

## A pulsed cloud of sex pheromone elicits upwind flight in male moths

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**ABSTRACT.** Male oriental fruit moths do not fly upwind in a continuous uniform cloud of pheromone, but readily do so when the cloud is pulsed at 1 or 0.5/s or when a plume from a point source of pheromone is placed within the continuous cloud. It is suggested that males of moth species that require such fluctuating pheromone stimulation for upwind flight will normally receive it from a filamentous, point-source-produced plume. However, we hypothesize that upwind progress may cease close to the source due to excessively high emission rates or inappropriate blend ratios, when fluctuating sensory output becomes attenuated, despite higher actual molecular concentration fluctuations.

**Key words.** Sex pheromone, *Grapholita molesta*, Lepidoptera, Tortricidae, orientation, upwind flight, intermittent stimulation, odour perception.

### Introduction

Hypotheses about how the sex pheromone blend ratios and emission rates of female moths attract males have centred around the concept of a behaviourally 'active space' (Bossert & Wilson, 1963), in which a time-averaged concentration of pheromone is above the response threshold for upwind flight. Recently there has been increasing evidence, however, that for some species the continuous presence of pheromone, regardless of average concentration, does not result in such behaviour (Kennedy *et al.*, 1980, 1981; Kennedy, 1982;

Willis & Baker, 1984). Rather, experiments have strongly suggested (Kennedy *et al.*, 1980, 1981; Willis & Baker, 1984) that upwind flight is elicited by significant amplitudes and frequencies of peak-to-trough fluctuations (Kennedy, 1983) in pheromone concentration created by a plume's filamentous structure (Wright, 1958).

We demonstrate here, more directly than in earlier studies, that fluctuating, discontinuous, pheromone stimulation produces upwind flight in male moths. In addition, we suggest that such flight, having begun far downwind under intermittent conditions characteristic of pheromone plumes, may cease close to the source when, as a result of an excessively high concentration or incorrect blend ratio, the fluctuating signal becomes too uniform.

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### Materials and Methods

Male oriental fruit moths, *Grapholita molesta* (Busck), were released three at a time from a 6.5×8 cm (height×diameter) screen cone, 30 cm from the downwind end of a previously described 1.8×0.6×0.6 m wind tunnel (Willis & Baker, 1984). Wind velocity was 0.5 m/s, created by means of a 1/5 h.p. rotary-blade blower (Dayton Corp.). All air in the tunnel was exhausted continuously via a 30 cm diameter duct connecting a fume hood to a 0.6 m long sheet-metal extension which sealed the downwind end of the tunnel's plexiglas working section. Air pressure was equalized by means of a valve in the exhaust connection so that when moths were introduced via a side door in the sheet-metal section, no perceptible disruption of the continuous or pulsed clouds occurred, as visualized by smoke.

The fine-mesh brass screening and metal strips used previously for uniform cloud formation (Willis & Baker, 1984) were left in place, but instead of rows of pheromone-impregnated rubber septa placed downwind of the strips, pheromone was introduced into the blower via an airstream blowing over septa housed in a 27×3.5 cm (length×diameter) glass tube. Each septum was impregnated with 1000 µg of the optimum *G. molesta* three-component pheromone blend: 6% (*E*)-8-dodecenyl acetate and 4% (*Z*)-8-dodecenyl alcohol (Cardé et al., 1979) in (*Z*)-8-dodecenyl acetate (Roelofs et al., 1969). The septa were placed on a rack 8 cm from the mouth of the tube and arranged so that they formed a disc 3 cm thick across the tube, whose mouth was 6 cm from the blower intake. A vacuum was drawn continuously from the base of the tube to prevent pheromone from being emitted. Either intermittent or continuous clouds were generated by opening a valve which released pressurized air into the base of the tube. The vacuum was thus overcome, creating an airstream blowing rapidly over the septa, thereby injecting pheromone-laden air into the blower. Only clean air entered the tunnel between emissions. The tempo of pulsing, either 1 or 0.5/s, was performed by manually opening and closing the pressurized air valve in time to an electronic tone beeping at 1/s. The duration of each burst of air through the tube was kept as short as possible with this mechanism, 0.25 s

on average. A point-source plume of pheromone was created using a rubber septum impregnated with 30 µg of the same blend used for clouds. The septum was suspended with nylon filament 20 cm above the floor, 10 cm from the tunnel's upwind end.

