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Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths

(neuroethology/orientation/electroantennogram/pheromone plume/filaments)

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ABSTRACT **We characterized single upwind surges of flying male *Heliothis virescens* moths in response to individual strands of pheromone generated experimentally in a wind tunnel. We then showed how this surge functions in this species as a basic 13.4-cm, 0.38-sec-long building block that is strung together repeatedly during typical male upwind flight in a normal pheromone plume. The template for a single iteration, complete with crosswind casting both before and after the straighter upwind surging portion, was exhibited by males flying upwind to pheromone and experiencing filament contacts just frequently enough to produce successful upwind flight to the source, as hypothesized by an earlier model. Also as predicted, with more frequent filament contact by males, only the straightest upwind portions of the surges were reiterated, producing direct upwind flight with little crosswind casting. Electroantennogram recordings made from males in free flight upwind in a normal point source pheromone plume further support the idea that a high frequency of filaments encountered under the usual pheromone plume conditions promotes only these repeated straight surges. In-flight electroantennogram recordings also showed that when filament contacts cease, the casting, counterturning program begins to be expressed after a latency period of 0.30 sec. Together these results provide a plausible explanation for how male and female moths, and maybe other insects, fly successfully upwind in an odor plume and locate the source of odor, using a surging–casting, phasic–tonic response to the onset and disappearance of each odor strand.**

In the quest for understanding how male moths fly upwind and locate females (1–5) there have been suggestions (6, 7) that all odor-mediated flight in moths, including host plant location by females, may be explained by two programs, optomotor anemotaxis (2) and self-steered counterturning (8), that are turned on and modulated by odor fluctuations. It has also been pointed out (9, 10) that many other kinds of insects flying upwind to odor exhibit behavior somewhat similar to moths', and these other insects may also use these same mechanisms in odor-source location. Knowledge about the mechanisms used by moths should therefore be important for understanding the neuroethology of olfaction, the evolution of pheromone and host–plant–insect systems, and the potential application of pheromones in insect control.

The physical structure of a pheromone plume created by a point source of odor is not a time-averaged homogeneous cloud. Turbulence causes the plume to break up into strands of odor-laden air (filaments) interspersed with pockets of clean air where little or no odor is present (11, 12). The physical intermittency of these filaments is a requirement for male moths to sustain their upwind progress; they will not continue to fly upwind upon entering a homogeneous cloud of pheromone (4, 13) but will if this cloud is alternated with swaths of clean air (14).

Recently, results from experiments investigating the high-speed behavioral and neurophysiological responses of male moths to filaments of pheromone were brought together with existing knowledge in an alternative explanation (7) of how moths successfully locate pheromone sources. The model proposed that the process of upwind flight in a plume of odor may be entirely reiterative and dependent upon two neuroethological systems, one being phasic (15) that responds to each filament of pheromone to produce upwind surges, the other being tonic, long-lasting neuronal excitation (16) that is expressed in each pocket of clean air to generate counterturn-driven casting flight back and forth across the wind line. The latter flight serves to make the males pause in their upwind progress but scan sideways back and forth across even very large pockets of clean air, often with many-second time gaps between filaments, with the result that they increase their chances of recontacting the shifting, fenestrated plume (6, 7, 17–19).

Following some initial experiments with single pulses of pheromone with *Heliothis virescens* (L.) (20), we (21) concluded that male moths' responses to single pheromone filaments need to be examined in detail to determine how a reiterative system (7) can result in successful upwind flight (20). The males' reaction latencies and durations in response to individual filaments of pheromone and their latency of response to clean air, combined with knowledge of the frequency of filaments that are encountered during upwind flight (7, 20, 21), are the key to understanding how upwind flight is sustained in order to locate females. We addressed these aspects of odor-mediated flight in a series of experiments designed to measure these latencies, durations, and filament frequencies.

