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Extreme Sensitivity in an Olfactory System

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Abstract

We recorded olfactory-induced cardiac responses to evaluate olfactory response thresholds to behaviourally relevant odours in a moth. Specific antennal receptor neurons enable insects to detect biologically meaningful odours such as sex pheromones and host-plant volatiles. The response threshold values demonstrated here are well below anything earlier reported in any organism. A heart response was triggered by less than six molecules of the most efficient odours hitting the antennae of the insect. The behavioural significance of this extreme sensitivity most likely lies in the creation of awareness and readiness to respond behaviourally at higher concentration levels.

Key words: moth, sex pheromone, plant odours, cardiac response

Introduction

The olfactory system has become an important model system regarding sensory detection and integration. In this context, insects represent an important source of information regarding fundamental principles of olfactory detection (Ziegelberger, 1995; Clyne *et al.*, 1999; de Bruyne *et al.*, 1999, 2001; Vosshall *et al.*, 1999; Störtkuhl and Kettler, 2001) and central nervous information processing (Hansson *et al.*, 1991, 1992; Stopfer *et al.*, 1997; Ito *et al.*, 1998; Christensen *et al.*, 2000; Dubnau *et al.*, 2001; McGuire *et al.*, 2001). Extensive investigations of the anatomy and function of the olfactory system of moths have provided a very useful model for neuroethological studies (Hartlieb and Anderson, 1999). Moth olfactory receptor neurons (ORN) exhibit a high degree of selectivity and sensitivity to both pheromone and non-pheromone volatiles, thus supplying the brain with high-quality information on odour identity, intensity and spatiotemporal distribution (Hansson *et al.*, 1992; Hansson, 1995; Christensen *et al.*, 1996, 2000; Hansson and Christensen, 1999; Vickers *et al.*, 2001). For instance, the high sensitivity to the female-produced sex pheromone exhibited by the silk moth (*Bombyx mori*) male allows it to respond behaviourally when just 85 ORNs/antenna intercept one molecule each per second (Kaissling and Priesner, 1970; Kaissling, 1971). On the other hand, much higher stimulus intensities are generally reported as minimum values needed for recording significant activity from ORNs (Kaissling, 1987), CNS neurons (Hansson and Christensen, 1999), as well as for driving an appropriate insect behaviour (Todd and Baker, 1999).

Odour detection induces cardiac responses that have been described as a sensitive tool for testing insect olfactory reactivity (Queinnec and Campan, 1976). Analogous responses also occur following stimulation of types of sensory receptors, such as visual (Thon, 1982), gustatory (Angioy, 1988) and mechanical (Ai and Kuwasawa, 1995). On the basis of facilitatory influence on motor activity, heart responses to visual stimulation were suggested to play a preparatory role for ensuing behaviour (Thon, 1982). Short cardiac response latencies (<1 s) suggest that sensory input activates a reflex control mechanism (Thon, 1982; Angioy, 1988; Angioy *et al.*, 1987) along cardiac innervation (Davis *et al.*, 2001). In blowflies, an immediate arrest of a fast phase activity and a prompt setting in of a slower one occur after olfactory stimulation with several kinds of volatiles (Angioy *et al.*, 1987). In *Heliothis virescens* moths, a sudden shift from a low-frequency phase of cardiac activity to a high-frequency one follows stimulation with sex-pheromone or 1-hexanol molecules at concentrations below threshold values eliciting behavioural responses (Angioy *et al.*, 1998).

Here we show an extremely high sensitivity of cardiac responsiveness to sex pheromone and plant odour information in both sexes of the cotton leaf worm moth, *Spodoptera littoralis*, an olfactory sensitivity higher than ever reported before. *S. littoralis* is a noctuid moth that has been very well

investigated concerning olfactory function and olfactory induced behaviour. Specific receptor neurons tuned to the odours used in the present study have been identified on the antenna. Unlike most other moths, the female also possess a well-developed sense for the sex pheromone components produced by herself. The function of this autodetection is so far unknown.

