MEASURED BEHAVIOURAL LATENCY IN RESPONSE TO SEX-PHEROMONE LOSS IN THE LARGE SILK MOTH *ANTHERAEA POLYPHEMUS*

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Summary

Males of the giant silk moth *Antheraea polyphemus* Cramer (Lepidoptera: Saturniidae) were video-recorded in a sustained-flight wind tunnel in a constant plume of sex pheromone. The plume was experimentally truncated, and the moths, on losing pheromone stimulus, rapidly changed their behaviour from up-tunnel zig-zag flight to lateral casting flight. The latency of this change was in the range 300–500 ms. Video and computer analysis of flight tracks indicates that these moths effect this switch by increasing their course angle to the wind while decreasing their air speed. Combined with previous physiological and biochemical data concerning pheromone processing within this species, this behavioural study supports the argument that the temporal limit for this behavioural response latency is determined at the level of genetically coded kinetic processes located within the peripheral sensory hairs.

Introduction

The males of numerous moth species have been shown to utilize two distinct behaviour patterns during sex-pheromone-mediated flight. In the presence of pheromone they zig-zag upwind, making forward progress over the ground, and in the abrupt absence of pheromone they switch to a side-to-side or lateral casting flight, making little or no forward or backward progress (Kennedy & Marsh, 1974; Kennedy, 1983; David, Kennedy & Ludlow, 1983; Baker, 1986). This switch to casting flight is thought to be a behavioural strategy for relocating a lost pheromone plume in turbulent air (David *et al.* 1983; Baker & Haynes, 1987). For two moth species, *Bombyx mori* during walking (Kramer, 1975) and *Plodia*

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interpunctella during flight (Marsh, Kennedy & Ludlow, 1981), the response latency for this behavioural switch on loss of pheromone was in the range 0·5–1·0s. For a third species, *Grapholita molesta*, this latency was even shorter, 0·15s, in achieving a significant increase in the course angle to wind (Baker & Haynes, 1987). These short-latency responses have supported the idea that pheromone-sensitive sensory hairs possess mechanisms for the rapid inactivation of stimulus molecules (Kaissling, 1974, 1986a; Vogt & Riddiford, 1981; Vogt, Riddiford & Prestwich, 1985; Vogt, 1987). Rapid inactivation would act as a noise filter within the sensory hair, eliminating residual pheromone molecules from the space surrounding the sensory dendrite, allowing the moth to respond physiologically and behaviourally to rapid fluctuations in ambient pheromone levels.

Little is known about the details of sex-pheromone-mediated flight in the large saturniid moths; it is not known whether they exhibit the two characteristic behaviours of forward zig-zag flight and lateral casting flight, or what the latency might be of switching behaviour on pheromone loss. The giant silkmoth *Antheraea polyphemus* is the only species for which there is considerable sensory physiological and sensory biochemical data within the context of pheromone-elicited behaviour. Thus, to correlate the physiological and biochemical kinetics for pheromone inactivation with the latency for switching behaviour within the same species, we flew male *A. polyphemus* moths in a wind tunnel using freshly prepared pheromone gland extracts as an attractant odour source. Moths which flew upwind in response to sex pheromone repeatedly exhibited a rapid switch to casting flight upon flying out of an experimentally truncated pheromone plume.

Materials and methods

Antheraea polyphemus were obtained as pupae (H. W. Hartman), stored at 4°C, and reared to adults at 25°C on a 16h:8h light:dark cycle. One- to three-day-old males were transferred to the wind tunnel room during their photophase, and lights were dimmed to experimental levels at the normal time of lights off. All experiments were conducted during the first 4h of the moth's scotophase.

Males were flown in a large sustained-flight wind tunnel constructed of clear polycarbonate plastic with a working section 3·6m long, 1m high and 1m wide at ground level (Kuenen & Baker, 1982, 1983). Wind at 0·5ms^{-1} was provided by a 746-W voltage-regulated fan, and air turbulence was reduced by passing the air through layers of muslin (supported by a mesh screen) and finally through a layer of fine-mesh polyester fabric. Light intensity was less than 5lx.