MATERIALS AND METHODS

Insects. *H. virescens* larvae were reared on a pinto bean diet (22). Males were separated from females at the pupal stage and were allowed to eclose in a separate environmental chamber at 25°C on a 16 hr:8 hr light:dark cycle. In all experiments males used were between the ages of 3 and 10 days.

Wind Tunnel. The wind tunnels used were based upon the design of Miller and Roelofs (23). The flying electroantennogram (EAG) and single pulse experiments were conducted in a 3.5 × 1.0 × 1.0 m wind tunnel at the University of California, Riverside (UCR). The responses of male moths to different pulse frequencies were ascertained in a wind tunnel of the same construction at Iowa State University, but 1.22 m shorter than the UCR wind tunnel. Since the video analysis fields of view were the same distance from the source in all experiments, the differences in tunnel length do not signifi-

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Abbreviation: EAG, electroantennogram.

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cantly affect the moths' behaviors that we analyzed. Wind speed in all experiments was 0.6 m/sec and light intensity, composed of a mixture of red and white light, was 0.5 lux.

Pheromone. In all experiments the complete blend of six behaviorally active components (24) in an appropriate ratio (25), relative to the major component, Z11-16:Ald, was used. For experiments utilizing the pulsing device (single and multiple pulse), 100 μg of Z11-16:Ald was loaded onto filter paper strips (Whatman no. 1, 3.5 × 0.5 cm) held inside glass pipettes (Fisher Scientific, no. 13-678-6A). For the flying EAG, 10 mg of Z11-16:Ald was loaded onto a rubber septum (A. H. Thomas, no. 8753 D22, sleeve type, 5 × 9 mm).

Single and Multiple Pulse Experiments. To examine the latency of response to filaments and clean air, *H. virescens* males were induced to fly upwind in an experimentally pulsed plume that mimicked the intermittency of a plume from a continuously emitting point source (20, 21). A pulsed plume of 10 filaments per sec was generated using air puffed through two pheromone-containing cartridges by an air-pulsing device (Syntech, Hilversum, The Netherlands, model SFC-2). The flight tracks of males that flew upwind were recorded by two video cameras (8, 26) located beneath the transparent, Plexiglas floor of the wind tunnel. As the males entered the cameras' 0.5 × 0.3 m field of view, 1–1.5 m downwind of the source, the plume was truncated by turning the pulsing device off. At the same instant a series of sequentially flashing red lights was initiated. The lights flashed down the wind tunnel at a velocity calibrated to match the wind speed. Hence the final filament and the lights traveled down the wind tunnel at the same speed. Single pulses were then generated by the pulsing device, each odor pulse demarcated by the sequentially flashing lights traveling down the wind tunnel (20). Males' responses to the truncation of the plume and responses to the interception of single filaments, both events timed according to the lights moving down tunnel past the moth, were recorded on videotape for later review (20).

An experiment was then conducted to measure the flight responses of male *H. virescens* to multiple pulses as might occur in a normal plume. Multiple filaments were extruded from the same pipettes as used in the single pulse experiment at pulse rates of 1, 2, 4, 5, and 10 pulses per sec. Pulses were 0.02 sec in duration and the air flow rate was held constant at 5 ml/sec. Flight tracks were recorded on videotape for later analysis.

Flying EAG. Fine Teflon-coated silver wires (diameter 0.3 mm) were inserted into the ends of an excised male *H. virescens* antenna. A small triangle of Velcro (fuzzy side) was attached to the wires, close to the antenna. When complete the antennal preparation was taken to the wind tunnel where males had been acclimating to ambient scotophase conditions for at least 1 hr, having had a small triangle of Velcro (hook-side up) attached to their thorax just prior to scotophase. The other ends of the silver wires (up to 1 m in length) were connected to an amplifier. A single male, with both antennae intact, was then selected and the antennal preparation was attached to the male by hooking the opposing pieces of Velcro on the EAG wires and the thorax of the male together. This (third) antenna was positioned so that it lay laterally across the head of the transporting male, allowing EAG depolarizations to occur regardless of which one of the two intact antennae might be struck with a pheromone filament.