Materials and methods

Insects

Experiments were performed on 2–5-day-old adults of *S. littoralis*. Larvae were obtained from the Swedish University of Agricultural Sciences in Alnarp, Sweden and reared on a semi-synthetic diet (Hinks and Byers, 1976) using potatoes instead of beans. Moths were separated by sex at the pupal stage, put into emergence boxes and kept in a cabinet at 24°C, 70–80% relative humidity, 7/17 night/day cycle. Adults were kept without food, but were provided with water *ad libitum* during the 24 h prior to experiments.

Moths were fixed dorsal side up on a strip of low melting point dental wax; both wings and legs were immobilized. Using soft wet paper, cuticular scales were removed from small areas of the mesothoracic and abdominal dorsal body surfaces to allow positioning of the electrodes. Each moth was placed on a microscope stage in the visual field of a stereomicroscope (Wild M5A; Wild Leitz Ltd, Heerbrugg, Switzerland) within a Faraday shield on an antivibration surface.

Cardiac activity recording

Monopolar extracellular electrocardiograms (ECGs) were performed on intact specimens using a pair of metal electrodes (Ag–AgCl wires, 250 μm diameter) in contact with the insect cuticle by means of a conductive ECG gel. The active electrode, connected to an amplifier (Altech Electronics, Italy), was positioned on the fourth abdominal segment. The ground electrode was placed on the mesothorax. Signals were displayed on the screen of an oscilloscope (Tektronix 5111A; Tektronix Inc. Beaverton, OR), stored on a modified video recorder (Vetter; A.R. Vetter Co. Inc. Rebesburg, PA) and later analysed with an integrated system of hardware and software (MacLab System; AD Instruments Ltd, Castle Hill, Australia).

Olfactory stimulation

A main flow of humidified and charcoal-filtered air (1.70 l/min) was continuously delivered through a glass tube (i.d. 8 mm), ending 2 cm in front of the moth antennae. The tip of a Pasteur pipette, containing a 7 × 15 mm piece of filter paper with (stimulus) or without (control) an olfactory stimulus, was inserted into a small opening in the glass tube, 70 mm from the antennae. By means of a mechanical puffing device (Altech Electronics, Italy), a 1 s air pulse (0.50 l/min) was then sent through the Pasteur pipette. A glass funnel (i.d. 5 cm) connected to an air suction line was positioned close to the preparation to take away the odour-carrying air after stimulation. The following odour stimuli were tested: three plant odours, geraniol, indole and ±linalool; female-emitted sex pheromone volatiles—the minor component [(*Z*,*E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12–14:OAc)], the major component [(*Z*,*E*)-9,11-tetradecadienyl acetate (*Z*9,*E*11–14:OAc)] and their blend in the proportion of 1:99, equivalent to that measured in the natural sex pheromone by Kehat and Dunkelblum (Kehat and Dunkelblum, 1993). Chemicals were diluted in hexane in decadic steps and tested in order of increasing concentration after solvent evaporation. The solubilization of indole in hexane was obtained by stirring and slightly heating the solution in sealed vials. Plant odours and pheromone compounds were tested on separate groups of specimens, each group comprising a minimum of 10 moths of each sex.

Experimental procedure

Cardiac activity was monitored after a 15 min period of recovery from electrode positioning. If heart activity was still affected by spontaneous movements of the insect (Thon, 1982), additional recovery time was allowed until regular cardiac activity was observed. Continuous ECG recording started three cardiac cycles before delivery of the main air flow. This air flow permanently flushed the antennae until the end of tests on each moth. Control and blank stimulations were performed both at the beginning and at the end of the experimental session by using empty pipettes and solvent-loaded pipettes after solvent evaporation, respectively. Animals displaying a response to background stimuli were removed from the experimental group. A single olfactory stimulation was delivered during a cardiac cycle, at the beginning of a fast or slow phase. For each experimental group, a given chemical was tested in increasing order of concentration until a cardiac response was measured. Subsequent stimulations were separated by a 3 min pause to avoid receptor adaptation (Kaissling, 1987). Sequences of stimulation with different odours were separated by 15 min intervals to limit possible habituation phenomena (Thon, 1982).