Our ability to produce a continuous uniform cloud of pheromone or a 'pulsed' cloud interrupted by periods of cleaner air was verified visually using TiCl<sub>4</sub> smoke emanating from dispensers in the pulse-generating device, by infra-red photocell recordings of the smoke pattern (Fig. 1A), and by recording electro-antennogram (EAG) responses to pulsed and continuous pheromone clouds (Fig. 1A) using the same concentrations as in the behavioural experiments. To the eye, the continuous cloud of smoke from the pulse-generator appeared as a uniformly grey fog, and the pulses of smoke during intermittent clouds appeared as more or less rectangular swaths of c. 0.4 m length travelling down the tunnel, sandwiched between swaths of nearly-clean air.

The photometer was placed on a circular metal ring (12 cm diameter) opposite an infra-red light-emitting diode. The ring was positioned perpendicularly to the wind, at the same position and height as the males' take-off point. Smoke interrupting the light beam was registered as a decrease in light intensity, and this fluctuation was amplified and recorded on a Gould Brush 220 strip chart recorder. EAG preparations were made using intact males (Kuenen & Baker, 1981) and were recorded at the position and height of the males' take-off point. The same pheromone septa and concentrations used during behavioural recordings were used for EAGs. Responses were recorded on a Hewlett-Packard 3390A integrator used as a strip-chart recorder.

All experiments were conducted 0–2 h before lights-off; the optimal time of sexual activity for this species (Baker & Cardé, 1979), at 880 lux fluorescent and incandescent lighting intensity. Several seconds after the pheromone regime had begun, the three males were placed, point of cone down, in a ring-stand (8.4 cm diameter) located 20 cm above the floor in the centre of the tunnel, 30 cm from the downwind end. Distance of up- or down-tunnel flight was scored as the first point at which males touched the tunnel's walls, whereupon observations were terminated. Flight

tracks of these males were also video-recorded and analysed using video-recording equipment and computer analyses of digitized tracks described previously (Kuenen & Baker, 1982; Willis & Baker, 1984). The video-camera's field of view encompassed the entire width of the tunnel and from 30 cm downwind to 70 cm upwind of the release cone.

### Results

A significantly greater percentage of males took flight in the point-source plume (88%<sup>b</sup>,  $n=60$ ) and in the clouds of pheromone pulsed at 0.5/s ('slow') (100%<sup>a</sup>,  $n=59$ ) and 1/s ('fast') (92%<sup>ab</sup>,  $n=60$ ) than when the cloud was continuously presented to males (67%<sup>c</sup>,  $n=60$ ) or in clean air (45%<sup>d</sup>,  $n=60$ ) (percentages having no letters in common are significantly different according to the Ryan (1960) test;  $P<0.05$ ). Additionally, a greater percentage of the males that took flight flew upwind in the pulsed clouds (56%<sup>a</sup>, fast; 63%<sup>a</sup> slow;  $n=55$  and 59, respectively) than in the continuous one (10%<sup>b</sup>,  $n=40$ ), and their distances of upwind progress were significantly greater than those of males in the continuous cloud. The latter males did not fly significantly farther upwind than those flying in the absence of pheromone ('clean air') (Fig. 1E).