The males were given a minute to recover from the handling procedure necessary for the attachment of the EAG and were then placed on a platform, 1.5 m downwind and in line with a point source of pheromone. Flights were recorded by a video camera located above the wind tunnel, looking downward with a field of view of 90 × 75 cm, and the EAGs on the oscilloscope screen were recorded by a second camera. The two images were synchronized to within 0.01 sec by a pair of simultaneously activated time–date generators.

RESULTS

In the single pulse experiment, all males responded to the truncation of the plume by entering into casting flight. Males that were still flying upwind when the last filament passed by the moth in the field of view (and hence probably intercepted one of the last possible filaments produced by the pulsing device) began casting within an average of 0.27 ± 0.1 sec (±SD). About 32% (n = 192) of these casting males subsequently responded to a single filament by making a short upwind surge followed by a lapse into casting flight. Of these responding males, only 13 (or 7%) of the tracks could be used because the surge was started and finished within the cameras' field of view. The surge was deemed to begin when the track angles of the vectors attained an upwind value of 60° or less (0° is directly upwind) and to have ended when this average decayed to >60° (more crosswind). No casting males exhibited any upwind displacement in the control situation when no filament was generated.

Three of these complete surges are shown in Fig. 1*A*. The latency between filament interception and the surge averaged 0.30 ± 0.16 sec (±SD, n = 13) (Fig. 1*Bi*) while the surge itself had a duration of 0.38 ± 0.12 sec (±SD). The males covered an average of 13.4 ± 6.2 cm (±SD) in the upwind direction during this time. Using the tracks of all 13 males, we calculated an average cast–surge–cast track that then served as a template for later comparisons (Fig. 1*C*). This was accomplished by combining the information from average track angles (Fig. 1*Bi*) and average ground speeds (Fig. 1*Bii*) for each 1/30th of a sec, synchronized by the passage of the filament past each moth (odor-on signal). Values for counterturning frequency, 2.38 ± 0.45 (±SD) counterturns per sec before and after and 3.33 sec ± 0.51 (±SD) during the surge, were also integrated into the cast–surge–cast template.

In the multiple pulse experiments, we showed that males were not able to fly upwind in plumes consisting of <4 filaments per sec, although they did become activated and cast for short periods of time out of the camera's field of view. This is in close agreement with our previous observational account (20, 21). The tracks in response to 4 filaments per sec (Fig. 2*A*) were more tortuous in shape than at 10 per sec (Fig. 2*B*), where many tracks had extended periods of nearly straight upwind flight and fewer periods of crosswind casting. These differences are reflected in the distributions of track angles of all of the 1/30th sec vectors, which were bimodally distributed around ±90° (Fig. 2*A*) as compared to a unimodal distribution centered around 0° (Fig. 2*B*), indicative of more crosswind casting flight at 4 per sec than at 10 per sec. At 5 per sec (not shown) the flight tracks and distribution of track angles were slightly more upwind and intermediate in appearance between those of the 4 per sec and 10 per sec filament frequencies. The more straight upwind distribution of track angles cannot be due to a compensatory response to changes in wind velocity, since only filament frequency was varied in these experiments.

Many of the actual flight tracks produced in response to 4 filaments per sec, a sufficient frequency to sustain upwind flight, are composed of visible templates of an average moth responding to a single pulse (surge) followed by clean air (cast) strung together in succession (Fig. 2*A*). Importantly, whenever males did cease upwind progress at the higher pulse frequencies before resuming upwind movement (presumably due to recontacting pheromone pulses since upwind movement was never observed during casting in clean air), the initial part of the surge following odor contact also resembled that of the template (Fig. 2*B*). Thereafter the tracks became canalized directly upwind, which can be explained by the repeated evocation of only the middle, upwind-most section of the surge by faster contact with filaments than casting could be evoked by pockets of clean