Sex pheromone was prepared from freshly excised female pheromone glands extracted in methylene chloride. The solvent was evaporated under nitrogen gas, and pheromone was subsequently dissolved in hexane. One gland equivalent (20μl hexane) was applied to filter paper attached to the end of a thread hanging from the tunnel ceiling. This odour source was positioned 15cm above the axial centre of the floor and 3cm from the upwind end of the wind tunnel. The filter paper functioned as a point source of pheromone release and yielded a tightly structured

plume travelling in a straight line down the tunnel parallel with its walls with a maximum cross-sectional diameter of approximately 5 cm as estimated by $TiCl_4$ release. Pheromone was removed from the tunnel by a 30-cm diameter exhaust tube (air speed at centre $2.9\,m\,s^{-1}$) aligned with the centre of the plume. The remainder of the air passing through the tunnel was recirculated through the room.

Moths were released into the tunnel at the downwind end. When a moth was observed to orient its upwind flight along the centre line of the tunnel at the height of the filter paper, the upwind observer quickly raised the filter paper by pulling on the thread. This caused an abrupt truncation of the pheromone plume at the flight level of the insect. A moth flying upwind in the pheromone plume was thus subjected to a sudden loss of stimulus as it flew out of the truncated plume. The upwind observer coordinated stimulus removal with moth position so that loss of stimulus occurred in the field of view of a video camera. A strip of light-emitting diodes (LEDs), activated sequentially according to the wind speed measured using $TiCl_4$ smoke, was used to display the down-tunnel progression of the plume's truncated upwind end (J. S. Kennedy & C. T. David, unpublished). Thus the precise moment of last possible contact with pheromone was observed from the video record as the point where the light pulse travelling down the tunnel with the wind met the moth travelling up the tunnel.

Flights were video-recorded from above in plan view, as described by Kuenen & Baker (1983). The camera's field of view spanned 105 cm, from 65 to 170 cm downwind of the pheromone source, and 40 cm on either side of the plume axis. Individual flight tracks were re-recorded onto a Sony SVM-1010 motion analyser. Frame-by-frame playback from this system gave the moth's consecutive 1/30 s positions, which were marked by an ink dot on a Mylar (clear plastic) sheet taped to the screen of the analyser (see Fig. 1). These tracks were digitized onto a microcomputer for further analysis. For each complete flight track, the track angle (with respect to the wind direction) and ground speed were calculated by computer for each 1/30-s vector, and the course angle, air speed, drift angle and other values were calculated by computer using the triangle of velocities formula (Kennedy, 1940; Marsh, Kennedy & Ludlow, 1978). The grand means of these 1/30-s values were calculated over 0.1 s intervals before and after pheromone loss for the six tracks that were analysed (see Fig. 2). The plots presented in Fig. 2 represent these grand means, averaged over the six flight tracks represented in Fig. 1. Time zero (Fig. 2) corresponds to the moment of last possible contact with pheromone, which is indicated for each flight track by arrows (Fig. 1).

To record electroantennograms (EAG) (Fig. 3) within the wind tunnel, a single male *A. polyphemus* antenna was positioned vertically, anterior side upwind, on the upwind edge of a mobile mount (T. C. Baker & K. F. Haynes, in preparation). The antennal base was impaled by a silver chloride plated silver wire, and the cut distal end was placed in contact with a saline/AgCl electrode. The antenna was positioned within the pheromone plume, 1 or 2 m downwind from the pheromone source. Signals were processed through a d.c. amplifier (100× gain) directly onto a

strip chart recorder. Stimulus conditions were identical to those employed in the flight studies, except that the stimulus was $10 \mu g$ of synthetic pheromone component (E,Z)-6,11-hexadecadienyl acetate (Kochansky *et al.* 1975; synthesized by H. J. Bestmann, courtesy of K.-E. Kaissling) applied to the filter paper.

