

Neurophysiological responses of pheromone-sensitive receptor neurons on the antenna of *Trichoplusia ni* (Hübner) to pulsed and continuous stimulation regimens

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Abstract. Both the frequency and the temporal pattern of action potential production in an insect olfactory receptor neuron are strongly affected by odorant composition and the time course over which stimulus concentration varies. To investigate the temporal characteristics of the neurophysiological responses of these neurons, we devised a stimulus delivery system that allows us to repeatedly present well-mixed, constant concentration odor pulses with relatively sharp onsets and offsets. Here we compare neurophysiological responses to several different stimulation regimens, including pulses of different durations and repetition rates. During stimulation with high concentrations of pheromone, the temporal pattern of neural activity from olfactory receptor neurons on the antenna of *Trichoplusia ni* (Hübner) is characterized by an initial phasic period (100–200 ms), followed by a tonic period which is typically maintained for the remaining duration of the stimulus. Different olfactory receptor neurons appear to vary among themselves in the relative distribution between the phasic and tonic portions of the overall discharge. During stimulation regimens involving rapid repeated pulses of odorants, a portion of the phasic response levels is preserved during each pulse. Consequently, *T.ni* males probably detect much of the fluctuation in concentration of pheromone that may normally occur downwind from the site of pheromone release.

Introduction

The influence of the structure of the pheromone plume on the mate-seeking behavior of insects has been a question of growing importance since the initial observations that some moths respond differently under continuous and intermittent pheromone stimulation (Kennedy, 1982; Baker *et al.*, 1985; Payne *et al.*, 1986). Enough is now known about the pheromonal dispersal process and the insect olfactory system to develop some hypotheses about the physical and neurophysiological bases of these effects (e.g. Mayer and Mankin, 1985; Light, 1986). The plume downwind from a point-source of pheromone is thought to be detected as a series of stimulus pulses of different durations and concentrations (Mankin *et al.*, 1980; Murlis, 1986). The receptor neurons produce action potentials at a rate proportional to pheromone intensity. This pheromone-evoked response is superimposed upon a random Poisson distribution of spontaneous activity, which contributes significantly to the total response at low stimulus intensities (Mankin *et al.*, 1987; Kaissling, 1987). The responses from all the pheromone receptor neurons on the antenna are combined in the macroglomerulus of the deutocerebrum (Boeckh *et al.*, 1984). The response is extracted from the spontaneous activity and information about key features of the stimulus pattern (Christensen and Hildebrand, 1987) is passed to higher centers of the brain (Olberg, 1983) that control the rate of counter-turning, the speed of flight and other aspects of orientation to the pheromone plume (Baker *et al.*, 1985).

While this broad outline of the pheromone detection-orientation process is generally accepted, a number of details are poorly understood, including the question of how precisely the peripheral receptor neurons resolve the fine structure of a pheromone plume. For example, Baker *et al.* (1985) found that pheromone pulses emitted in a wind tunnel could be resolved easily in *Grapholita molesta* (Busck) electroantennograms where the pulse rate was 0.5/s, but not so easily when the rate was 1/s. Kaissling (1986, 1987) found that *Antheraea polyphemus* (Cramer) receptor neurons sensitive to a minor component, (*E*)-4, (*Z*)-9-tetradecadien-1-ol acetate, could resolve pulses as fast as 10/s, but the neurons sensitive to the major component, (*E*)-6, (*Z*)-11-hexadecadien-1-ol acetate, could not resolve pulses emitted at rates above 2/s. Differences between neurons also were observed in comparisons of the patterns by which the impulse frequency rose and fell during stimulation.

Historically, the measure used to quantify responses from insect olfactory receptors has been the mean frequency of action potentials discharged during a defined stimulus period. Notwithstanding, the CNS may extract additional information from other parameters such as those involving the temporal distribution of impulses, e.g. instantaneous frequencies, interspike intervals, or differences among the initial response latencies of different neurons. Because such response measures are strongly dependent on the pattern and intensity of the instantaneous stimulus concentration applied to the receptor neuron, the parameters of the stimulus must be tightly controlled before their effects can be interpreted reliably. Unfortunately, these stimulus parameters cannot be monitored directly because the concentrations which are effective physiologically are well below those that can be measured even with the most sophisticated analytical instrumentation. Consequently, most studies have been unable to apportion the measured differences in the temporal pattern of the observed response to either the stimulus regimen or to the intrinsic properties of the receptor neuron.