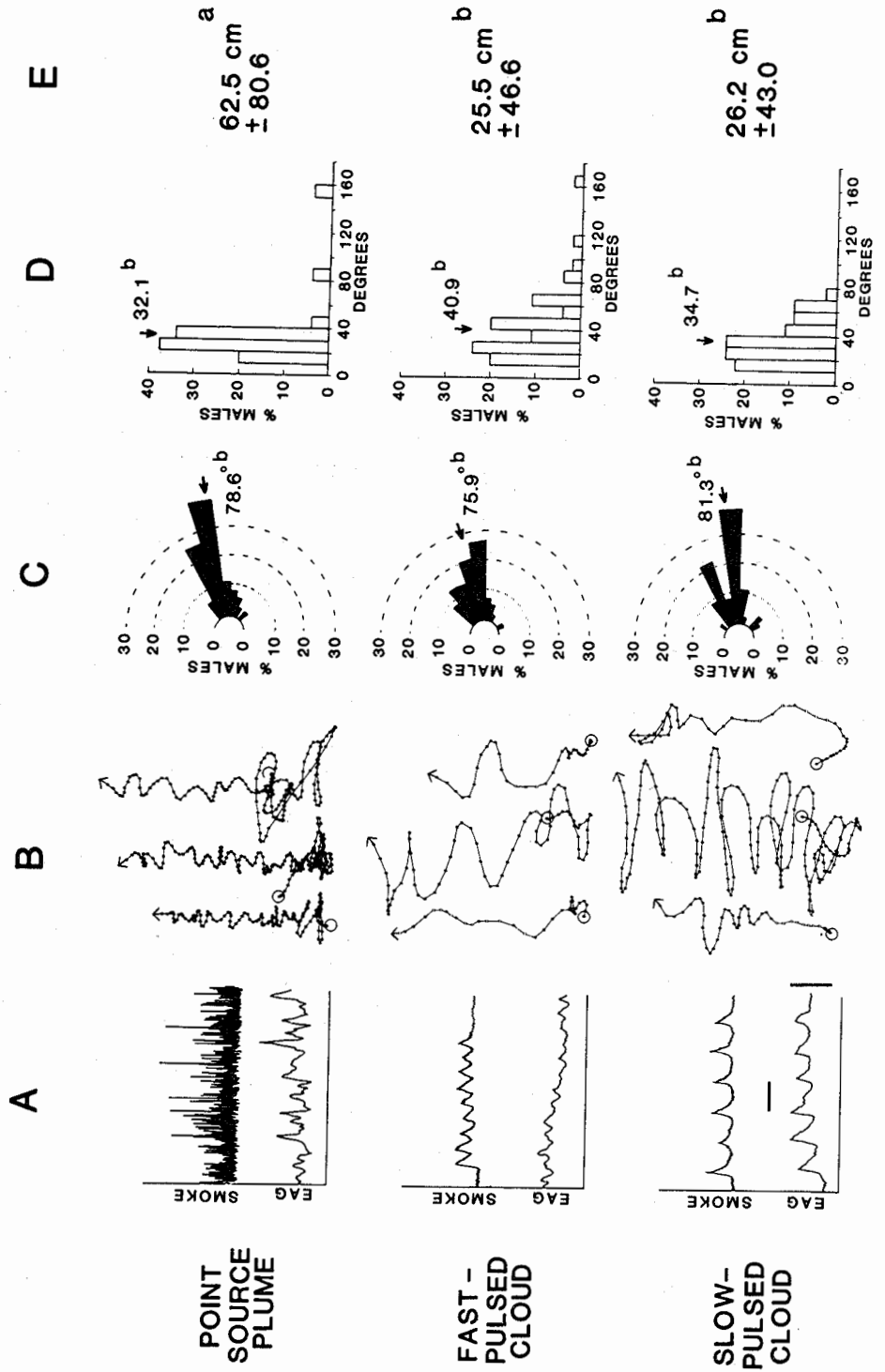
Analysis of video-recorded flight tracks revealed that the males flying in the pulsed clouds counterturned across the windline with inter-reversal track angles (Marsh *et al.*, 1978; Kuenen & Baker, 1983) and across-wind counterturn symmetries not significantly different from males flying to the point-source plume (Figs. 1C and 1D, respectively). The latter values were measured by taking successive pairs of inter-reversal track angles from each flight track and calculating for each pair the deviation of the second angle from a perfectly symmetrically performed track angle; one that matches the first but on the other side of the windline. We view the symmetry of successive across-wind inter-reversal track angles as reflecting the degree of integration of the self-steered counterturning programme with the polarizing effect of optomotor anemotaxis.

On the other hand, males flying in the continuous cloud exhibited wide, 90° cross-wind reversals characteristic of casting after

pheromone loss (Marsh *et al.*, 1978; Kuenen & Baker, 1983), but these counterturns were performed as symmetrically across the windline as the other males' upwind zigzags, despite their larger magnitude (Fig. 1D). In contrast, the few males that took flight when released into clean air displaced predominantly downwind with very few across-wind counterturns. These were performed significantly less symmetrically than those observed in response to any of the pheromone treatments (Fig. 1D).