Results and discussion

Analysis of flight tracks

The tracks analysed come from six independent approaches to the pheromone source by a single male (Fig. 1). Ten unrecorded males appeared to behave in the same way. While in the pheromone plume, the moths flew slowly and zig-zagged narrowly, all the time progressing upwind and up-tunnel. As they approached the field of view of the video recorder, the pheromone source was raised and the pheromone plume truncated. Computer analysis of the video recordings (Fig. 2) indicated that by 0·5 s after the last possible contact with pheromone (pheromone loss), the moth's track angle had already increased significantly, becoming crosswind casting at approx. 90° by 0·8 s (Fig. 2A). Casting lasted approximately 10 s, by which time the moth had drifted down-tunnel while remaining at the same height. The pheromone source was then repositioned and the moth re-attracted. In the case of the moth whose tracks were analysed, this cycle was repeated six times, after which the moth landed on the floor.

The moth accomplished this increase in track angle by a combination of increasing its steered course angle to wind (Fig. 2B) and reducing its air speed (Fig. 2C). Prior to pheromone loss, the moth steered a course at a mean of $11·4° \pm 3·9°$ ($\pm$s.d.) while flying upwind during the $-1·0$ to $+0·1$ s time interval (Fig. 2B) (0° = upwind; absolute values of angles were used for means). The course angle increased to $20·1° \pm 0·9°$ during the 0·4–0·7 s interval, and to $36·0° \pm 2·8°$ during the 0·8–1·5 s interval following last possible contact with pheromone. Air speed had decreased significantly by 0·5 s after last possible pheromone contact (Fig. 2C). The decreasing air speed, already noticeable by 0·3 s following pheromone loss, combined with the increasing course angle, resulted in the observed significant increase in the resultant track angle to wind apparent by 0·5 s after pheromone loss (Fig. 2A).