Here, we describe the responses of olfactory receptor neurons on the antenna of male *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), the cabbage looper moth, to stimulation with an individual pheromone component using a new delivery system that generates a uniform stimulus pulse with a well-defined onset and offset. The receptor neurons studied were those sensitive to (*Z*)-7-dodecen-1-ol acetate (*Z*7-12:AC), the major pheromone component, and designated as A neurons in HS sensilla (O'Connell *et al.*, 1983; Mayer and Mankin, 1985; Grant and O'Connell, 1986). The extensive studies previously conducted on *T.ni* pheromonal chemistry (Bjostad and Roelofs, 1983; Bjostad *et al.*, 1984), behavior (Leppä, 1983; Linn *et al.*, 1984, 1988; Landolt and Heath, 1987), antennal morphology (Mayer *et al.*, 1981) and neurophysiology (Mayer, 1973; O'Connell *et al.*, 1983; O'Connell, 1985, 1986; Grant and O'Connell, 1986; Grant *et al.*, 1988) make this insect an ideal subject for an in-depth study of temporal patterns of response.

Materials and methods

Insects and chemicals

Male *T.ni* pupae were taken from colonies maintained at the Insect Attractants laboratory and held at 75–85% relative humidity, 24–26°C under a 14:10 h light:dark regimen.

Neurophysiological tests were conducted 24–72 h after eclosion.

The Z7-12:AC was analyzed on a CPS-2 50-m capillary column by R.R.Heath of this laboratory and found to be greater than 99.9% pure. Aliquots of either 1 or 2 μg in 50 μl hexane were pipetted into the wells of rubber septa (5 \times 9 mm; A.H.Thomas Co., Philadelphia, PA), previously refluxed in methylene chloride to remove contaminants (Heath *et al.*, 1986; Steck *et al.*, 1979). The septa were air-dried at room temperature for 2 days prior to use and were used no more than 7 days following dosing. Septa dosed in this manner usually elicited appropriate responses from receptor neurons for periods of at least 2–4 weeks (half-life: 34.8–35.9 days; Butler and McDonough, 1979). Stimulation by the 2 μg septum was used to provide stimulation that elicited a low- to mid-range response, 20–25 imp/s, from the HS(A) neurons. The response to the 1 μg stimulus, \sim 6 imp/s, was below mid-range, and at least two orders of magnitude above the unstimulated spontaneous activity (Grant and O'Connell, 1986; Mankin *et al.*, 1987) and the behavioral threshold (R.Mankin, unpublished data).

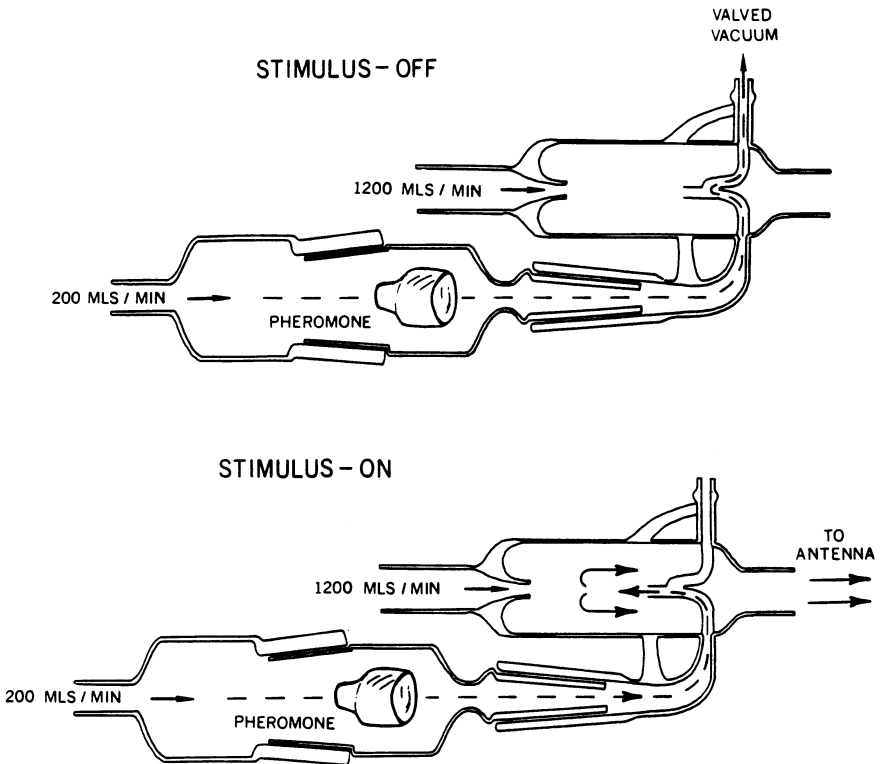


Fig. 1. Schematic diagram of the stimulus delivery system. The antennal preparation is bathed in a constant stream of carrier air (1200 ml/min). The pheromone emitted from a rubber septum is carried in a stream of stimulus air (200 ml/min) which can be diverted either to a vacuum line (\sim 250 ml/min) (Stimulus-Off), or into a mixing chamber where it combines with the carrier air and is directed over the preparation (Stimulus-On).