The flight tracks of males flying upwind in the pulsed clouds did not appear to be as regular in zigzagging tempo or width as those of males flying in the plume (Fig. 1B). Tracks of males flying at least 25 cm upwind in the pulsed clouds exhibited zigzags approximately twice as wide, on average, as those of males flying upwind in the plume ( $10.4 \pm 6.0$  cm SD,  $n=28$  males, fast-pulsed;  $12.0 \pm 8.8$  cm SD,  $n=29$ , slow-pulsed;  $5.3 \pm 1.4$  cm SD,  $n=25$ , plume). This difference may have been due, in part, to our inability to generate concentration fluctuations in our pulsed clouds that were as rapid or as sharply rising and falling as those in the plume, as indicated by smoke and EAG (Fig. 1A). Previous work (Kuenen & Baker, 1982) established that plumes from  $1 \mu\text{g}$  sources elicited zigzags in *G. molesta* males approximately twice the width of those performed in plumes from sources loaded with 10 or  $30 \mu\text{g}$ . Thus our pulsed clouds may have been mimicking the types of fluctuating stimulation found in weaker point-source plumes. Alternatively, the more shallowly and less sharply fluctuating stimulation might be similar to that found at greater distances (10–15 m) downwind from a stronger point-source (Murlis & Jones, 1981). The tracks of moths flying at great distances from a pheromone source have never been analysed in detail, although some published tracks of male gypsy moths (David *et al.*, 1983) suggest a wider variety of zigzagging widths under such conditions than in a wind tunnel (Cardé & Hagaman, 1979), perhaps similar to the flight variation seen in our pulsed clouds.

Male *G. molesta*'s lack of upwind progress in the continuous cloud of pheromone was not due to an excessively high concentration that was then diluted by pulsing because, in a second experiment, a significantly greater percentage of males that took flight progressed



