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Winging It: Moth Flight Behavior and Responses of Olfactory Neurons Are Shaped by Pheromone Plume Dynamics

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Abstract

Terrestrial odor plumes have a physical structure that results from turbulence in the fluid environment. The rapidity of insect flight maneuvers within a plume indicates that their responses are dictated by fleeting (<1 s) rather than longer (>1 s) exposures to odor imposed by physical variables that distribute odor molecules in time and space. Even though encounters with pheromone filaments are brief, male moths responding to female-produced pheromones are remarkably able to extract information relating to the biological properties of these olfactory signals. These properties include the types of molecule present and their relative abundances. Thus, peripheral and central olfactory neurons are capable of representing these biological properties of a pheromone plume within the context of a temporally irregular and unpredictable signal. The mechanisms underlying olfactory processing of these signals with respect to their biological and physical properties are discussed in the context of a behavioral framework.

Key words: Lepidoptera, neuroethology, odor plume, olfaction

Introduction

The biochemical machinery that drives life results in the formation of molecules as by-products or end products. Some of these molecules either inevitably escape or are purposefully emitted into the environment where they can then be detected by other organisms. For many animals, the various and specific resources that are required for survival are often scattered throughout the environment and can be accurately identified and located in part through the characteristic volatiles that they emit. In many instances, odorous molecular mixtures diffuse from their point of origin and are transported away from their source by prevailing fluid currents. Under physical conditions where turbulence dominates over diffusion, shedding and vortices act quickly upon the nascent plume and create a highly intermittent signal that meanders downstream. This intermittency can be maintained over considerable distances from the plume origin. By recognizing specific chemical mixtures animals can initiate locomotory activities to either avoid the organism generating the signal (e.g., predators or dominant competitors) or seek out the odor source (e.g., resources such as food, refugia, and mates). In the latter case of resource discovery, animals at a variety of scales in terrestrial and aquatic environments have evolved behavioral algorithms that allow them to succeed at following the fluctuating, sporadic odor signals present in turbulent plumes to their origin. Some animals actively sample odor-bearing air by drawing it over the olfactory epithelium while other organisms appear to sample the existing plume structure passively.

The intent of this contribution is to first examine the influence of chemical and physical properties of odor plumes on the behavior of animals and then consider how these properties are represented by peripheral and central olfactory neurons. These issues will be addressed with a focus on moths, which sample their odor environment while flying. These animals offer extensive insights into odor-mediated orientation and the constraints imposed by a dynamic, fluctuating signal on the underlying olfactory processing.

Information content of odor plumes

The nature of turbulent odor plumes means that they contain two basic sources of information: 1) the chemical content of the signal and 2) the distribution of these odorous molecules in time and space. Both play an important role in shaping the odor-mediated behavioral responses of organisms. The chemical content of the signal comprises two basic

aspects: 1) quality—the different molecules that are produced and released into the environment by an organism and 2) quantity—the relative ratios of the different molecular species in the odor. I refer to these as biological properties because they are the result of living processes. The second source of information results from the physical forces that distribute odorous molecules in time and space once they have been emitted by an organism. These forces are a reflection of the physical properties of the fluid environment in which a particular odor plume is created (see Weissburg, 2000; Koehl, 2005). In turbulent odor plumes where the role of diffusion is diminished, the quantity of molecules present in a given space at a given time (intensity/concentration) and the flux of those molecules (the rate at which concentration changes over time) can both be important factors in modulating the responses of orienting animals. The structure of odor plumes both in terrestrial and aquatic environments has been measured most frequently through the detection of surrogate molecules that simulate the presence or absence of actual odor molecules. Ionized air and an ion detector have been used both in wind tunnel and field situations to examine plume properties (Murlis and Jones, 1981; Murlis *et al.*, 1990, 1992, 2000). More recently, a tracer gas (propylene) and miniphotoionization detector (mini-PID) were used to mimic and measure odor plume dynamics (Justus *et al.*, 2002a, 2002b, 2005). These studies have revealed that plumes are highly intermittent and that their filamentous structure is maintained far downwind of the point of origin. In aquatic environments such as a laboratory flume, fine-scale structure of an odor plume was measured using dopamine-sensitive carbon fiber microelectrodes (Moore *et al.*, 1989; Moore and Atema, 1991). Other studies have characterized odor plume structure in an estuarine tidal creek (which presents relatively simple hydrodynamic conditions) using a fluorometric device (Zimmer-Faust *et al.*, 1995; Finelli *et al.*, 1999). These studies have provided useful information about plume structure and the parameters that show systematic variation across the transverse and longitudinal axes of mass fluid movement (see Vickers, 2000). Behavioral studies indicate that both biological properties as well as the physical distribution of odor molecules in time and space are important for organisms that orient in odor plumes. This implies that the olfactory system must extract and preserve information relating to these properties from the odor signal.

Pheromone-mediated moth flight behavior

In many moth species, females manufacture and release a specific blend of odorants that together comprise the pheromone—a mixture that is highly attractive to males of the same species (Witzgall *et al.*, 2004). Some of the individual odorants (each referred to as a pheromone component) may inhibit the behavioral responses of closely related or sympatric species thereby preventing potential mating mistakes and maintaining reproductive isolation. Males respond to the correct pheromone blend by flying upwind and locating the source—a behavior that can occur over distances of tens of meters or more (David *et al.*, 1983; Vickers and Baker, 1997a). This is a simplistic description of a complex behavior. In fact, the behavioral strategies that males employ have been extensively studied, debated (reviewed in Arbas *et al.*, 1993; Cardé and Minks, 1997), and modeled (Belanger and Willis, 1996). It is generally accepted that upwind flight results from the integration of at least two mechanisms: optomotor anemotaxis and counterturning. The former mechanism refers to orientation responses with respect to the direction of fluid flow (in this case air in the form of wind) that are guided by visual feedback. The latter mechanism refers to an internally generated behavior that causes the moth to turn back and forth across the wind line at regular intervals. This is evident in the zigzagging upwind flight tracks that male moths make as they progress upwind toward a pheromone source (Figure 1a). The frequency of turning ranges from about 2 to 6 Hz depending upon the moth species. Larger moths such as *Manduca sexta* tend to turn less frequently compared to smaller moths such as *Grapholita molesta* which exhibit higher rates of counterturning (Willis and Arbas, 1991; Willis and Baker, 1988). Counterturning is most evident, however, when an individual male loses contact with the pheromone plume and plunges into clean air. Under these conditions, a male will cease upwind progress and turn its track across the wind line, executing a series of turns perpendicular to the wind direction vector (Figure 1b). This behavior is known as casting, and it often lasts for several seconds as the male systematically scans horizontal and vertical air space seeking the lost plume. Importantly, zigzagging upwind and crosswind casting flight both require the moth to steer with respect to the wind direction and thus entail the integration of both visual feedback (optomotor anemotaxis) and counterturning. As time progresses, males typically drift downwind and under certain field conditions will turn and actively fly downwind (Baker and Haynes, 1996).