Delivery system

To provide a uniform stimulus with a well-defined onset and offset a dual-stage pheromone delivery device (Figure 1) was constructed. The stimulator consists of a glass mixing chamber through which filtered air (carrier air; 1200 ml/min) continuously passes to an outlet positioned ~ 5 mm above the antennal preparation. A smaller diameter (ID = 1 mm) glass tube connects one end to the pheromone source and passes through the mixing chamber to a vacuum line (~ 250 ml/min). This tube also has an outlet in the mixing chamber. The dispenser containing the pheromone is attached to the stimulation system by a ground glass joint and is continuously purged with an airstream (stimulus air; 200 ml/min). To initiate pheromone stimulation, a three-way solenoid valve (General Valve Corp., Fairfield, NJ) connected to the vacuum line is activated. The activation of this valve diverts the pheromone-laden stimulus airstream into the mixing chamber, counter-current to the carrier airstream (1200 ml/min) which insures effective mixing. The resulting combined airstreams are directed over the antennal preparation. The stimulus period is terminated by deactivating the vacuum valve thereby redirecting the pheromone-laden air from the mixing chamber to the exhaust vacuum line. Precise timing for activation and deactivation of the valve is controlled by a microcomputer. A second vacuum source is positioned under the antenna to remove pheromone-contaminated air from the area surrounding the preparations. The airstreams are delivered from high purity compressed air cylinders and filtered by passing them, in turn, through silica gel, charcoal and indicator silica gel. After leaving the compressed air cylinders, both airstreams contact only teflon and glass tubing. The stimulator always contained a single dose of purified odorant. Since the septum is purged at a fixed rate this stimulator should produce a stimulus pulse that is nearly rectangular in odorant concentration. At worst, we feel the rate of increase at the leading edge of the stimulus may be reduced due to adsorption of pheromone to the glass surfaces of the mixing chamber and the outlet of the system.

Recording

Insects were secured for single-sensillum recordings with low-melting point wax and one antenna was positioned to allow access with the microelectrodes. The microelectrodes, composed of electrolytically sharpened tungsten wire (tip diameter $< 1 \mu\text{m}$), were held by micromanipulators (E.Leitz, Inc., Rochwell, NJ) and positioned under a compound microscope (Leitz, Inc; maximum magnification $900\times$). The indifferent electrode was inserted into the lumen of a flagellar subsegment in the distal third of the antenna and the recording electrode was positioned at the base of a sensillum trichodeum located along the distal margin of a more proximal subsegment. The signals from the microelectrodes were amplified by a Grass P-18[®] preamplifier and displayed on a Tektronix Model 5113[®] storage oscilloscope. The signals were further amplified and sent to a Digital PDP-11/23[®] microcomputer for data acquisition and analysis (Mankin *et al.*, 1987).

Following penetration of the sensillum by the recording electrode, the stimulus outlet was positioned over the preparation to isolate it in the filtered carrier air for 10–15 min prior to the first stimulus presentation. The spontaneous activity of each neuron in the

sensillum was measured before any stimulus was presented and also was monitored at intervals during the recording session to assess the status of the preparation.

Stimulus regimen

The antennae were exposed to several different patterns of stimulation including: (i) one continuous 650-s pulse; (ii) multiple 3-s pulses spaced 3 min apart for 180 min; and (iii) multiple 0.3-s pulses spaced 0.7 s apart for 10 s. In one series of tests, six different sensilla preparations from four insects were alternately tested under two different regimens to determine whether the total number of action potentials elicited by a continuous 3-s pulse were comparable to the number elicited by 10 0.3-s pulses. A minimum of five replicates were obtained under each regimen for each preparation. Event-time histograms were calculated for the mean responses of neurons stimulated under each regimen ($n = 48$ preparations) and also for replications on individual preparations ($n = 5-10$).