Upwind flight to an odour source by moths is thought to be controlled by optomotor anemotaxis, where the moth monitors its direction and speed of progress over the ground visually, compensating for wind-induced drift by adjusting its course angle and air speed (Marsh *et al.* 1978; Kennedy, 1983, 1986). In addition, the counterturns associated with zig-zagging and casting are thought to be strictly internally controlled (Kennedy, 1983; Baker, 1986). In our experiments, regular counterturns were often not apparent in the moth progressing upwind in pheromone. As best could be determined, they occurred at intervals of $0·43 \pm 0·20$ s ($\pm$s.d. average of 19 counterturns). However, following plume loss obvious counterturns occurred at increasing intervals of $0·59 \pm 0·25$ s, $1·03 \pm 0·15$ s
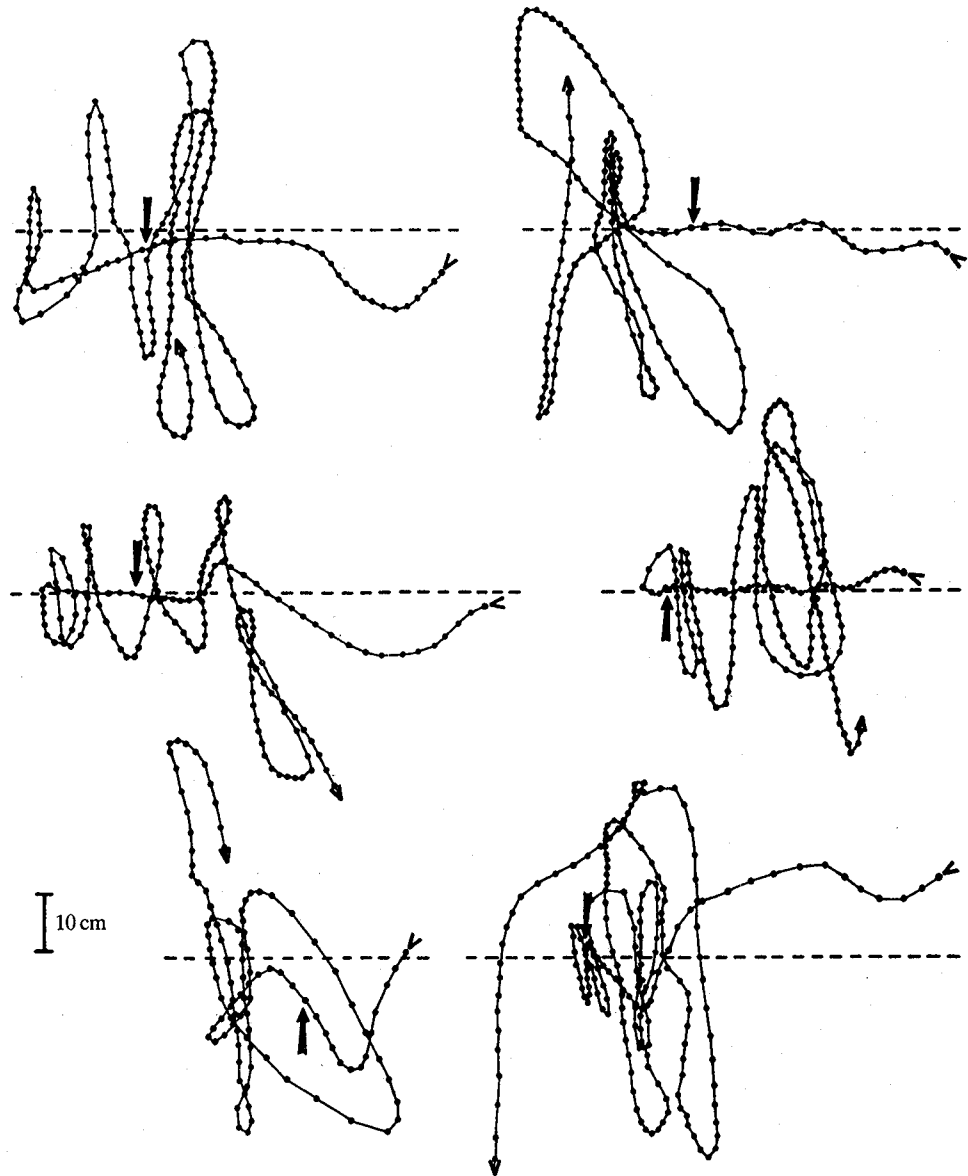
Fig. 1. Flight tracks of a single *Antheraea polyphemus* male flying in a wind tunnel and making six consecutive approaches towards a pheromone source, videotaped from above. For each track, the moth entered the field of view from the right, and wind was from the left. At the arrows the moth flew out of the experimentally truncated plume (last possible contact with pheromone) and switched his flight behaviour to lateral casting. Dots represent the moth's positions at 1/15 s intervals. Alternate dots from the original 1/30 s data tracings have been omitted for clarity.
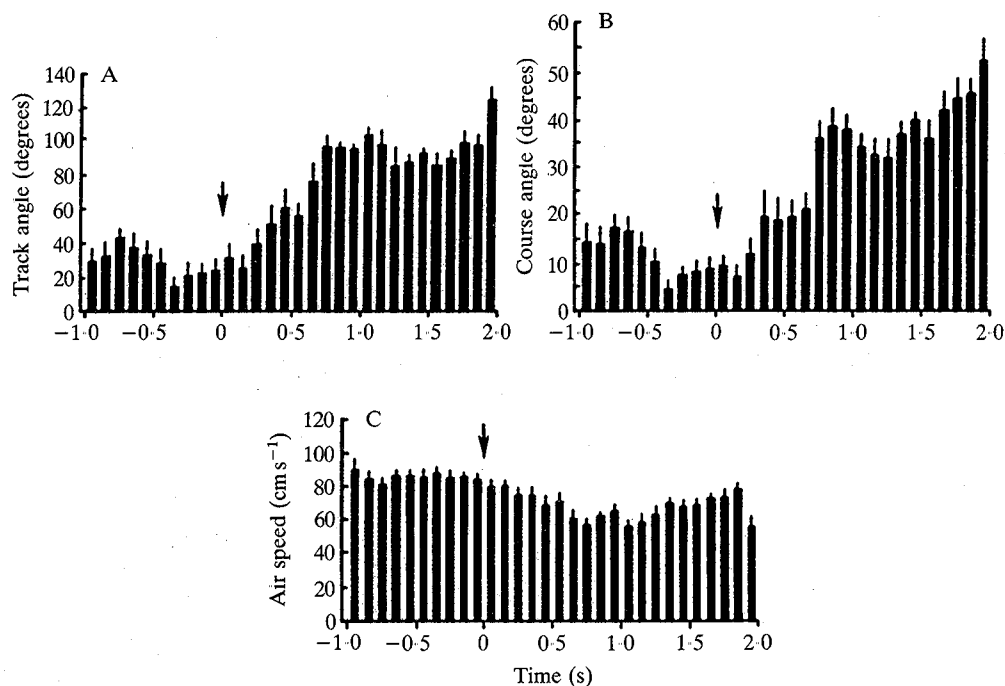
Fig. 2. Analysis of the flight tracks depicted in Fig. 1. The switch from up-tunnel flight to lateral casting on loss of pheromone (marked with an arrow) is seen as an increase in track angle (A) off the wind vector (0°). This was accomplished by an increase in course angle (B) accompanying a decrease in air speed (C). Values were calculated for each $1/30$ s vector for 1 s preceding and 2 s after pheromone loss, 0 s corresponding to the arrows in Fig. 1. Calculated data were pooled for all six tracks over each 0·1 s interval, and are presented as a mean absolute value $\pm$ s.e.