Data analysis

Regular cardiac activity of male ($n = 11$) and female ($n = 19$) moths in the absence of stimulation was evaluated by measuring cardiac phase duration and signal frequency at the beginning, at the end and at each quarter of phase duration. Values are expressed as means ± SE. Levels of significance of sex-related differences were determined by an unpaired Student's *t*-test between groups. For each experimental group and odour tested, the cardiac response threshold was set to the amount of odour applied on a filter paper to which >50% of the specimens tested showed a cardiac response. Latency of the cardiac response was

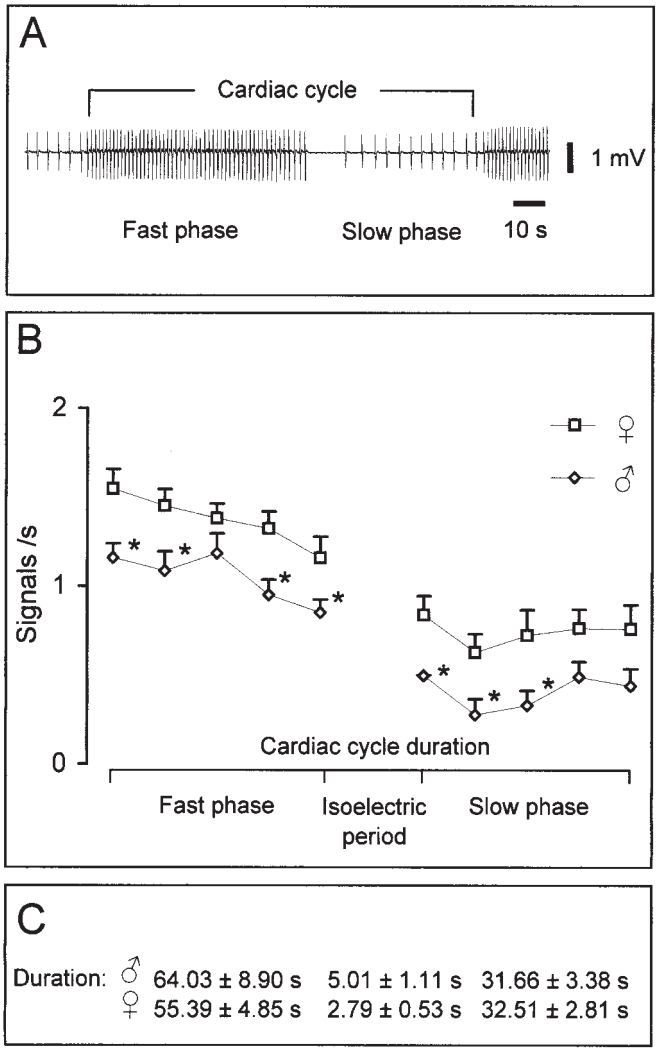

Figure 1 Regular heart activity of *Spodoptera littoralis* in absence of sensory stimulation. **(A)** Electrophysiological recording on an intact moth preparation. **(B)** Mean values ± SE of myocardiac spike frequency during the fast and slow phase of the regular cycle in females (n = 19) and males (n = 11). On the abscissa, the duration of each cardiac phase is represented by five points, each corresponding to a 1 s time interval at the beginning of the phase, at 1/4, 1/2, 3/4 and at the end of the phase. **(C)** Mean values ± SE of duration of the fast phase, the isoelectric period and the slow phase in males and females. Asterisks denote significant differences between corresponding values of the two sexes (Student's t-test).