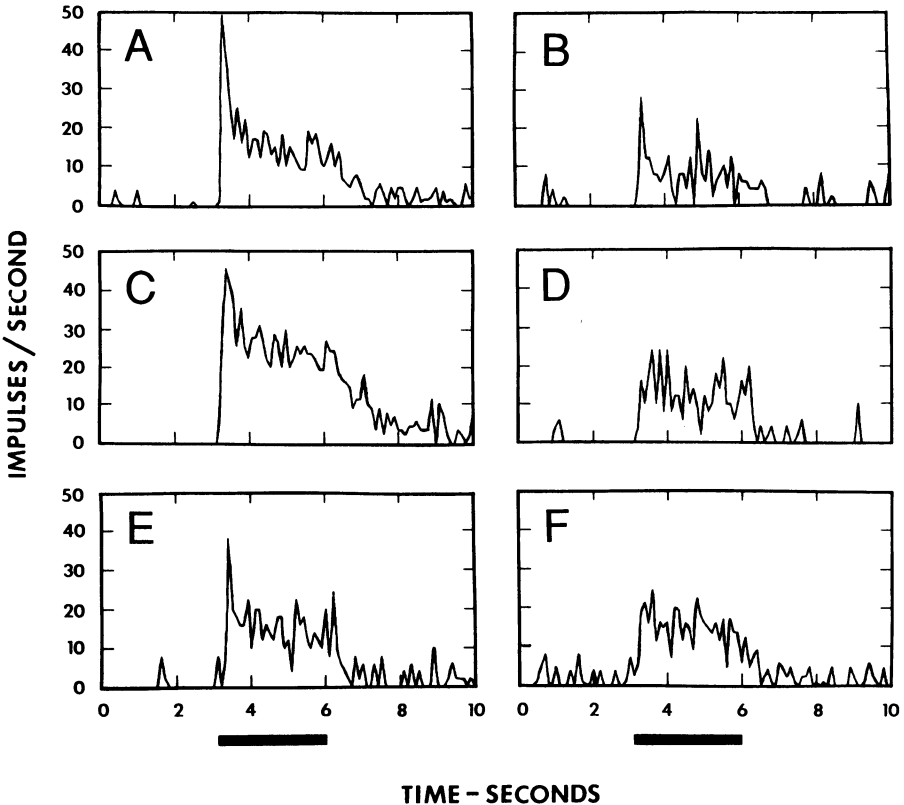


Fig. 2. Averaged event-time histograms for six individual antennal preparations (A-F). Each histogram represents an averaged response to repeated stimulations of $2 \mu\text{g}$ of Z7-12:AC. The solid bar under each histogram indicates the timing and duration of activation of the valve controlling the stimulus. The actual odorant reaches the antenna ~ 0.15 s after the valve is triggered.

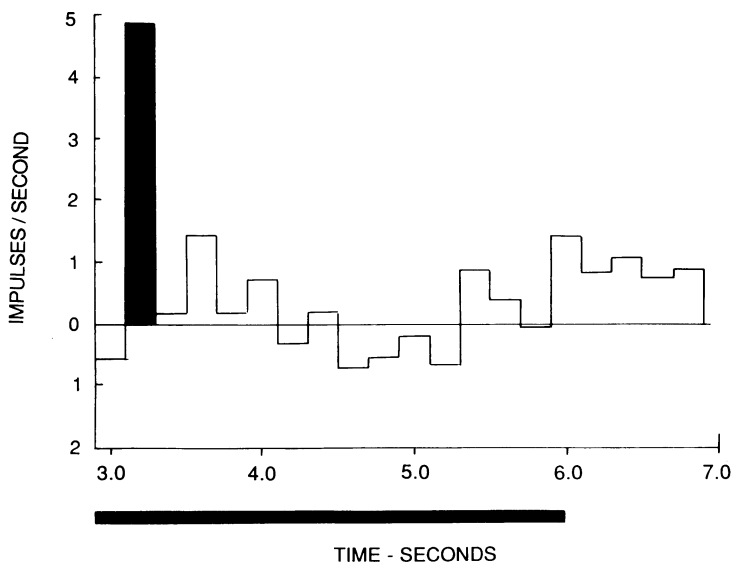


Fig. 3. An event-time histogram representing the difference in average response between two receptor neurons to the same doses of Z7-12:AC. Both neurons had similar tonic discharge frequencies (14.1 and 13.8 imp/s). The solid bar indicates a statistical difference between the two averaged responses for that time interval ($\alpha = 0.01$; 2-tailed sign test; Siegal, 1956).