Effects of biological plume properties on behavior

Many odors comprise complex mixtures of different molecules that together give the overall perception of a specific entity (e.g., Axel, 1995). Many species of moth utilize mixtures as their pheromone but these mixtures are often relatively simple, comprising two to five biochemically related odorants (Witzgall *et al.*, 2004). The most abundant pheromone component in a mixture is referred to as the primary (or major) component, whereas other components are secondary (or minor). It is important to note that in order to be considered part of the necessary attractive pheromone mixture, each odorant must have demonstrated positive behavioral activity in males, and thus, both primary and secondary pheromone components are crucial in conferring biological activity on the odor. Indeed, male moths have

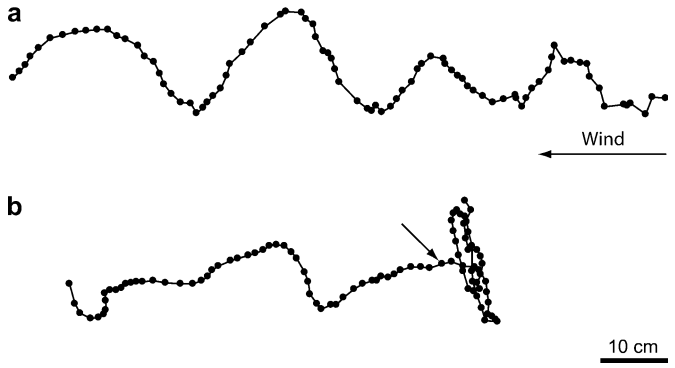

Figure 1 **(a)** Upwind flight by a male *Heliothis virescens* moth responding to a wind-borne pheromone plume is the product of two mechanisms: optomotor anemotaxis which allows the moth to steer with respect to the wind direction and counterturning which produces the regular track reversals across the wind line. **(b)** Following loss of the pheromone plume, indicated by the arrow, and ensuing flight into clean air, a male *H. virescens* moth ceases upwind flight after a short latency and begins a series of turns back and forth across the wind line, a behavior known as casting. The moth continues to both steer with respect to the wind direction and counterturn during this behavior. Dots along each track are separated by 1/30th s.

the lowest behavioral threshold to the complete pheromone blend (Linn *et al.*, 1986) indicating that detection of the blend as an entity is important. Consequently, male moth behavior is readily altered by removing individual pheromone components from the blend—sometimes this results in complete failure of the males to orient upwind (often in the case of removal of the primary pheromone component), whereas the effects of removal of minor components can be more variable ranging, for example, from a failure to orient to a reduction in either the number of males able to locate the pheromone source or the efficiency with which they do so (Linn *et al.*, 1984). Furthermore, changes in the ratios of different pheromone components can have substantial effects on the attractiveness of a particular pheromone blend (Roelofs *et al.*, 1987; Willis and Baker, 1988). One example features two sibling moth species of European corn borer, *Ostrinia nubilalis*, that rely on the same two pheromone components (i.e., same pheromone quality), (*E*)-11-tetradecenyl acetate and (*Z*)-11-tetradecenyl acetate. The *E*-strain responds positively to a 99:1 mixture of the *E*:*Z* isomers, whereas the *Z*-strain responds optimally to a 3:97 mixture (Roelofs *et al.*, 1987). There is no cross-attraction between the strain-specific blends, and male response quickly diminishes as the optimal isomeric ratios are changed along a linear axis. Additionally, the concentration of pheromone loaded onto a dispenser can influence the flight performance of male moths (Kuenen and Baker, 1982; Linn *et al.*, 1988; Charlton *et al.*, 1993).

Effects of physical plume properties on behavior

There is no question that the distribution of odor molecules in time and space in a plume play an important role in shaping behavioral responses of male moths. Initially, male attraction to female moths was hypothesized to occur inside an "active space," a spatial envelope within which the time-averaged concentration of the plume was sufficient to elicit a behavioral response (Bossert and Wilson, 1963). However, subsequent experiments with a number of different moth species demonstrated that even gross plume structure played a significant role in shaping behavior. For example, *Adoxophyes orana* and *G. molesta* males exposed to continuous, uniform clouds of pheromone (or miasma) after a brief upwind movement ceased upwind flight and began crosswind casting, the typical response to loss of odor. Importantly, when a more concentrated plume was superimposed upon the cloud background, males resumed upwind flight indicating that fluctuations in concentration were necessary for the maintenance of upwind flight (Kennedy *et al.*, 1981; Willis and Baker, 1984). Additionally, *G. molesta* males were able to sustain upwind flight when a continuous cloud was pulsed to produce bands or swaths of pheromone (Baker *et al.*, 1985). These results suggested that intermittent, fluctuating pheromone stimulation was important in allowing males of most species to maintain upwind flight although there appear to be some exceptions (e.g., Justus and Cardé, 2002; Justus *et al.*, 2002b). This idea was built upon by Baker (1990) and Kaissling and Kramer (1990) who proposed that male moths respond to encounters with the wisp-like filaments of pheromone that constitute the plume by surging upwind and quickly lapsing into crosswind casting in the absence of further stimulation. Experiments designed to test this hypothesis subsequently demonstrated that the fine-scale, filamentous structure of a pheromone plume was important in shaping flight responses. After intercepting individual filaments of pheromone, male *Heliothis virescens* that had previously been casting in clean air were shown to respond with a brief upwind surge before lapsing into crosswind, casting flight once again (Vickers and Baker, 1994, 1996). The surge comprised a short upwind movement resulting from the male turning into the wind and increasing its airspeed. This was accompanied by a short-lived increase in turning frequency. Thus, an encounter with a single pheromone filament altered the visually guided anemotactic response as well as the counterturning program. Concurrent, but independent, studies of another species, *Cadra cautella*, similarly revealed the ability to respond to encounters with individual pheromone filaments (Mafra-Neto and Cardé, 1994, 1995a,b, 1996). Flight in plumes in which the frequency of filaments was controlled by a puffing device revealed that upwind flight by *H. virescens* males was not sustainable in response to plumes that were created at a frequency of less than four filaments per second. At this threshold frequency flight trajectories were a mixture of upwind movements (surges) interspersed with crosswind casting. However, as pulse frequency was increased, male flight trajectories became oriented much more directly upwind with little crosswind flight (Vickers and Baker, 1994, 1996). Similar