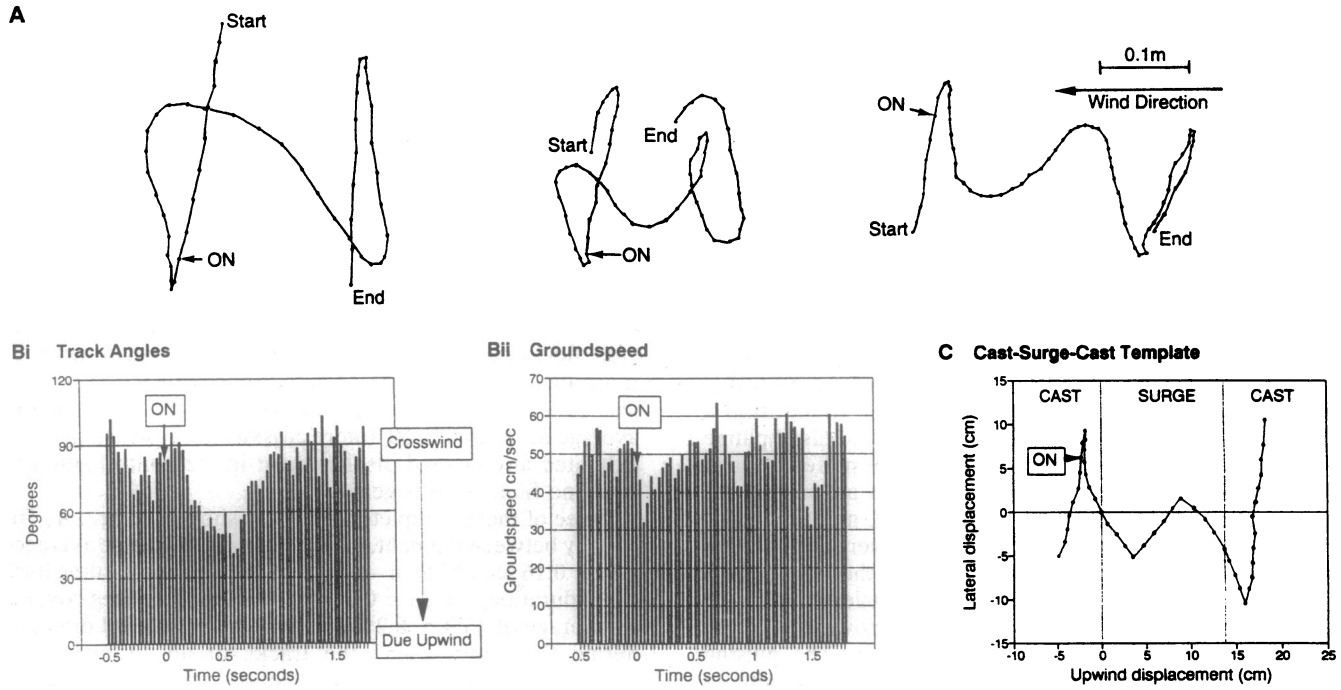

FIG. 1. (*A*) Three responses of flying *H. virescens* males to the passage of a single strand of conspecific pheromone (ON). The many casting track legs at the beginning and end of the tracks have been omitted in the drawings to clarify the cast–surge–cast transitions. Dots are 1/30 sec apart. (*B*) Average track angles (*i*) and ground speeds (*ii*) of 13 males that responded to contact with a single filament of pheromone by making an upwind surge. Tracks are synchronized by the passage of the filament at the ON signal and each bar represents the average for 1/30th of a sec. (*C*) A combination of the average track angles and ground speeds (*Bi* and *Bii*, respectively) plus counterturning frequency produces a cast–surge–cast template.

air. This latter supposition was supported by results from the EAG experiments.