Results

Temporal pattern of response

In spite of the fact that the stimulator is thought to produce a nearly rectangular pulse of odor, some of the receptor neurons sampled produced action potential responses with an initial phasic component. In these receptor neurons, the temporal pattern of action potential discharge generated in response to pheromone stimulation is characterized by two distinct periods of activity; an initial phasic response period with a duration of 100–200 ms, followed by a second tonic response period typically with a lower frequency of action potential production. The relative distribution of activity in these two periods is dependent on stimulus concentration. At lower pheromone concentration levels, the phasic component of the response is reduced or absent (Mankin *et al.*, 1987). The characteristic phasic-tonic pattern is apparent in several of the histograms displayed in Figure 2 which shows the average response during successive 100 ms intervals for five or 10 repeated pheromone stimulations of six different preparations. The possibility exists that those neurons producing a large phasic component in their response were doing so in response to mechanical stimulation caused simply by changes in flowrate (1200 ml/min to 1400 ml/min). However, control experiments, during which 200 ml of clean air were added to 1200 ml of carrier air, did not change the spontaneous activity. These results indicate that the cells producing a phasic response are not simply intrinsically sensitive to the mechanical stimulation associated with changes in flowrate.

The distinction between the different discharge patterns is demonstrated by the difference histogram in Figure 3, in which the average response in 2F during successive

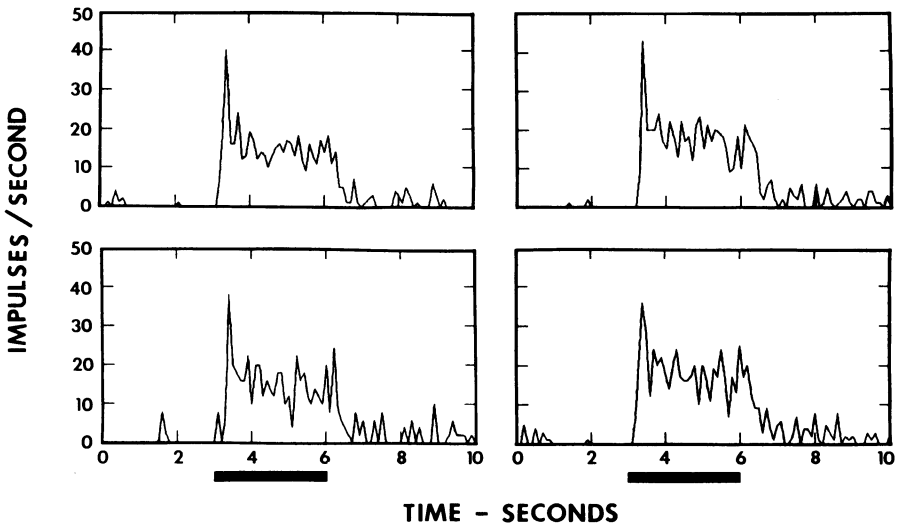


Fig. 4. Four averaged event-time histograms from an individual preparation in which the stimulus delivery system was repositioned after each stimuli was presented. The solid bar under the histograms indicates the on and off of the valve which controls the stimulus.

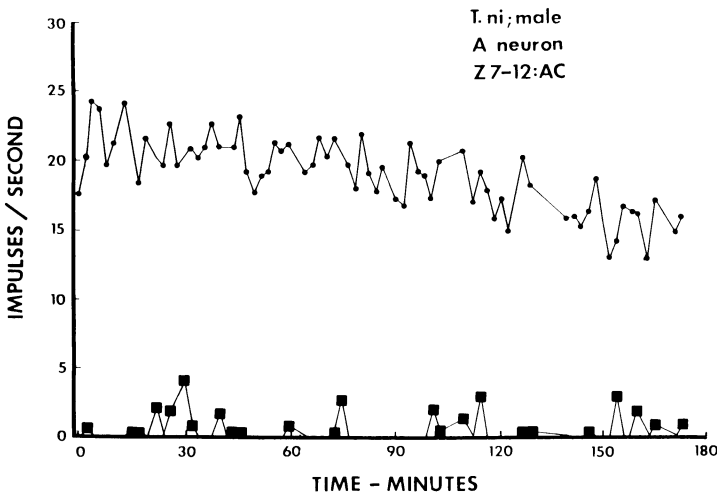


Fig. 5. The mean frequency response from one neuron stimulated with 3 s pulses of $2 \mu\text{g}$ of Z7-12:AC at 3 min intervals for 180 min (closed circles). The stimulus delivery system was not moved during the testing period. The closed squares are estimates of the spontaneous activity as calculated from the 3 s interval immediately preceding the stimulus period.