and $1·53 \pm 0·54$ s on the first to third, fourth to sixth, and seventh to ninth track legs, respectively.

Responses to fine-scale discontinuities in plume structure

A naturally occurring pheromone plume has a highly turbulent and discontinuous structure (Wright, 1958; Murlis & Jones, 1981; David et al. 1983; Murlis, 1986). From the perspective of a stationary observer these discontinuities can occur at time scales of milliseconds, seconds or minutes, depending on their cause (Murlis, 1986). Clearly, our experiments support other observations that moths will respond to interruptions of the stimulus if those interruptions are of sufficiently long duration. We would like to know how brief an interruption of the stimulus can be and still be detected and acted upon.

The fine-scale discontinuities in plume structure can be inferred from stationary electroantennogram (EAG) recordings made within the wind tunnel (Fig. 3). Although air flow in our wind tunnel was reasonably laminar, turbulence induced by the filter paper source caused the plume to leave in the form of a spiral, which
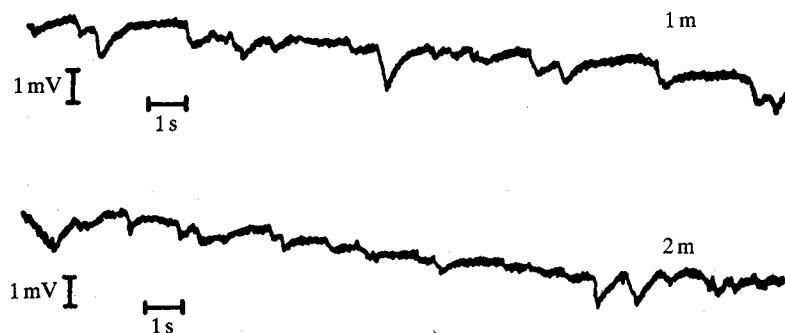
Fig. 3. Electroantennogram recordings of an isolated *Antheraea polyphemus* antenna positioned 1 m or 2 m downwind from the pheromone source.