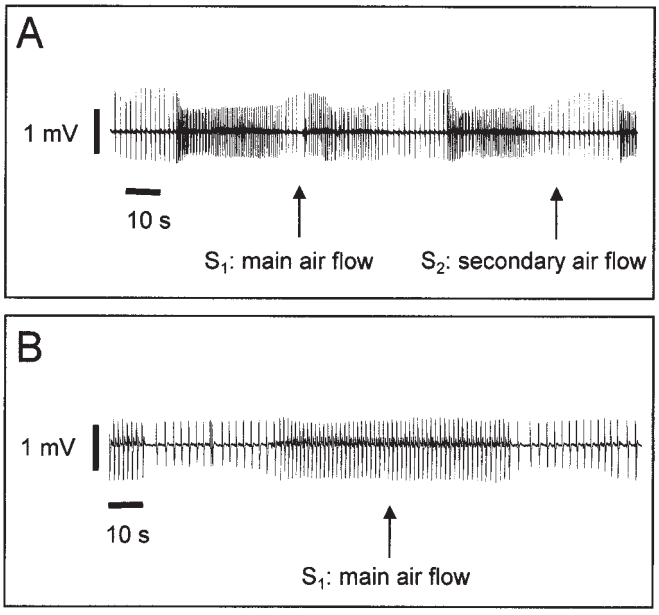

Figure 2 Variations in cardiac activity following antennal mechanosensory stimulation on an intact *Spodoptera* moth. **(A)** Initiation of the continuous flow of pure air (S_1: 1.70 l/min) during a slow phase was followed by an immediate increase in signal frequency. This effect was transitory and the alternation between regular fast and slow phases returned even though the air flow continued to reach the insect's antennae. The addition of a secondary pure air flow from an empty stimulus pipette (S_2: 0.50 l/min; control test) did not induce any heart frequency alteration in the subsequent slow phase. **(B)** Initiation of the continuous flow of pure air (S_1: 1.70 l/min) during the fast phase did not induce any variation in cardiac activity.

measured as the time interval between the initiation of the stimulus and the occurrence of variations in activity, as shown in the electrocardiogram.

Approximate numbers of molecules added to the stimulus filter paper were calculated by multiplying the amount added in grams with Avogadro's number (1 mol = 6×10^{23} molecules) and dividing the number arrived at by the mol. wt of the substance added.

Results

In absence of stimulation, electrocardiograph activity of *S. littoralis* took the form of a cyclic alternation between high- and low-frequency phasic bursts (Figure 1A). These phases were termed the fast phase and the slow phase, respectively (Angioy *et al.*, 1998). Analysis of signal patterns recorded in 19 females and 11 males showed that the heartbeat pattern did not differ between the sexes, except that females generally exhibited a higher frequency of cardiac signals (Figure 1B). An isoelectric period lasting several seconds was recorded at the end of the fast phase in a limited number of males (three) and females (four).

On initiating the main and continuous flow of pure air across the animal's antennae at the beginning of a slow phase, an immediate increase in the frequency of cardiac signals was observed (S_1 in Figure 2A). The slow phase reverted to a characteristic fast phase. Therefore, in *Spodoptera,* as well as in other insect species (Angioy *et al.*, 1998), tachycardia results from antennal mechanosensory stimulation caused by the arrival of pure air. This effect was transitory and a regular slow phase subsequently set in even when the main air flow continued to reach the moth's antennae, a result most probably due to mechanoreceptor adaptation. Addition of a secondary air flow did not modify the subsequent slow phase (S_2 in Figure 2A). In all insects tested (>100) mechanoreceptor stimulation with the air flow was followed by a tachycardia response only when stimu-