Intervals between successive hits with pheromone, as indicated by EAG activity, showed that males making relatively steady upwind progress with no overt casting intercepted >5 filaments per sec (mean interval, 0.19 ± 0.12 sec; ±SD, n = 28), which was greater than for males flying crosswind (<4 filaments per sec; mean interval, 0.28 ± 0.23 sec; ±SD, n = 19), or for antennae held stationary in the plume before flight (3.4 ± 0.74 filaments per sec; ±SD, n = 4 antennae).

On average, males that were flying upwind turned crosswind if no further filaments were contacted in 0.30 ± 0.17 sec (±SD, n = 15). Males that were flying crosswind upon encountering a filament turned their tracks upwind in an average of 0.23 ± 0.11 sec (±SD, n = 12) following contact. These results using a typical pheromone plume emitted by a standard dispenser correspond closely to the results from the single pulse experiment indicating that the surge duration in response to pheromone was 0.38 sec before decaying into casting flight and that casting males responded to a filament itself within 0.30 sec.

In Fig. 3 the track of one male (Fig. 3*A*) shows that it is hit by one filament just before and two filaments just as it takes flight, producing an upwind trajectory. During this surge, the male is hit by three filaments (gaps between filaments are F–1, 0.18 sec; 1–2, 0.18 sec; and 2–3, 0.08 sec). Filament 1 appears to have no effect on the behavior because after the average 0.23-sec latency period, now 0.26 sec later (just after no. 3) the moth begins to go into casting flight across wind to the left. Filaments 2 and 3 occurred too late in the surge to prevent this cast, but 0.27 sec later (and before casting is fully established) the moth does begin another upwind surge. The male then contacts three more filaments in quick succession, filaments 4 (0.17 sec after no. 3), 5 (0.23 sec after no. 4), and 6 (0.13 sec after no. 5), and these are apparently sufficiently frequent to sustain the upwind movement. Following filament 6, the moth turns its track across the wind line after 0.5 sec without filament contact.

In Fig. 3*B* the male initiates flight in the upwind direction. Almost at takeoff he receives a filament and then again shortly after taking flight (0.27 sec, no. 1). Upwind flight appears to be sustained owing to the sequential arrival of filaments with short gaps between them (0.20 sec, nos. 1–2; 0.07 sec, nos. 2–3; 0.08 sec, nos. 3–4; 0.17 sec, nos. 4–5). After filament 5, there is a latency period of 0.43 sec without further contact with pheromone filaments before the male turns its track crosswind on a casting leg.

DISCUSSION

The results of the present study provide direct evidence that a fast-acting phasic–tonic, surging–casting response system, acting reiteratively in response to pheromone filaments, is responsible for creating sustained upwind flight in odor plumes by moths. Single surges consisted of a straighter upwind portion that decayed into casting as counterturning frequency declined and the anemotactic system shifted to allow more crosswind, instead of upwind, movement (Fig. 1). Faster filament pulse rates created straighter upwind tracks with the straighter portion being evoked more repeatedly, there being no time for casting to be expressed (Fig. 2). EAG recordings from males flying upwind in a typical pheromone plume (generated from a rubber septum impregnated with pheromone) confirmed that encounters with a train of filaments averaged 5.4 per sec and produced fairly straight upwind flight. These typical, somewhat zigzag-shaped, tracks were thus produced by a filament contact rate greater than the 2 per sec rate that produced no upwind flight, only casting, in the pulsed filament experiment or than the 4 per sec pulse rate that produced upwind flight and source location, but with casting, tortuous flight tracks (Fig. 2). At this latter experimentally pulsed frequency, during which upwind flight was only minimally sustained, the beginning and the

