

Incorporation of behavioral results with known neurophysiological data has benefited both disciplines previously (Baker *et al.*, 1988; Olberg and Willis, 1990; Almaas *et al.*, 1991; Christensen *et al.*, 1991; Vickers *et al.*, 1991). Obviously more experimentation is needed in order to investigate the effects of filament frequency and concentration on male flight behavior. Nevertheless, our system, in concert with neurophysiology, provides a starting point for picking apart the many possible interactions among the intermittent fine structure of a plume, the pheromone quality, and the concentration that are involved in successful upwind flight to the source.

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