responses to manipulations of pheromone plume structure were also reported for *C. cautella* males (Mafra-Neto and Cardé, 1995a, 1998).

Integrating biological and physical plume properties

Clearly, both biological and physical properties of plumes can independently influence moth flight behavior, and indeed, experimental manipulation of these features has provided a great deal of information about the behavioral mechanisms that moths employ to maneuver in wind-borne pheromone plumes. Additional experiments that have examined the interface between these biological and physical properties have also illuminated our understanding. For example, Vickers and Baker (1992) used a pulsing device in which the two necessary pheromone components of the *H. virescens* pheromone were delivered in alternating filaments. Each filament's odor quality was thus suboptimal, and significantly fewer males responded to staggered component filaments in contrast to the comparable blend filament treatment (9% vs. 30% source location) (Vickers and Baker, 1992). Furthermore, interception of single pheromone filaments tainted with a behavioral antagonist elicited suboptimal upwind surges in *H. virescens* males due to reductions in both anemotactic and counterturning reponses (Vickers and Baker, 1997b). In contrast, experiments with a related species, *Helicoverpa zea*, showed that the anemotactic and counterturning components of the upwind surge were unaltered by the presence of a behavioral antagonist mixed with the pheromone but that the latency to the surge response was greater. This delayed reaction time might account for the pronounced antagonistic effect of this compound in point-source pheromone plumes, where frequent and rapid encounters with filaments would occur (Quero *et al.*, 2001). Additional experiments with *H. zea* in which plumes were created by simultaneously pulsed odor cartridges (one emitting an attractive binary pheromone blend and the other a known antagonist) separated by as little as 1 mm resulted in greater attraction than a confluent strand emitted from both cartridges. This revealed the remarkable faculty of the olfactory system of these insects to discriminate filaments separated by as little as 1–3 ms (Fadamiro and Baker, 1997; Baker *et al.*, 1998; Fadamiro *et al.*, 1999). These studies argue that at least one aspect of the biological property set (odor quality) is assessed within the physical spatiotemporal context—the individual pheromone components must be synchronized in time and space for blend recognition and optimal behavioral responses. Thus, it seems reasonable to speculate that the interplay between these features should be represented within the olfactory system.

The moth olfactory system

The moth olfactory system is relatively simple but representative of higher vertebrates making it a useful model (Hildebrand and Shepherd, 1997; Eisthen, 2002). Thousands of cuticular hairs (sensilla) cover the adult antenna, and many of these contain the dendrites of one or more olfactory receptor neurons (ORNs) (reviewed in Hansson, 1995; Keil, 1999). Axons of ORNs coalesce in the lumen of the antenna to form the antennal nerve and project to the antennal lobe, the primary processing center for olfactory information in the insect brain—analogous to the vertebrate olfactory bulb. The antennal lobe is organized into spheroidal knots of neuropil known as glomeruli that are a common anatomical feature of the olfactory systems of a broad diversity of animal taxa (Hildebrand and Shepherd, 1997; Eisthen, 2002). Each ORN axon projects to a single glomerulus wherein synaptic connections are made with central interneurons (Hansson *et al.*, 1992; Anton and Homberg, 1999). Males possess a sexually dimorphic subset of long sensilla that contain ORNs tuned to pheromone components or behavioral antagonists (often pheromone components of closely related or sympatric species). Activity in these ORNs is processed within a specialized subset of enlarged glomeruli that occupy a position close to the entrance of the antennal nerve into the antennal lobe—the macroglomerular complex (MGC). At least three different types of neuron populate the antennal lobe neuropil: intrinisic local interneurons that often arborize within many glomeruli, projection neurons (PNs) that send axons to higher brain centers via one of three main output tracts, and centrifugal neurons that typically exhibit dendritic arborizations in other areas of the brain with axonal connections in the antennal lobe (Hansson, 1995; Anton and Homberg, 1999). The moth olfactory system is amenable to electrophysiological recording which can be performed from both sensory and central neurons providing insights into their ability to respond to the biological and physical properties of odor plumes. Additionally, both peripheral and central neurons can be stained using a variety of techniques to reveal their morphological characteristics which can then be correlated with their physiological properties. Local interneurons appear to modulate activity between glomeruli via γ-aminobutyric acid inhibitory synaptic connections. PNs are analogous to the mitral/tufted cells of the vertebrate olfactory bulb, and many pheromone-specific PNs exhibit dendritic arborizations restricted to a single glomerulus (uniglomerular) although some arborize in more than one glomerulus (multiglomerular). There is substantial physiological and morphological evidence that ORNs and PNs that comingle within a single glomerulus generally also respond to the same narrow set of odorants (in the case of pheromones, often only a single odorant e.g., *H. virescens* and *M. sexta*). Thus, odor quality of multicomponent pheromone blends in these animals is represented by activity of PNs with dendritic arborizations housed across an array of individual glomeruli. Such combinatorial codes have now been implicated in olfactory research on a variety of organisms (e.g., Friedrich and Korsching, 1997; Joerges *et al.*, 1997) with moth pheromonal systems representing the more specialized end of the spectrum (Vickers *et al.*, 1998).