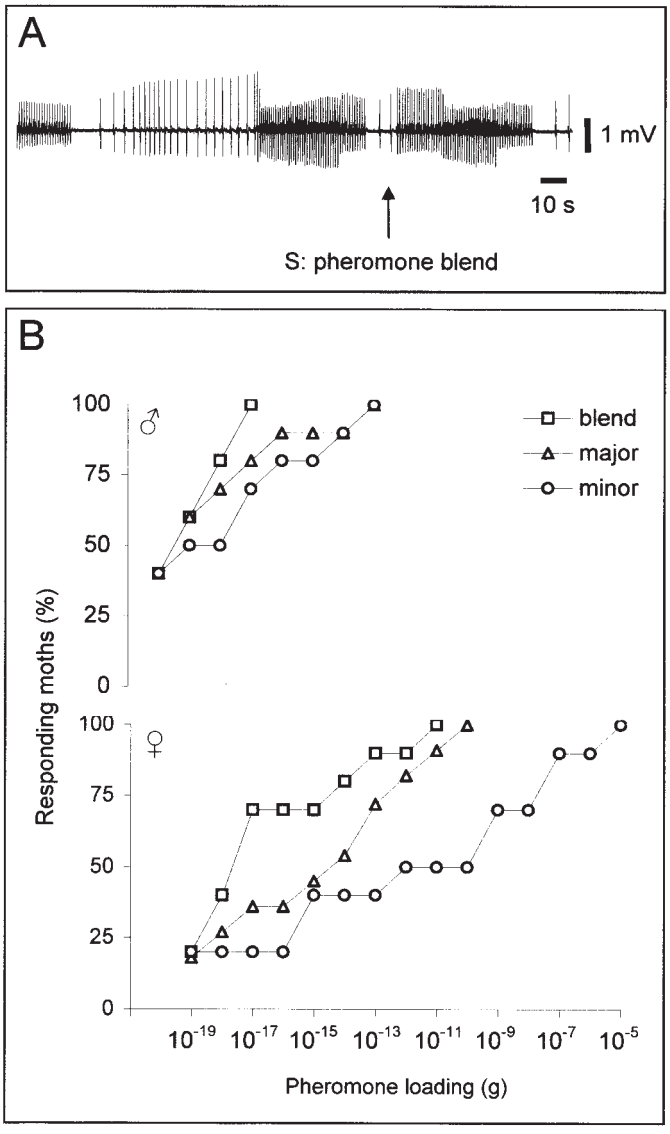

Figure 3 Changes in cardiac activity following antennal olfactory stimulation with sex-pheromone-related odours in intact *Spodoptera* moths. **(A)** An immediate increase in signal frequency of the slow phase followed olfactory stimulation (S: 1 s pulse of 10^{-18} g of the 1:99 blend of the minor and major pheromone components). The moth antennae were uninterruptedly in the main flow of pure air starting from 5 min prior to application of the stimulus. **(B)** Relation between dose of odour and percentage of male (n = 11) and female (n = 19) moths, showing cardiac response to a single stimulation with increasing amounts of a pheromone component (*Z*9,*E*12–14:OAc or *Z*9,*E*11–14:OAc) or their 1:99 blend.