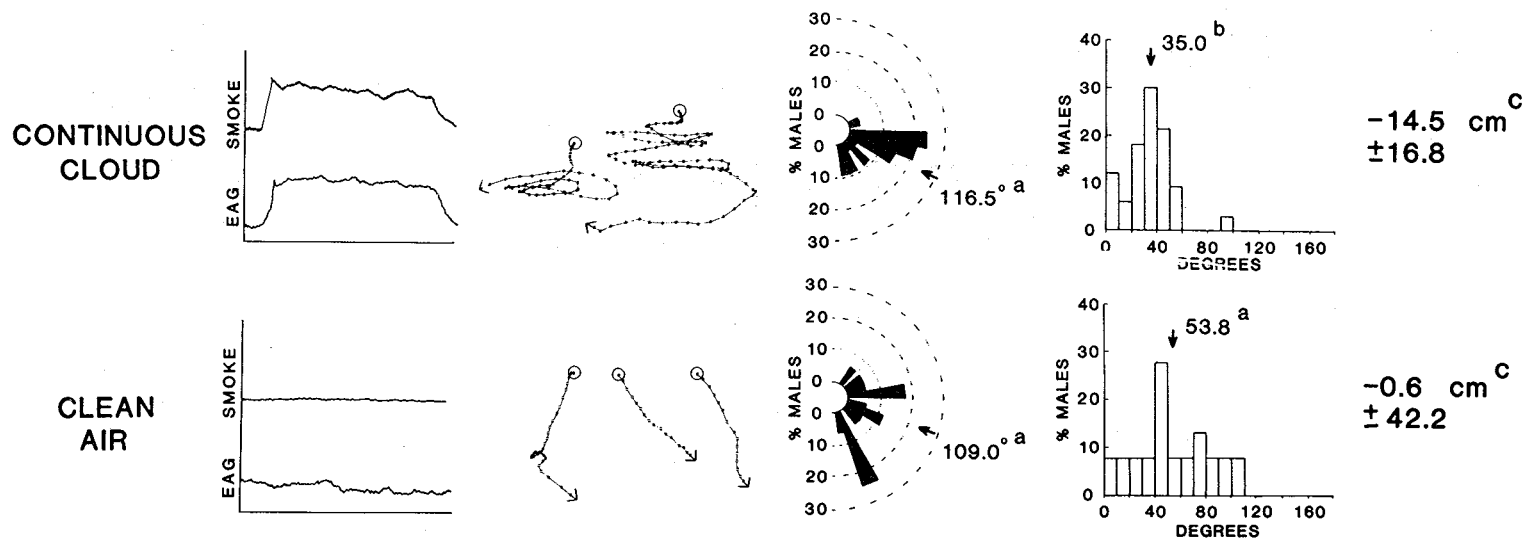


FIG. 1. (A) Measurements of stimulus patterns at point of take-off using recordings of  $\text{TiCl}_4$  smoke made with a photocell ('smoke'), and of pheromone using an EAG preparation ('EAG'). Vertical and horizontal bars in slow-pulsed figure denote for all figures 1 mV EAG response and 2 s respectively. Smoke and pheromone used in creating continuous cloud for illustration were turned off after 10 s. (B) Representative flight tracks of males video-recorded from above flying in response to different pheromone stimulation regimes. Wind was from the top in each figure, and open circles at beginning of each track denote the take-off point, which has been shifted to various locations in the figure only for the purpose of illustrating several tracks at once. Dots along tracks denote 1/30 s intervals. (C) Distributions and grand means of mean inter-reversal track angles of all males that took flight to different pheromone regimes and completed at least one turn ( $n$  (males) and SD, from top, are 36, 16.3; 44, 20.7; 45, 23.6; 33, 25.5; 17, 40.3). (D) Distributions and grand means of across-wind counterturn symmetries of all males flying in different pheromone regimes that completed at least two turns ( $n$  (males) and SD, from top, are 36, 32.1; 44, 23.6; 45, 16.7; 30, 18.1; 14, 27.9). (E) Mean upwind flight distances  $\pm$ SD of all males that took flight in response to different regimes. In (C), (D) and (E), means in each column having no letters in common are significantly different according to Duncan's multiple range test ( $P < 0.05$ ).

upwind when a plume from a 30  $\mu\text{g}$  source was placed within the cloud (42%,  $n=48$ ; 50%,  $n=48$  for plume in clean air) than when this plume was absent (17%,  $n=42$ ;  $P<0.05$ , Ryan's (1960) test for adjusted significance levels). Moreover, flying males progressed nearly as far upwind in the plume placed in the cloud as in the plume in clean air ( $29.4\pm 66.3$  cm SD,  $n=48$ ;  $43.3\pm 74.4$  cm SD,  $n=48$ , respectively;  $P>0.05$ ), and significantly farther than in the cloud alone ( $-14.5\pm 15.8$  cm SD,  $n=42$ ;  $P<0.05$ , Duncan's Multiple Range Test (Steel & Torrie, 1960)).

### Discussion

There are three species of moths for which it is known that continuous stimulation with uniform pheromone clouds does not evoke sustained upwind flight: *Adoxophyes orana* (Kennedy et al., 1980, 1981), *G. molesta* (Willis & Baker, 1984; this paper), and *Lymantria dispar* (C. T. David, personal communication). In both *A. orana* and *G. molesta* the onset of a cloud elicits a brief upwind surge, but the continuous stimulation subsequently fails to sustain it. Kennedy et al. (1980, 1981) and Kennedy (1983) hypothesized that it is the ups and downs in concentration from a plume's filaments that cause sustained upwind flight, and showed that a plume placed in a uniform cloud readily evoked such flight when the cloud had failed to do so. Willis & Baker (1984) showed that *G. molesta* males flew upwind along either vertical or horizontal edges of a clean-air-pheromone cloud interface, but not more than briefly after being engulfed by a cloud. They hypothesized that such progress was due to the intermittency of stimulation that males received by their movement in and out of the cloud, plus a slight inhomogeneity of concentration at the edge due to turbulent eddies. Now we have shown more directly than in previous studies that it is the fluctuations alone, apart from any other possible spatial gradient cues, that sustain upwind flight. The same cloud that previously failed to cause upwind flight now, when pulsed, did so.

Despite the fact that we have demonstrated directly that pulsed pheromone concentration is necessary to sustain upwind flight in moths, we do not propose that females of the several

moth species known to rhythmically extrude their pheromone glands (Cardé & Roelofs, 1973; Cardé et al., 1984; Conner et al., 1980) gain signal value and promote such upwind flight in males by this pulsed emission pattern. On the contrary, the lack thus far of a demonstrable effect of pulsing of point source plumes upon male response (Cardé et al., 1984) is most likely because the plume's filaments already provide sufficiently intermittent stimulation to elicit upwind flight. A further enhancement in perceived fluctuation and in attraction may be possible due to the pulsing of glands, but this has not yet been demonstrated behaviourally. Should an increase in attraction eventually be demonstrated in such species, we think it would not likely be due to a coded, species-specific pulsing pattern, but rather to an enhancement of intermittency. There is recent evidence that a major reason for gland pulsation in arctiids may be to increase the emission rate of pheromone from within the invaginated tubes of these complex glands (Schal & Cardé, 1985).