Responses of antennal sensory structures to pheromone

Olfactory receptor neurons

Male antennal ORNs are often specifically tuned to single components of the pheromone blend. For example, there are three different physiological sensillar types on male *H. virescens* antennae (Hansson *et al.*, 1995; Baker *et al.*, 2004). Two of these sensilla house an ORN that respond specifically to either one of the two essential components of the pheromone blend, (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal. The third sensillar type contains two ORNs, one of which is tuned to (*Z*)-11-hexadecenyl acetate, an odorant that inhibits male flight when combined with the normal pheromone blend. Thus, it is clear that these ORNs are primarily responsive to the qualitative properties of the odor striking the antenna—odor quality is disassembled by these narrowly tuned ORNs. Indeed, this representation is maintained by the antennal lobe output neurons, such that PNs with arborizations restricted to individual MGC glomeruli are primarily activated by single odorants (see below).

A few studies provide evidence that odor mixtures can modulate the electrophysiological responses of pheromone-component specific ORNs in some moth species. While such effects do not appear to be a universal phenomenon, their occurrence is relevant here. In *Trichoplusia ni*, responses of ORNs tuned to the major pheromone component were enhanced by the presence of certain additional pheromone components (O'Connell *et al.*, 1986). Additionally, coincident stimulation with plant volatiles influenced the responses of ORNs tuned to pheromone components in males of different species (suppression: Den Otter *et al.*, 1978; van der Pers *et al.*, 1980; synergism: Ochieng *et al.*, 2002). The mechanisms underlying these various mixture-induced effects are not well understood, but changes in peripheral activity stemming from fluctuating odor quality may play a significant role in shaping the responses of higher order neurons.

In many experimental situations ORN physiological profiles are established by pulsing odor stimuli directly over the antenna while recording from sensilla housing ORN dendrites. Some studies have directly examined the maximum frequencies of pulse resolution by ORNs. In *M. sexta*, ORNs could follow 20-ms pulses up to 3 Hz, whereas those of *Antheraea polyphemus* were capable of resolving up to 5 Hz (Kaissling, 1986; Rumbo and Kaissling, 1989; Marion-Poll and Tobin, 1992; Kodadová, 1996). Additionally, experiments in which recordings from single sensilla have been made either within a laboratory wind tunnel (Baker *et al.*, 1988; Valeur *et al.*, 1999, 2000) or in the field (Van der Pers and Minks, 1993) have shown that ORNs respond to the arrival of pheromone filaments created in synthetic as well as female-emitted pheromone plumes.

The ability of ORNs to follow and transduce pulses of odor as they arrive at the antenna will clearly affect the responses of any central olfactory neurons in the antennal lobe which are reliant upon synaptic activation by these peripheral inputs. Any limitations on the transduction process could affect the detected structure—thus rates of adaptation and disadaptation will influence the ability of ORNs to follow the arrival of odor filaments on the antenna and report their temporal intermittency accurately to higher brain centers. Baker *et al.* (1988) demonstrated that the majority of single ORNs tuned to (*Z*)-5-decenyl acetate on the antennae of male *Agrotis segetum* adapted to intermittent arrival of pheromone when placed in a plume generated by a source with a high loading concentration (300 μg). Interestingly, ORNs tuned to a second component, (*Z*)-7-dodecenyl acetate, were not adapted in this same plume, suggesting that the peripheral representation of the blend ratio was altered. Previous behavioral studies had shown that males ceased upwind flight in plumes of the same dosage (Löfstedt *et al.*, 1985). Valeur *et al.* (2000) also correlated in-flight arrestment of *A. segetum* males with maximal spiking activity in *Z*5-12:OAc–specific neurons. In addition, other physical variables can affect these invertebrate creatures—colder temperatures significantly decreased the ability of sensory neurons to follow the arrival of individual pheromone pulses along the antenna of *G. molesta* and *A. polyphemus* males (Baker *et al.*, 1988; Kodadová, 1996). Cool temperatures (17°C) hampered the ability of *G. molesta* antennal ORNs tuned to (*Z*)-8-dodecenyl acetate to follow the arrival of pulsatile stimuli compared to warmer conditions (23°C). A similar temperature drop had been shown to interfere with the ability of *G. molesta* males to fly upwind and locate a pheromone source (Linn *et al.*, 1988).

Electroantennograms

A useful technique for measuring activity along the length of the antenna is the electroantennogram (EAG), whereby electrodes are connected across each end of an intact or excised antenna, and the differential signal across the electrodes is amplified and recorded. The EAG signal represents the summed potential of all electrical activity along the antenna without respect to the behavioral significance of the activity. EAGs have been used for many years as a tool to aid in the identification of biologically active molecules using a spectrum of techniques (see Bjostad, 1998). However, because the male EAG is an extremely sensitive method for detecting the presence or absence of pheromone, this technique has also proven useful as a method for measuring the spatiotemporal structure and dynamics of pheromone plumes. Indeed, because the antenna is the actual sensory apparatus used to transduce odor signals and any EAG activity reflects inherent biological limits, it can be argued that the EAG activity is the most relevant measure of plume structure (Baker and Haynes, 1989).