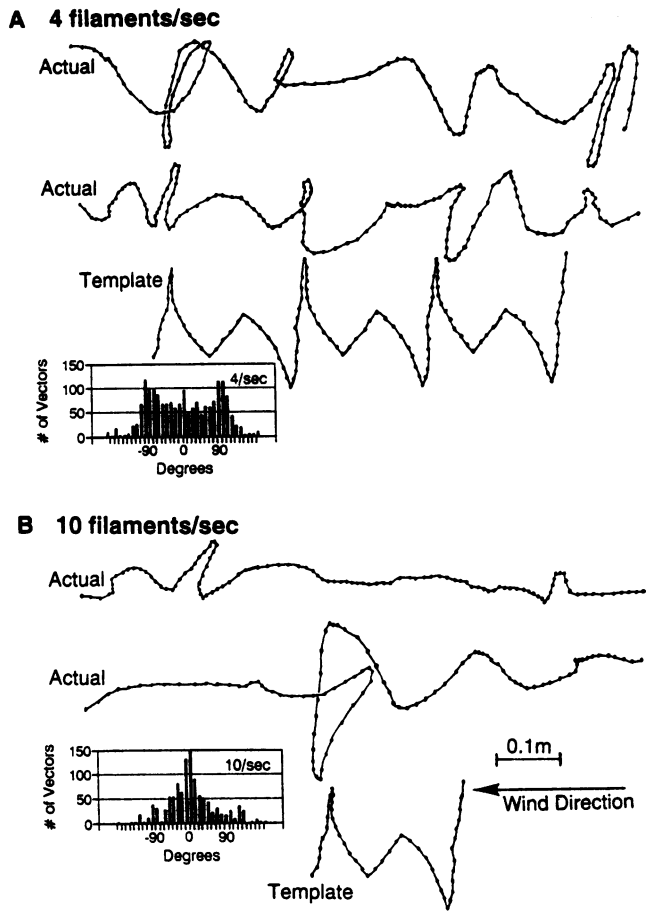

FIG. 2. (*A*) Flight tracks of two male *H. virescens* in response to a pulsed plume generated at 4 filaments per sec. The cast–surge–cast template (Fig. 1*C*) laid end to end indicates that the upwind progress of these males is due to the repetitive evocation of single surges interspersed by casting. (*B*) Flight tracks of two males in response to a plume generated at a rate of 10 filaments per sec. The middle, upwind-most part of the surge is reiteratively evoked by fast filament contact resulting in straighter upwind tracks compared to 4 filaments per sec. When males did lose the train of pulses at these higher production rates (lower track), the initial part of the recovery following pulse recontact resembled the first part of the cast–surge–cast template, before becoming canalized more upwind once again.