tended to expand slightly during its down-tunnel movement. Such induced turbulences are common in nature. At 1 m from the pheromone source the antenna responded with depolarizations (bursts) of $1 \cdot 13 \pm 0 \cdot 83$ s ($\pm$ s.D., averaged over 53 s; burst detection threshold was $0 \cdot 2$ mV) (Fig. 3). The burst frequency was similar at 2 m $1 \cdot 09 \pm 0 \cdot 9$ s (averaged over 70 s). A male with two antennae, instead of the one used for these EAGs, would span approximately 3 cm and probably encounter plume filaments at a somewhat higher frequency because of the doubled antennal area presented. Likewise, males flying at 80 cm s^{-1} air speed instead of being stationary as with the EAG preparation (where air speed was 50 cm s^{-1}) should encounter filaments at approximately a 50% higher frequency, or approximately $1 \cdot 6$ bursts s^{-1}. Such an increase in burst frequency with air speed has been observed in EAGs from the moth *Grapholita molesta*, where the antennae were pushed at various velocities directly upwind in a pheromone plume (T. C. Baker & K. F. Haynes, unpublished results).

Kramer (1986) utilized a walking assay to present males of the silk moth *Bombyx mori* with pulsed pheromone at varying pulse durations and intervals. When the interval between pheromone pulses exceeded $0 \cdot 6$ s the animals responded with a noticeable change to a more obliquely cross-wind track. This result is temporally similar to those from our wind tunnel studies. However, although *B. mori* responded to constant pheromone stimulation with upwind movement, its response was significantly enhanced when the stimulus was pulsed at $0 \cdot 3$ s intervals. Thus, *B. mori* males responded to pulses at intervals exceeding $0 \cdot 6$ s and sometimes to pulses at intervals of $0 \cdot 3$ s, but apparently fused pulses separated by shorter intervals. Kaissling (1969) measured by EAG the physiological response characteristics of *B. mori* antennae, and showed that the half-time for decay in electrical output following stimulus loss was about $0 \cdot 5$ s. This temporal correlation between antennal physiology and behaviour suggests that the frequency limits may be determined peripherally in the antenna, rather than in the central nervous system.

Such a correlation can also be made for *A. polyphemus*. Our own experiments

have demonstrated that a flying *A. polyphemus* male can display a significant change in behaviour by 0·4–0·5 s following pheromone loss (Fig. 2), although full casting was not obvious until 0·7 s. Kaissling (1986*b*) has reported that the neurones in single sensory hairs of *A. polyphemus* antennae can respond with action potentials to each repeated odour pulse of 20 ms duration, when presented at a rate of up to 3·4 Hz. At 4·2 Hz not every odour pulse evoked action potentials. These experiments demonstrated that, at least at pulse frequencies below 4 Hz, *A. polyphemus* receptors accurately relay the frequency of arriving odour bursts to higher centres. However, at frequencies above 4 Hz, these antennal sensory hairs no longer record the pulses as distinct and separate. As in *B. mori*, frequency limits appear to be set at the periphery, in the sensory hairs themselves.

Biochemical correlates of the behavioural response

These findings suggest that a moth's ability to detect temporal discontinuities in pheromone stimulus is limited by processes within the pheromone-sensitive sensory hairs, whatever else might be occurring within the central nervous system. Pheromone molecules are thought to enter the sensory hairs through thousands of small pores which penetrate the outer cuticular hair-wall (Steinbrecht, 1980, 1987; Keil, 1984). The pheromone molecules then pass through a lumen containing a proteinaceous fluid consisting of pheromone-binding proteins and pheromone-degrading enzymes (Vogt & Riddiford, 1981; Klein, 1987; Vogt, 1987), arriving at the sensory dendrite membrane where they presumably initiate an electrical impulse in the sensory neurone by interacting with a membrane-bound receptor protein (Vogt, Prestwich & Riddiford, 1988).