Stationary EAGs

EAG preparations held stationary in plumes generated by a point source in a wind tunnel can be used to sample the

frequency and magnitude of electrical events passively occurring along the antenna. Such measurements confirm that the biological appearance of the plume is highly intermittent (Baker *et al.*, 1985) and compare well with other techniques for measuring turbulent plume structure including the use of fluorescein dyes in aquatic environments (Zimmer-Faust *et al.*, 1995) or electrochemical detection of a tracer such as dopamine (Moore *et al.*, 1989; Moore and Atema, 1991). Vickers *et al.* (2001) utilized an EAG preparation (*H. virescens* male antenna) to sample cross-sectional plume structure in a wind tunnel at different wind speeds and determined that there was a steep decline in filament frequency at the edges of the active space particularly at slower wind speeds where the plume appeared less dispersed. EAG amplitude also differed over a pheromone plume's cross section with large amplitude depolarizations (bursts) occurring in the central region particularly at slower wind speeds. Recordings from stationary EAG preparations (*G. molesta* male antenna) in the field revealed that EAG burst frequencies were about 1/s even up to 30 m downwind of the pheromone source. These frequencies were low because the pheromone plume meandered in horizontal and vertical planes and made only seldom contacts with the stationary preparation. However, burst amplitude diminished as distance increased (Baker and Haynes, 1989). In a recent study, EAGs were recorded from the antennae of three different moth species in response to pulsatile plumes created at frequencies up to 33 Hz. Lower pulse frequencies (under 5 Hz) were clearly resolvable by the antenna with almost complete return to baseline in between EAG bursts (Bau *et al.*, 2002). Fourier analysis of the EAG waveforms recorded at higher frequencies of pulse delivery revealed distinct peaks in the power spectrum corresponding to these faster pulse delivery rates, suggesting that some neural elements on the antenna (i.e., ORNs) were capable of responding to pulse arrival at rates between 25 and 33 Hz (Bau *et al.*, 2002). Such rapid activity (pulse delivery at >10 Hz) creates a fusion of EAG activity such that there is no recovery to baseline—a condition that was noted to be correlated with cessation of upwind flight in *G. molesta* (Baker and Haynes, 1989). However, one of the species utilized in the study of Bau *et al.* (2002), *C. cautella*, appears to be capable of responding within homogeneous pheromone clouds with directed upwind flight at least for short periods (Justus and Cardé, 2002; Schofield *et al.*, 2003). Measurements of ion plumes coupled with simultaneous pheromone plume EAG recordings (male *Lymantria dispar* antenna) in open field and forest revealed that there was a correlation between some variables of these independent measures of plume structure (Murlis *et al.*, 2000). Recently, EAG responses from male antennae of two different species were correlated with coincident mini-PID measurements of concentration from a tracer gas (propylene) plume produced in a wind tunnel (Justus *et al.*, 2005). The results demonstrated that application of a low-pass filter to signal recorded from the PID approximated the EAG signal of each species but also that the response dynamics of the two species were not equivalent.

Moving EAGs

In order to simulate the plume structure encountered by flying male moths, an EAG preparation mounted on a moving platform was utilized to measure plume structure while being pushed upwind toward the pheromone source (Baker and Haynes, 1989). This is the only mechanism by which the encounter rate with pheromone filaments can be increased when the filaments are being produced at a constant rate (Baker and Vickers, 1994). The results revealed that EAG burst frequency increased from 1.6 to 3.8 Hz as higher moth airspeeds (from 30–80 cm/s) were simulated by pushing the mounted preparation faster up the wind tunnel but peak-to-trough EAG burst amplitudes were unchanged over the range of speeds tested.

Flying EAGs

Using a pair of fine chloridized silver wires inserted into either end of an excised antenna provided a technique for allowing an EAG preparation to be mounted with velcro and transported by a flying *H. virescens* male (Vickers and Baker, 1994; Vickers *et al.*, 2001). Mounting an EAG on a freely behaving insect demonstrated that males did indeed encounter an intermittent and fluctuating pheromone signal during upwind flight (Figure 2a,b; Vickers *et al.*, 2001). This same EAG technique had been used in some of the stationary recordings described above (Vickers *et al.*, 2001), facilitating a comparison between various EAG parameters under the two differing conditions. Not surprisingly, the transported EAG registered activity from encounters with pheromone filaments most frequently along the plume centerline with a decline in encounter rate toward the edges of the plume mirroring the results from the stationary EAG (Figure 2c). However, the relative EAG amplitudes were two to three times higher than those measured from the stationary preparation. This was not the result of EAG amplitude increasing as the males approached the pheromone source as these did not change significantly with distance from the source (Vickers *et al.*, 2001). Contributions that might be made to the differences measured between stationary and flying EAGs from wing beating or antennal flicking versus simple movement through the plume were not determined in this experiment but might be important (Koehl, 2005). Nonetheless, these results demonstrated that a freely moving and behaving animal will experience a different sensory environment than a stationary one. It remains to be seen how sensory activity might change, if at all, in response to specific maneuvers such as crosswind casting versus upwind surging, particularly if the changes in wing kinematics that accompany these maneuvers actually increase the airflow through the antennae (Koehl, 2005). It is possible that the sensory activity evoked by a filament encountered during a transverse sweep across the plume (casting) may be different from the