200 ms intervals has been subtracted from the corresponding responses in 2A. These two neurons were selected for comparison because they have similar levels of activity in the tonic portions of their response (2A-14.1 imp/s, 2F-13.8 imp/s). Their average responses differed significantly only in the magnitudes of their phasic components during

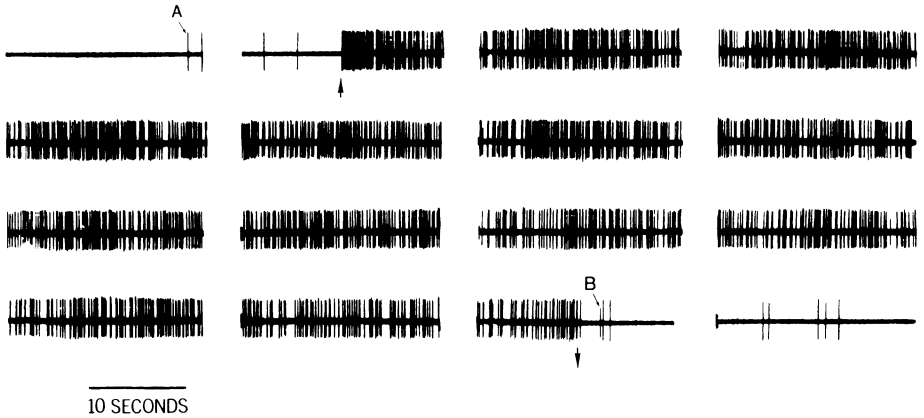


Fig. 6. Sixteen 20-s segments of an electrophysiological trace illustrating the response of a single neuron to continuous stimulation with $1 \mu\text{g}$ of Z7-12:AC for 650 s. Each segment represents 20 s of neural activity and is separated from the adjacent segment by a 30-s gap. Arrows indicate the onset and offset of the stimulus. In this series of records impulses generated by the A neuron are predominant; the B impulse is seen infrequently.

the first 200 ms interval ($\alpha = 0.01$; 2-tail sign test; Siegal, 1956). The difference among responses in Figure 2 apparently are intrinsic features of the neurons and are not simply artifacts of stimulation variation. This is supported by the responses in the test where the stimulus delivery device was removed and repositioned over the antenna several times on a single preparation (Figure 4). The frequency histograms of the responses from this single neuron in these tests do not exhibit the level of variation observed in Figure 2, which suggests that the position of the dispenser is not the determining factor to account for the differences in the phasic-tonic responses observed from different preparations.

Responses to repeated stimulations

The stability in the concentration delivered by the stimulator and the reliability of the preparation were evaluated by repeatedly exposing a neuron to 3 s pulses from the $2 \mu\text{g}$ septum at ~ 3 min intervals for 180 min (Figure 5). The flow through the dispenser was maintained throughout the 180 min session. The impulse frequency in response to pheromone stimulation remained relatively constant at 20.7 imp/s during the first half of the session. There was a slight decrease in response to 17.4 imp/s during the second half. Not all preparations are stable for this duration; however we have observed preparations that produced consistent responses over even longer periods of 12–24 h.

Responses to prolonged stimulation

Receptor neurons exposed to constant stimulation from a $1 \mu\text{g}$ septum for periods up to 650 s exhibited relatively constant levels of neural activity throughout the stimulus period. In the example of Figure 6, the stimulus began during the second 20-s recorded interval and ended during the 15th interval. The mean impulse frequencies for each recorded interval are plotted in Figure 7. After an initial phasic burst at stimulus onset, the response frequency decreased from 6.4 to 5.1 imp/s throughout the remainder of

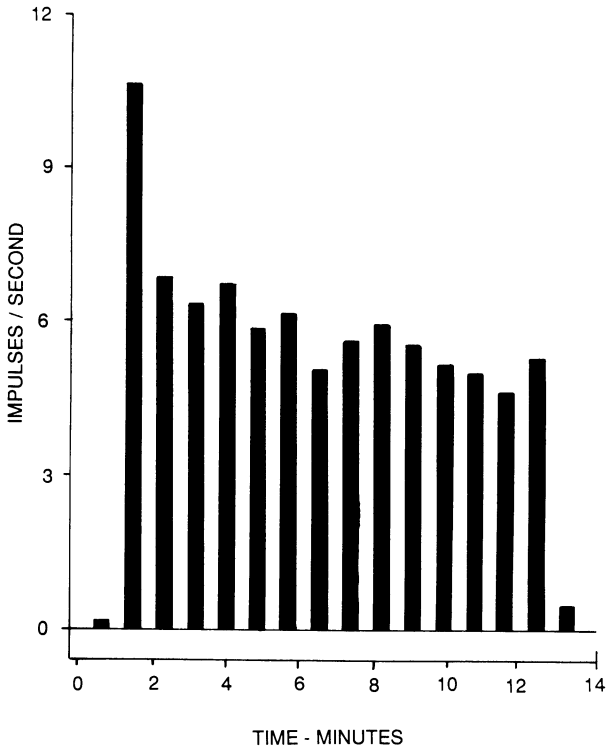


Fig. 7. A response histogram for the neuron shown in Figure 6. Each bar represents the overall action potential frequency for a 20-s period with 30 s separating each bar.

the stimulus period. Prior to stimulus onset, the spontaneous activity was 0.17 imp/s. After stimulus offset, the activity declined to 0.4 imp/s.