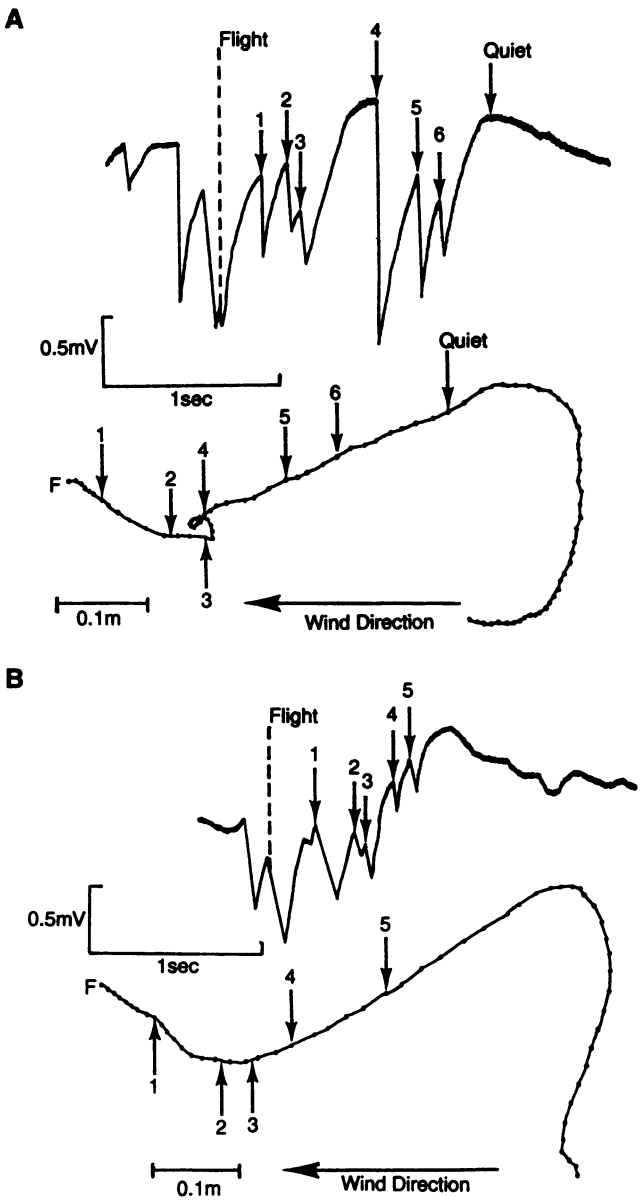

FIG. 3. Tracks of two males plus the EAG traces recorded from an excised antenna mounted on each of their thoraces during flight. Numbers along each track refer to the successive EAG depolarizations that occurred as each moth flew.

end (casting) of the surge template was repeatedly revealed (Fig. 2*A*). In the flying EAG experiment, the middle, straighter upwind portion was apparently expressed repeatedly and frequently enough to prevent casting flight from occurring. Failure to contact a filament, however, eventually resulted in a turn across the wind line (commencement of casting flight) 0.30 sec later.

The surge duration can be viewed as the latency to casting upon entering clean air, hence the need for a highly phasic neuroethological response as part of the entire system. The male must react quickly to the filament by surging upwind, thereby advancing directly toward the source (18), but he must also react to the pocket of clean air behind it to keep from plunging too far into a large void of clean air caused by a significant change in wind direction at the source (6, 7). To react too slowly and continue upwind in such a pocket would mean going astray from the toward-source direction and also from the strands of pheromone that have swung away from the male (6, 7).

Although it is now apparent that measuring reaction times to odor onset and loss is critical to understanding odor-mediated orientation by moths (6, 7), the reaction times to odor loss have only been estimated for a few species (19, 20, 26–29). Moreover, in only two species have the response times (latencies and durations) to pheromone onset been measured: *Grapholita molesta* (Busck) (19) and *H. virescens* (ref. 20; this study). Males of the latter species can fly very straight upwind both in the field and in the laboratory (20). Our findings that *H. virescens* males react more slowly to clean air and hence have longer-duration surges than *G. molesta* males support the phasic–tonic model for upwind flight and explain why *H. virescens* males can have much straighter tracks than *G. molesta*, whose tracks are nearly always zigzag-shaped. *H. virescens* males will be less likely to go into casting flight and zigzag like *G. molesta* in plumes of similar structure (and filament frequency) because *H. virescens*' longer-duration surges carry them into filaments more often before casting can be fully expressed. Recent results from another species, *Cadra cautella* (Walker), show relationships between track straightness and response to single filaments (30) remarkably similar to those found in *H. virescens* (ref. 20; this study).

The findings of the present study may be important for understanding all odor-mediated flight in moths and the neuroethology of moth olfaction, in light of phasic and tonic response systems (7). Two studies have demonstrated many remarkable similarities between the flight tracks of female moths responding to host odor with the tracks of males of the same species responding to sex pheromone (26, 29), including the presence of counterturning upon odor loss, as well as optomotor anemotaxis, which must be used by all flying moths in order to advance upwind (6, 17). The likelihood that similar, if not identical, phasic–tonic, surging–casting systems are employed by both males and females in upwind flight to odor is indicated even more strikingly in reviewing the results of antennal transplantation experiments performed by Schneiderman *et al.* (31). They showed that female *Manduca sexta* (L.) possessing male antennae, transplanted as imaginal discs during pupal development, flew upwind in response to sex pheromone plumes in a manner similar to that of pheromone-stimulated males, including the typical zigzagging flight. Taken together, these results coupled with our current findings point to the likelihood that male and female upwind flight is governed by the same phasic–tonic, surging–casting systems, both behaviorally and neurophysiologically.

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