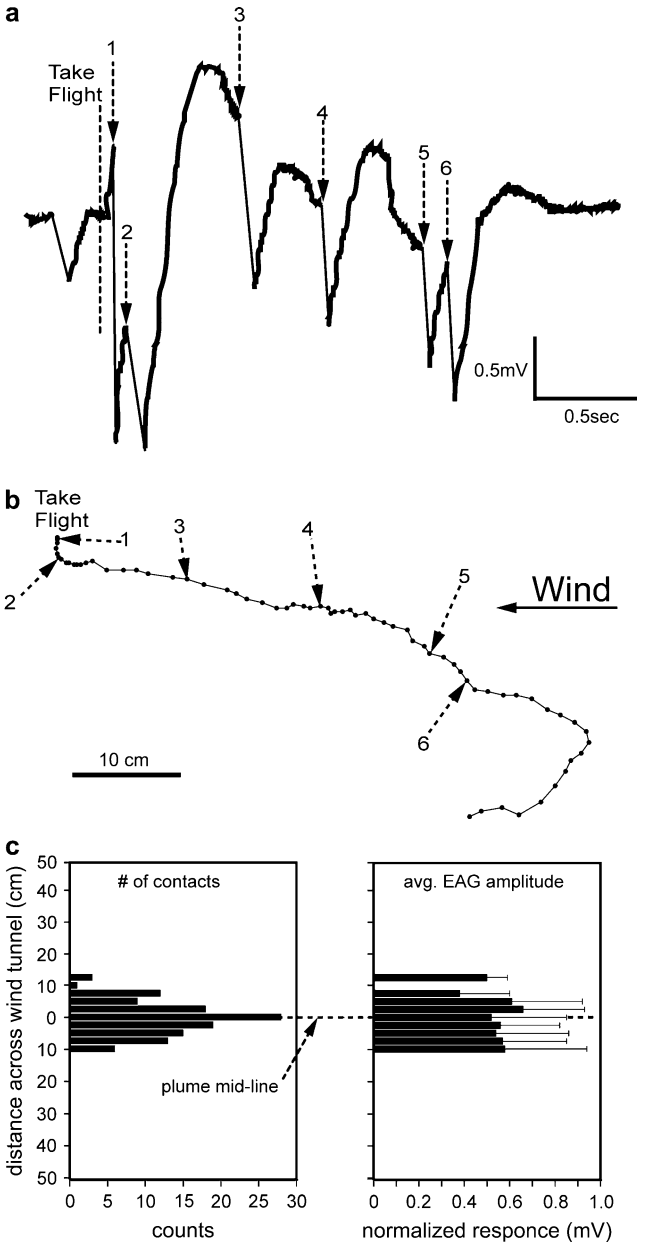

Figure 2 Upwind flight by male moths is sustained by repeated, intermittent contacts with a pheromone plume. **(a, b)** EAG trace recorded from an excised antenna transported on the thorax of a male *Heliothis virescens* engaged in upwind flight in a pheromone plume. Numbers along the EAG trace and corresponding flight track indicate the points at which EAG bursts (swift downward deflections in the trace) occurred. **(c)** Data from 20 such flights revealed that most EAG bursts occurred along the plume's horizontal midline with a rapid decline in activity to either side. However, EAG amplitudes were similar regardless of the proximity to the plume midline. Figure modified from Vickers *et al.* (2001) and reproduced with permission of Macmillan Magazines Ltd.

same filament encountered either during direct upwind flight (surging) or even downwind flight, which moths in the field have been shown to do following loss of a pheromone plume (Baker and Haynes, 1996). Thus, during a surge, various factors may contribute to maximizing the velocity of sensory appendages relative to wind-borne pheromone filaments thereby constituting an effective behavioral sniff.

Responses of single central neurons

Odor pulses

The responses of single central olfactory PNs to stimulation of the ipsilateral antenna with pulses of pheromone components or mixtures thereof have been investigated in a number of species. In fact, this is the normal method for identifying the physiological, odorant-driven response properties of central PNs. However, these studies are most useful in understanding the effects of odor plume dynamics on neuronal responses when the odor pulse stimulus regime is such that it mimics the type of plume structure that the animals normally encounter and require in order to sustain upwind flight, that is, brief pulses at a frequency of 2 Hz or greater. Longer pulses, greater than 100–200 ms in duration, delivered at a slow frequency might be informative about certain cellular or network properties such as synaptic connectivity or rates of adaptation (e.g., Christensen *et al.*, 1998) but provide little information about how these neurons handle the temporal dynamics under which they are required to operate during flight. In male *M. sexta* central olfactory output neurons tuned to pheromonal odorants can be categorized into two broad physiological types based upon their responses: generalists and specialists (Christensen *et al.*, 1989, 1996). To a greater or lesser extent these categories can be identified in all other species of moth that have been examined to date.

Pheromone-specialist PNs are specifically excited only by a single odorant and have a variable ability to follow intermittent stimuli. Anatomically, these PNs usually have a dendritic arbor restricted to a single glomerulus (i.e., they are uniglomerular). In *M. sexta*, the temporal responses of some central PNs were sharpened by the presence of both components of the pheromone mixture. Investigations of the underlying synaptic mechanisms determined that this response was due to a mixture of inhibition driven by one pheromone component and excitation evoked by the other pheromone component. Indeed the strength of inhibition, as measured by the magnitude of the inhibitory postsynaptic potential (IPSP), was strongly correlated with the ability of individual PNs to follow the arrival of pulsatile stimuli up to a frequency of 10 Hz (Christensen and Hildebrand, 1997; Heinbockel *et al.*, 1999, 2004). Furthermore, varying stimulus duration was best represented in those PNs that exhibited the strongest IPSPs (Christensen and Hildebrand, 1997). Many PNs were capable of following pulses delivered at a rate of 3–5 Hz, certainly within the range expected in a natural odor plume and around the rate required to sustain upwind flight in *H. virescens*. Other PNs were capable of following pulse frequencies up to 10 Hz and may be important close to a pheromone source where higher pulse frequencies are likely to be encountered. The mixed excitatory and inhibitory

synaptic inputs driven by different pheromone components evidenced in such PNs provide a physiological mechanism that brings together a representation of the biological and physical (temporal) properties of the pheromone plume. Furthermore, when the ratio of the two components used to elicit these responses was changed from 1:1 to 2:1, the PNs were no longer able to follow the arrival of pulsatile stimuli because the inhibitory input from the elevated component caused prolonged hyperpolarization such that the excitatory postsynaptic potentials in response to the unchanged component could no longer bring the PN to threshold (Christensen and Hildebrand, 1997). More recent experiments with further ratios and dosages showed that *M. sexta* olfactory PNs were highly sensitive to the specific blend presented (Heinbockel *et al.*, 2004). Thus, two important dimensions of the biological property set (quality and quantity) were necessary to facilitate representation of the temporal structure of the stimulus in some PNs. In other species, the ability of central PNs to follow the arrival of pulsatile stimuli does not always require the presence of the multicomponent pheromone. For example, in *A. segetum* biphasic excitatory–inhibitory responses of single PNs were observed but these occurred in the presence of only a single pheromone component (Lei and Hansson, 1999). Nonetheless, the ability of these PNs to follow pulsatile stimuli was a function of the magnitude of the inhibitory contribution to the biphasic response.