Vogt and colleagues (Vogt & Riddiford, 1986; Vogt *et al.* 1985, 1988; Vogt, 1987) have described some of the biochemical components that control sensory hair function in *A. polyphemus*, and have suggested that these components are organized and kinetically designed with the function of processing pheromone molecules in an efficient manner. One of these components is a potent sensilla-specific esterase which degrades pheromone *in situ* with an estimated half-life of 15 ms (Vogt *et al.* 1985). At such rates, this esterase could easily clear the sensory hair of 'old' stimulus rapidly enough to allow the hair to respond to external stimulus transients, down to a limiting frequency. These limits could be determined by how fast the esterase can function, by its kinetic properties. Additionally, the pheromone receptor proteins (Vogt *et al.* 1988) and associated transductory components may possess inherent refractory properties that limit sensory hair response. We therefore suggest that these biochemical components provide the molecular correlate which underlies the animals' ability to discern fine-scale discontinuities in pheromone plume structure. We are left with one question. Have these animals reached some physical limit which constrains the biochemical components of the sensory hairs from processing signal more quickly; or have they produced components which are optimally tuned in their kinetic properties to a particular level of efficiency, thus acting as genetically tuned signal filters? The

answer must await more information regarding the molecular-level properties of these sensory hairs, and the behavioural utilization of pulsed signals.

References

BAKER, T. C. (1986). Pheromone-modulated movements of flying moths. In *Mechanisms in Insect Olfaction* (ed. T. L. Payne, M. C. Birch & C. E. J. Kennedy), pp. 39–48. Oxford: Clarendon Press.

BAKER, T. C. & HAYNES, K. F. (1987). Manoeuvres used by flying male oriental fruit moths to relocate a sex pheromone plume in an experimentally shifted wind-field. *Physiol. Entomol.* **12**, 263–279.

DAVID, C. T., KENNEDY, J. S. & LUDLOW, A. R. (1983). Finding of a sex pheromone source by gypsy moths released in the field. *Nature, Lond.* **303**, 804–806.

KAISSLING, K. E. (1969). Kinetics of olfactory receptor potentials. In *International Symposium on Olfaction and Taste III* (ed. C. Pfaffmann), pp. 52–70. New York: Rockefeller University Press.

KAISSLING, K. E. (1974). Sensory transduction in insect olfactory receptors. In *Biochemistry of Sensory Functions* (ed. L. Jaenicke), pp. 243–273. Berlin: Springer Verlag.

KAISSLING, K. E. (1986a). Chemo-electrical transduction in insect olfactory receptors. *A. Rev. Neurosci.* **9**, 121–145.

KAISSLING, K. E. (1986b). Temporal characteristics of pheromone receptor cell responses in relation to orientation behavior of moths. In *Mechanisms in Insect Olfaction* (ed. T. L. Payne, M. C. Birch & C. E. J. Kennedy), pp. 193–199. Oxford: Clarendon Press.

KEIL, T. A. (1984). Reconstruction and morphometry of silkmoth olfactory hairs: A comparative study of sensilla trichodea on the antennae of male *Antheraea polyphemus* and *A. pernyi* (Insecta, Lepidoptera). *Zoomorphology* **104**, 147–156.

KENNEDY, J. S. (1940). The visual responses of flying mosquitoes. *Proc. zool. Soc. Lond.* A **109**, 221–242.

KENNEDY, J. S. (1983). Zig-zagging and casting as a programmed response to wind-born odour: a review. *Physiol. Entomol.* **8**, 109–120.

KENNEDY, J. S. (1986). Some current issues in orientation to odour sources. In *Mechanisms in Insect Olfaction* (ed. T. L. Payne, M. C. Birch & C. E. J. Kennedy), pp. 11–25. Oxford: Clarendon Press.

KENNEDY, J. S. & MARSH, D. (1974). Pheromone-regulated anemotaxis in flying moths. *Science* **184**, 999–1001.