Upwind flight in a plume can cease before the source is reached, becoming instead in-flight station-keeping (arrestment) if too much pheromone is emitted or if the blend ratio is slightly wrong (Baker & Roelofs, 1981; Baker et al., 1981; Linn & Roelofs, 1983). We suggest that the neuronal cause of arrestment in such cases may be a relative fusion of the 'flickering' receptor output generated by the plume's filaments. When arrestment occurs close to an excessively emitting source, the receptors may be unable to continue to reflect the actual peak filament concentrations as they increase past the receptors' capacities (Baker, 1985) but, also, the surplus molecules will take longer to locate unoccupied receptor sites and be cleared by enzymatic degradation (Kaissling, 1971). The receptors, therefore, might fire more continuously at significant rates even during intervals when the moth is actually in a pocket of clean air or 'outside' the plume during a reversal. The moth, earlier kept in the immediate vicinity of the plume by its programme of narrow counterturns to significantly flickering stimulation farther downwind, now reaches a zone where the increasingly narrow counterturns trap it even more and prevent excursions into clean air from lasting long enough to reduce fusion of

the neuronal signal. Wrong blend ratios at normal emission rates may also result in relatively fused signals. In *Argyrotaenia velutinana*, the slower time course required for clearing of one component, (*E*)-11-14:Ac (Roelofs & Comeau, 1971; Baker & Roelofs, 1976), suggests that too much of this component would be more likely to 'fill in', with neuronal firing, the troughs between peak stimulation by pheromone filaments. Lower frequencies of source location are observed in response to such high (*E*):(*Z*) ratios.

Recently, interneurons that respond phasically to changes in pheromone concentration have been discovered in *Bombyx mori* (Olberg, 1983). The interneurons exhibit two distinct firing frequencies, 'high' and 'low', and the neurons alternate between these states upon repeated presentations of pheromone. Usually increases in concentration, but sometimes also decreases, trigger changes in state. If such interneurons were present in *G. molesta* they would require fluctuating pheromone stimulation to change states, and the frequency of state changes may be what modulates the counterturning programme (Willis & Baker, 1984).

Such 'flip-flopping' interneurons (Olberg, 1983) will not change states if the receptors modulating them are not themselves providing significantly fluctuating output. In our study, EAGs in the three pheromone regimes that elicited upwind flight (the plume and slow- and fast-pulsed clouds) clearly changed amplitude. In contrast, EAGs did not fluctuate noticeably under constant stimulation by the continuous cloud, in which upwind flight was not observed, although they did remain constantly high, indicating that the receptors were not adapted. This lends support to the idea that it is not the overall magnitude of receptor output, but the magnitude of receptor output fluctuations which is important for evoking upwind flight.

For moths requiring fluctuating pheromone stimulation, the traditional view of above-threshold pheromone stimulation as a time-averaged concentration (Bossert & Wilson, 1963) needs revision to take into account more than the average concentration or even the peak pheromone flux (Elkinton & Cardé, 1984). Frequency and amplitude of peak-to-trough concentration changes may be sufficient

for describing such stimulation for many moths (Kennedy, 1983). However, for some species such as *G. molesta* which are arrested by high concentrations, there may be upper limits to abilities to process ever higher and sharper rises and falls, with the possibility that at some point before the source is reached the neuronal fluctuations become too attenuated to sustain upwind flight. Consequently, the neuronal thresholds of flickering and fusion may both play a significant part in defining the upwind flight active space for a particular blend and emission rate.

In light of these considerations, the plume's physical structure as created by the wind speed and large- and small-scale turbulence, among other factors, would all play a large role in defining the active space (Murlis & Jones, 1981). However, measuring molecular concentration changes using a perfect detector that responds virtually instantaneously to flux changes, and registers the actual peaks and troughs in pheromone concentration, will not give the entire picture of the active space. What will be important, as it is to a responding male in nature, are the fluctuations as registered by neuronal elements. Moth olfactory neurons thus far do not seem to be perfect detectors. Their limitations to registering actual fluctuations of concentration would most likely start at the receptor level, including receptor capacities and lag times in processing and clearing pheromone molecules (Kaissling, 1971). The properties of these imperfect detectors will determine the behaviourally relevant amount of pheromone fluctuation and help determine the active space for upwind flight.

Apart from the practical implications of these findings and those of Kennedy *et al.* (1980, 1981) for mating disruption strategies that would employ a diffuse cloud of pheromone instead of multiple discrete point-source plumes, they raise questions concerning odour perception in all animals. For instance, 'sniffing' short bursts of odour, instead of inhaling a continuous stream, has been suggested as a mechanism in humans and other mammals for preventing adaptation and a rapid waning of sensitivity (Stoddart, 1976). 'Flicking' of the olfactory organs by the spiny lobster enhances the detection of gradual concentration changes by receptors (Schmitt & Ache, 1979). Insects have no known mechan-

ism for sniffing odour, and hence the intermittent stimulation provided by odour plumes, perhaps enhanced by the insects' zigzagging flight paths, may result in the same effect.

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