Included among neurons in the pheromone-specialist category are blend-sensitive PNs that had little or no response to individual components but were either synergized or enhanced by the presence of an appropriate pheromone blend to produce a temporally coherent response. In *H. virescens*, blend-synergist PNs responded only to a complete blend presented as a stimulus, whereas blend-enhanced PNs, similar to those neurons described above in *M. sexta*, had a weak response to a single pheromone component that was greatly improved by the presence of a blend (as shown in Figure 3) (Vickers *et al.*, 1998). Blend responsive PNs have also been identified in other species including *A. segetum* and *Spodoptera littoralis* (Anton and Hansson, 1995; Hartlieb *et al.*, 1997; Wu *et al.*, 1996). The general occurrence of blend sensitivity in a subpopulation of antennal lobe PNs in a variety of moth species suggests that they play an important role in olfactory processing, perhaps in determining the simultaneity of arrival of individual pheromone components in a blend. Thus, in several species of moth, a spectrum of physiological profiles exists to convey varying combinations of odor quality and temporal information from the antennal lobe to higher brain centers.

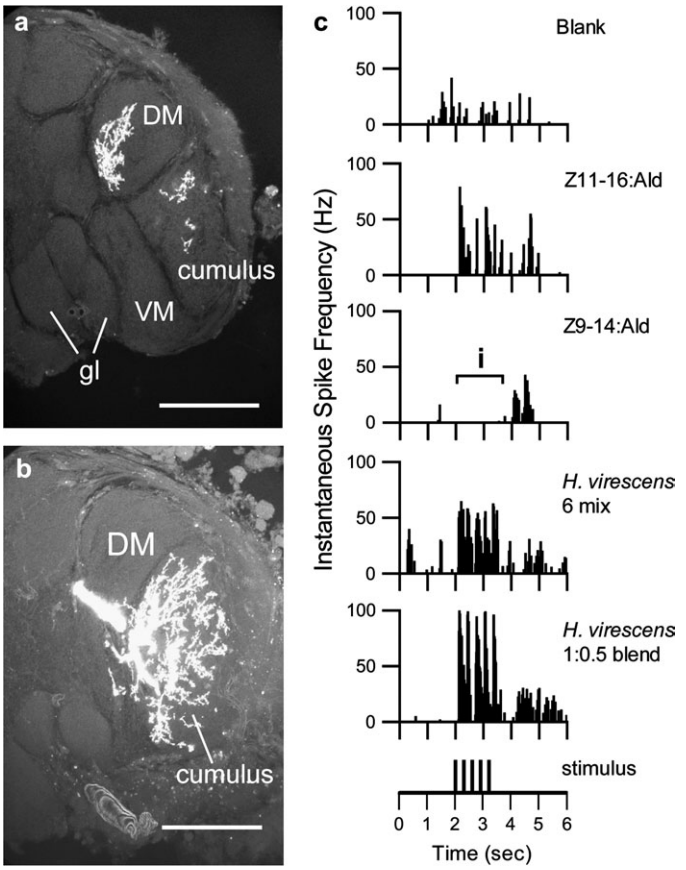

Figure 3 Morphological and physiological characteristics of an olfactory PN in the antennal lobe of a male *Heliothis virescens* that responded optimally to a blend of pheromone components. **(a, b)** Confocal images of sectioned material reveal that this PN had dendritic arborizations in two glomeruli [named dorsomedial (DM) and the cumulus]. Other ordinary glomeruli (gl, some of which are indicated) were not innervated by this PN. Scale bars = 50 μm. **(c)** Electrophysiological records revealed that this PN was depolarized by (*Z*)-11-hexadecenal (*Z*11-16:Ald) and inhibited by (*Z*)-9-tetradecenal (*Z*9-14:Ald) (period of inhibition indicated by bracket and "i"), responses likely mediated by arborizations in the cumulus and DM, respectively. Presentation of these pheromone components in a blend (*H. virescens* 6 mix and 1:0.5 blend) resulted in a stronger response that more accurately encoded the temporal dynamics of the stimulus than *Z*11-16:Ald presented alone. Each stimulus was presented as a series of 5 × 40–ms pulses. Scale bars: horizontal = 1 s, vertical = 50 mV. Figure modified from Vickers *et al.* (1998) and reproduced with permission of John Wiley & Sons, Inc.

Odor plumes

Relatively little is known about how central olfactory PNs respond to intermittent stimulation in a pheromone plume. Recordings from single olfactory PNs, while simultaneously monitoring pheromone-induced activity on the ipsilateral antenna (by means of an EAG), demonstrated that pheromone filaments arriving on the antenna were faithfully translated into activity of central PNs (Figure 4a). Both numbers of action potentials and maximum instantaneous frequency of spikes occurring in a burst were significantly correlated with the peak-to-trough amplitude of each EAG deflection (Figure 4b; Vickers *et al.*, 2001). However, the slope of the EAG onset was not a good predictor of PN activity (Figure 4b). Presumably, factors that change EAG parameters such as burst amplitude (and the underlying causal neuronal responses) will have a corresponding effect on the activity of central PNs. These recordings were made from individual

PNs that were tuned to individual pheromone components, but no difference was evident between PNs tuned to different pheromone components. These results are important because they demonstrate a direct correlation between the magnitudes of peripheral and central neuronal activity. However, these experiments were performed in a stationary preparation, and the consequences of animal movement within the plume upon central activity have not yet been investigated directly. Nonetheless, the flying EAG experiments described above have provided insights into the temporal structure of plumes encountered during flight and, given the correlation between EAG and central activity, it is not unrealistic to speculate on the central responses based upon these data. Given the fact that EAG amplitudes increased by two- to threefold for a flying male, one might predict that the activity of central PNs would be similarly affected. However, experiments in which the activity of single central olfactory PNs is recorded during flight pose technical challenges that have yet to be resolved.