Responses to pulsed stimulation

The responses elicited by 10 0.3-s pulses, each separated by 0.7-s intervals, were compared with the responses elicited by a single 3-s pulse from the 2 μ g septum. Extracellular impulse records from one of these sensilla preparations are illustrated in Figure 8C–D and histograms of the average response from six preparations under these two regimens are shown in Figure 8A–B. Under repetitive stimulation, the response to the initial pheromone pulse was identical to the phasic part of the response to the continuous pulse. Subsequent pulses elicited responses that were higher in frequency than the tonic portion of the response to the continuous pulse. Apparently, part of the phasic component is retained in the responses to each of the short pulses.

Discussion

To characterize a constantly varying pheromone plume from an insect's point of view first requires some knowledge about how precisely the receptor neuron responses mirror

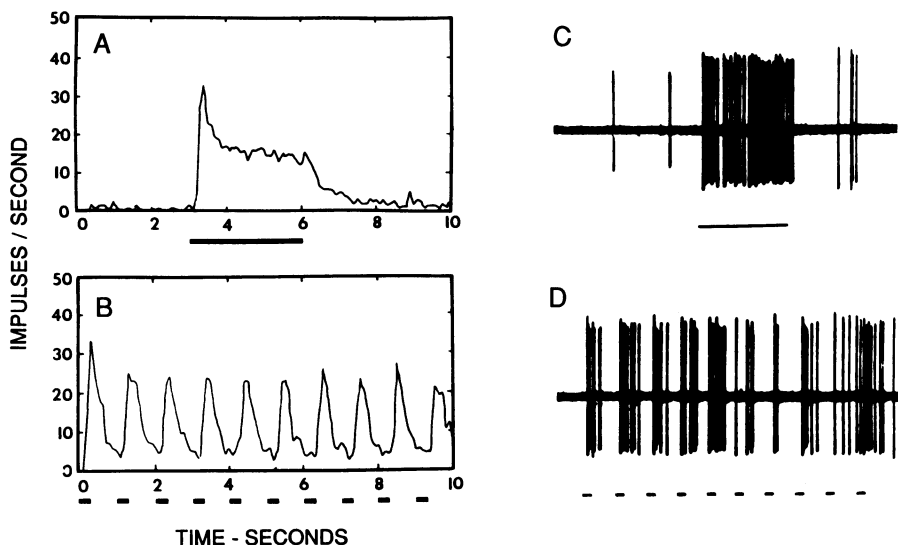


Fig. 8. Averaged responses from repeated stimulations by $2 \mu\text{g}$ of Z7-12:AC on six individual preparations. (A) The mean frequency of the phasic portion was 25.9 imp/s. The mean frequency of the tonic portion was 16.03 imp/s. (B) Stimulus period comprised 10 0.3-s pulses separated by 0.7 s. (C) Typical electrophysiological recordings which illustrate the response to a single 3-s pulse of Z7-12:AC. (D) Typical electrophysiological recordings which illustrate the response to 10 0.3-s pulses of Z7-12:AC. In all cases the onset and offset of the stimulus trigger is indicated below the response.

the quantitative and temporal variations of the plume. One goal of this study was to devise a stimulus delivery system that passed pheromone over the antenna at a constant intensity for precisely defined intervals. The stability of the responses that were obtained to multiple and continuous pheromone pulses suggest that this goal was largely achieved. In Figure 8A, for example, which combines responses from 48 stimulations, the response pattern is well-defined. The insect CNS integrates responses from about 50-fold more neurons; consequently, the representation that it obtains probably is even more well-defined.