KLEIN, U. (1987). Sensillum-lymph proteins from antennal olfactory hairs of the moth *Antheraea polyphemus* (Saturniidae). *Insect Biochem.* **17**, 1193–1204.

KOCHANSKY, J., TETTE, J., TASCHENBERG, E. F., CARDE, R. T., KAISSLING, K. E. & ROELOFS, W. L. (1975). Sex pheromone of the moth *Antheraea polyphemus*. *J. Insect Physiol.* **21**, 1977–1983.

KRAMER, E. (1975). Orientation of the male silkmoth to the sex attractant bombykol. In *Olfaction and Taste V* (ed. D. A. Denton & J. P. Coghlan), pp. 329–335. New York: Academic Press.

KRAMER, E. (1986). Turbulent diffusion and pheromone-triggered anemotaxis. In *Mechanisms in Insect Olfaction* (ed. T. L. Payne, M. C. Birch & C. E. J. Kennedy), pp. 59–67. Oxford: Clarendon Press.

KUENEN, L. P. S. & BAKER, T. C. (1982). Optomotor regulation of ground velocity in moths during flight to sex pheromone at different heights. *Physiol. Entomol.* **7**, 193–202.

KUENEN, L. P. S. & BAKER, T. C. (1983). A non-anemotactic mechanism used in pheromone source location by flying moths. *Physiol. Entomol.* **8**, 277–289.

MARSH, D., KENNEDY, J. S. & LUDLOW, A. R. (1978). An analysis of anemotactic zigzagging in male moths stimulated by pheromone. *Physiol. Entomol.* **3**, 221–240.

MARSH, D., KENNEDY, J. S. & LUDLOW, A. R. (1981). Analysis of zig-zagging flight in moths: a correction. *Physiol. Entomol.* **6**, 225.

MURLIS, J. (1986). The structure of odour plumes. In *Mechanisms in Insect Olfaction* (ed. T. L. Payne, M. C. Birch & C. E. J. Kennedy), pp. 27–38. Oxford: Clarendon Press.

MURLIS, J. & JONES, C. D. (1981). Fine-scale structure of odour plumes in relation to insect orientation to distant pheromone and other attractant sources. *Physiol. Entomol.* **6**, 71–86.

STEINBRECHT, R. A. (1980). Cryofixation without cryoprotectants. Freeze substitution and freeze etching of an insect olfactory receptor. *Tissue Cell* **12**, 73–100.

STEINBRECHT, R. A. (1987). Functional morphology of pheromone-sensitive sensilla. In *Pheromone Biochemistry* (ed. G. D. Prestwich & G. L. Blomquist), pp. 353–383. New York: Academic Press.

VOGT, R. G. (1987). The molecular basis of pheromone reception: its influence on behavior. In *Pheromone Biochemistry* (ed. G. D. Prestwich & G. L. Blomquist), pp. 385–431. New York: Academic Press.

VOGT, R. G., PRESTWICH, G. D. & RIDDIFORD, L. M. (1988). Sex-pheromone receptor proteins: visualization using a radiolabeled photoaffinity analog. *J. biol. Chem.* **263**, 3952–3959.

VOGT, R. G. & RIDDIFORD, L. M. (1981). Pheromone binding and inactivation by moth antennae. *Nature, Lond.* **293**, 161–163.

VOGT, R. G. & RIDDIFORD, L. M. (1986). Pheromone reception: a kinetic equilibrium. In *Mechanisms in Insect Olfaction* (ed. T. L. Payne, M. C. Birch & C. E. J. Kennedy), pp. 201–208. Oxford: Clarendon Press.

VOGT, R. G., RIDDIFORD, L. M. & PRESTWICH, G. D. (1985). Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proc. natn Acad. Sci. U.S.A.* **82**, 8827–8831.

WRIGHT, R. H. (1958). The olfactory guidance of flying insects. *Can. Entomol.* **90**, 81–89.