Responses of neuronal populations

It is obvious that neurons are not independent entities but are organized into circuits that facilitate interactions with other neurons in the population through synaptic

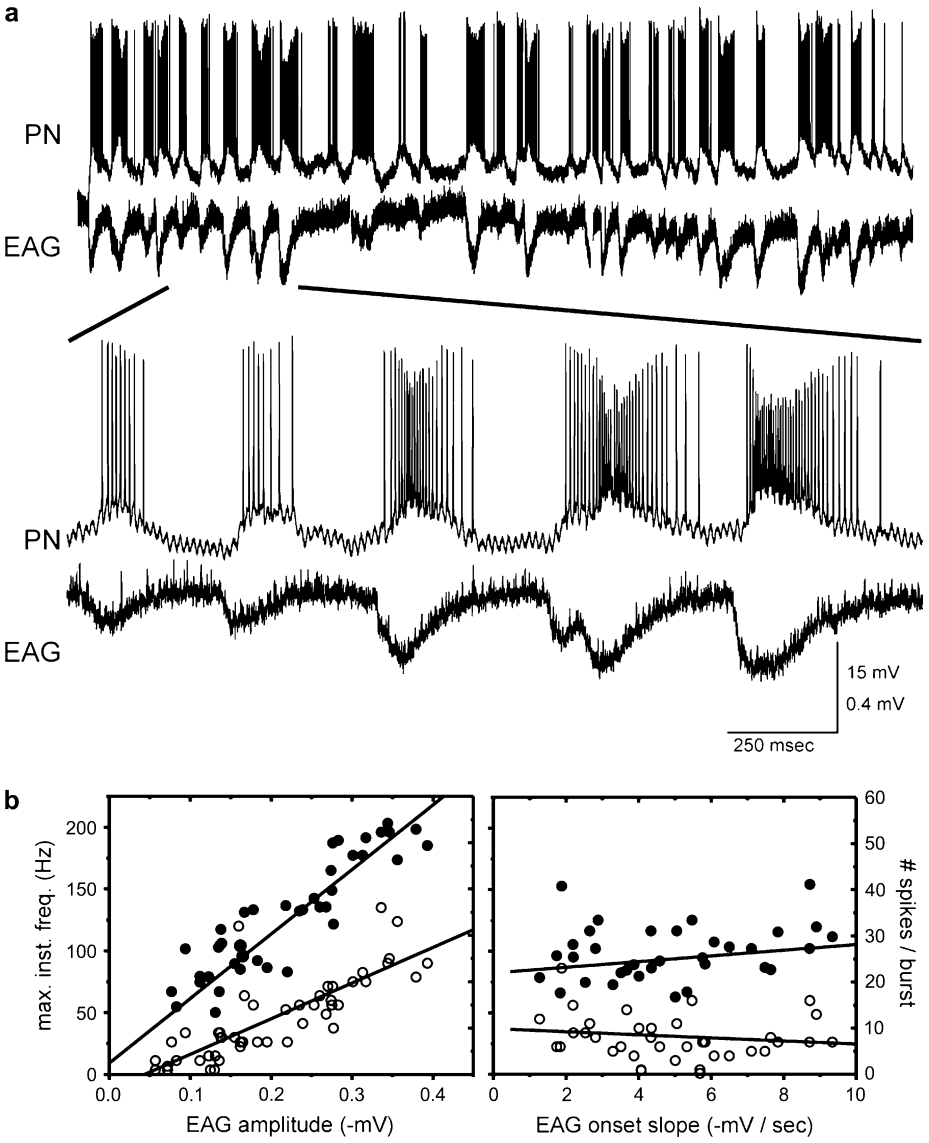

Figure 4 Temporal patterns of action potentials in olfactory PNs are strongly dependent upon the stimulus dynamics of the wind-borne pheromone plume. **(a)** A 15-s segment of activity recorded simultaneously from a PN and the ipsilateral antenna (EAG) in a *Heliothis virescens* male. Details of the indicated segment is shown in the lower traces. Scale bars apply to magnified traces only. **(b)** Intensity of pheromone stimulation as indicated by EAG amplitude (left) was tightly correlated to both the maximum instantaneous frequency of PN response (solid circles: $r^2 = 0.76$, $P < 0.0001$) and the mean number of spikes per pheromone-evoked burst (open circles: $r^2 = 0.65$, $P < 0.0001$). Other measures of peripheral activity such as EAG onset slope (right) did not serve as reliable predictors of central activity. Figure modified from Vickers *et al.* (2001) and reproduced with permission of Macmillan Magazines Ltd.

and electrical contacts. Several recent studies have sought to understand how the organization of connections between central neurons in the moth olfactory system contributes to the temporal structure of their activity. These studies have included recordings from pairs of olfactory PNs as well as multielectrode recordings from neuronal ensembles in the antennal lobe (Christensen *et al.*, 2000, 2003; Lei *et al.*, 2002, 2004). These studies have highlighted the important role that synchronization of action potential activity across neuronal populations might play in coding and discrimination of olfactory signals (i.e., recognition of the biological properties of a pheromone signal). It does not appear that such temporal mechanisms discard the ebb and flow of the olfactory signal but instead are proposed to actually preserve the instantaneous fluctuations in stimulus dynamics that occur in turbulent pheromone plumes for higher centers in the brain (Christensen *et al.*, 2003).

Conclusions

Plume dynamics are important in modulating the behavioral responses of male moths (reviewed here) and other animals (Koehl 2005). ORNs and central olfactory PNs are capable of following the arrival of individual pheromone filaments at frequencies that are likely to be encountered by male moths flying in a naturally intermittent plume. In addition to preserving a faithful representation of the plume's temporal fluctuations, the male moth olfactory system also extracts and encodes information regarding the biological properties of the pheromonal signal. The effects of the fluid environment on odor stimulus dynamics have important consequences for animal behavior in general. An approach that integrates an appreciation of these various aspects is essential to understanding how the olfactory system processes and discriminates among the many odors to which a given animal responds.

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