In many electrophysiological studies of insect olfaction, odorants are released from some substrates, such as glass, paper or rubber, into a small volume of still air. The stimulus is then delivered to the preparation by passing a large volume of air through this smaller volume thereby purging the space containing the pheromone. Since one would expect that volatile odorants such as pheromones would rapidly reach an equilibrium concentration in this small volume of still air, the stimulus pulse that is subsequently delivered to the antenna may well be characterized by an initial high-concentration burst followed by a lower concentration period. The delivery system used in the experiments described here has a constant stream of air flowing over the emission source which prevents the build up in concentration characteristic of pheromone delivery from a static system. Although actual concentrations of odorants delivered by this system over the time periods of interest could not be calibrated due to difficulties associated with measuring the extremely low concentration levels that are behaviorally relevant to insects, we feel that our system does reliably produce relatively-constant, discrete,

odor pulses with sharp onsets and offsets and is effectively prevented from producing odor pulses with an initial elevated concentration component. A formulation which releases pheromone at a constant rate in a system that produces a well-defined stimulus pulse is necessary in any attempt to separate intrinsic variation in the receptor neuron response from extrinsic variation in the stimulation presentation. This type of system permits one to observe inherent response differences among receptor neurons that might otherwise be indistinguishable from differences due to nonuniform stimulus presentations.

Phasic-tonic response patterns in chemoreceptors have been reported frequently (Kaissling, 1971, 1986, 1987; Mustaparta, 1975; Rumbo, 1981, 1983), but a few instances of tonic patterns have been reported as well. In most of the studies where tonic patterns were observed, the stimuli were high-vapor-pressure gases such as CO₂, N₂O or Xe (Kellog, 1970; Stange and Diesendorf, 1973). Kafka (1970) injected a uniform, saturated vapor of (*E*)-2-hexenal into a stimulus airstream and found that the neural response to this chemical also had no significant phasic component. In this study we found instances of both phasic and tonic responses, depending on the neuron tested, which indicate that at least part of the temporal pattern of response reflects intrinsic characteristics of the receptor neuron. This result recapitulates a finding reported frequently in the literature (see e.g., O'Connell, 1975; Rumbo, 1981; Grant and O'Connell, 1986; Kaissling, 1987), that considerable interneuronal differences may occur even among receptor neurons specialized to detect particular pheromone components. The problem of variability among receptor neurons of a given type also has been discussed with respect to contact chemoreceptor neurons (Dethier, 1974; Schoonhoven, 1975; van der Molen *et al.*, 1985; Blaney *et al.*, 1986).

We have demonstrated here that at lower concentrations (response values = 6 imp/s) receptor neurons continue to respond for long periods of time (up to 650 s) without a large degree of sensory adaptation. This may be an important characteristic of those sensory neurons which are presumed to be involved in long range orientation of males to females. It would be of little evolutionary advantage to incorporate sensory neurons into the orientation system that adapted quickly to pheromone thus interrupting the transmission of information to the CNS. Of course, such scenarios are almost entirely dependent on the concentration and composition of the pheromone in the plume downwind from the calling female.

Work by Murlis and Jones (1981) and Mankin *et al.* (1980) has shown that a downwind plume may actually be composed of discrete packets of pheromone, some of which may have relatively high concentrations. Additionally, studies by Conner *et al.* (1980) have demonstrated that certain Arctiid moths release their pheromone in pulses by rapidly everting and retracting their abdominal pheromone gland. In either case, the composition of the plume downwind from a 'calling' female may be encountered by a 'tracking' male as relatively discrete pulses of odorants. There is theoretical evidence to suggest that pulsing the odorant enhances the information content of the chemical signal (Bossert, 1968). Cardé *et al.* (1983) concluded from their wind tunnel tests with *Lymantria dispar* (L.) that pulsed pheromone stimuli did not lower the threshold as compared to continuous stimulation, suggesting that pulsed stimulation was of little advantage to the insect for odor detection. However, work by Baker *et al.* (1985) indicates that male *G. molesta* orient better to discrete rather than continuous pheromone clouds. We show in these

studies that with a pulsing sequence of 0.3 s on and 0.7 s off, a substantial portion of the phasic component is preserved during each pulse thus increasing the overall number of impulses generated. Conceivably a key behavioral response could be evoked in *T.ni* by this periodic waning and enhancement of the neurophysiological response. The resolution of such questions must await the complete understanding of *T.ni* orientation and the antecedent peripheral and CNS processes by which they are evoked.

Acknowledgements

We thank R.R.Heath for the preparation and calibration of the septa and R.J.O'Connell for discussion and comments on the manuscript. Additionally, we thank J.Sharp for technical assistance. A portion of this work was reported at the 1985 Association for Chemoreception Sciences annual meeting. We acknowledge support to A.J.G. during preparation of the manuscript from grants NS 14453 from NINCDS and from the Alden Trust awarded to R.J.O'Connell. Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the US Department of Agriculture or the State of Florida.

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Received on September 1, 1988; accepted on January 22, 1989