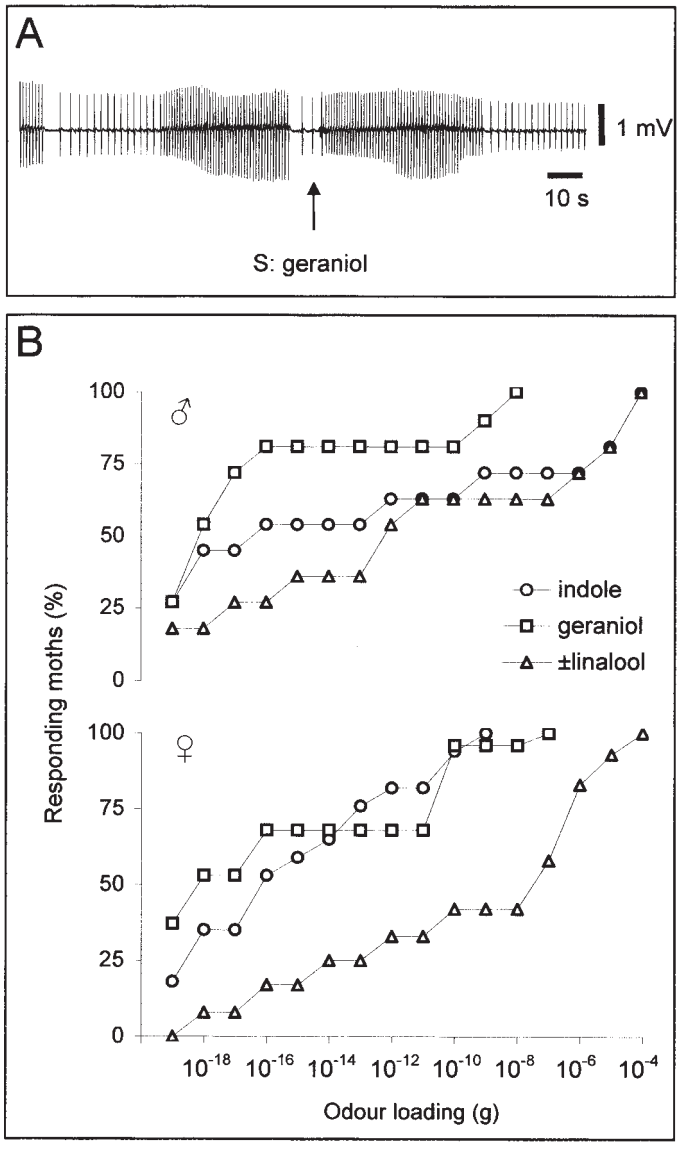

Figure 4 Changes in cardiac activity following antennal olfactory stimulation with plant-related odours in intact *Spodoptera* moths. **(A)** The frequency of slow phase signals reverts to a fast phase frequency immediately after olfactory stimulation (S: 1 s pulse of 10^{-16} g of geraniol). The moth antennae were placed in the pure airflow starting from 5 min prior to application of the stimulus. **(B)** Relation between dose of odour and percentage of male (n = 10) and female (n = 10) moths, showing the cardiac response to a single stimulation with increasing amounts of geraniol, indole or ±linalool.

lation was carried out during a slow phase. Stimulation during a fast phase had no effect (S_1 in Figure 2B).

As following primary mechanosensory stimulation, reversion of slow-phase activity resulted from olfactory stimulation with the tested sex pheromone- or plant-related chemicals (Figures 3A and 4A). The latency time from stimulation to response was ~1 s. No alteration in activity followed olfactory stimulation applied during a fast phase. The dose–response curves obtained after stimulating moths with increasing amounts of the major or minor component of the sex pheromone, or a behaviourally relevant blend of these, reveal extremely low response thresholds, but also sex-related differences in cardiac responsiveness (Figure 3B). In >50% of the male moths tested, the cardiac response occurred at doses of 10^{-19} g of the blend and of the major component (*Z*9,*E*11–14:OAc), and of 10^{-17} g of the minor component (*Z*9,*E*12–14:OAc). In female moths, doses of equivalent effectiveness were 10^{-17} g of the blend, 10^{-14} g of the major component and 10^{-9} g of the minor component.

In the female, threshold levels were thus 10^2, 10^5 and 10^8 times higher than in the male for the major component, minor component and blend stimulus, respectively.

As regards the plant odours tested, doses amounting to 10^{-18} g of geraniol and 10^{-16} g of indole evoked cardiac response in >50% of moths of both sexes (Figure 4B). Doses of ±linalool amounting to 10^{-12} and 10^{-7} g were necessary to induce a cardiac response in 50% of males and females, respectively. Only for ±linalool was a sex-related difference observed, where the male responded at 10^5 times lower concentration than did the female.

Discussion

Monitoring of olfactory-induced cardiac responses in order to evaluate detection levels of odours turned out to be an effective experimental strategy. The response of the *Spodoptera* heart accurately indicates odour perception, even when volatiles are delivered in amounts substantially below those required for obtaining an observable behavioural response (Anderson *et al.*, 1993). Extremely low amounts of sex pheromone compounds were sufficient to induce responses in a significant number of males. 10^{-19} g, i.e. ~240 molecules, of the pheromone blend applied to the stimulus filter paper were sufficient to elicit a response in >50% of the males. At an absolute maximum, 10% of the molecules, i.e. ~24 molecules, will leave the filter paper during the stimulus period. The antennae cover ~25% of the outlet of the stimulus tube, bringing the number of molecules potentially hitting an olfactory sensillum on the antenna down to about six. The detection level is thus of a magnitude well below what has earlier been calculated as being necessary to induce a behavioural response in the silk moth (Kaissling and Priesner, 1970) and at a magnitude almost impossible to imagine. We therefore repeated our series of experiments three times on separate groups of 10 insects each, but arrived at the same result each time. The difference between response thresholds recorded in *Bombyx* and *Spodoptera* may depend on a higher sensitivity of the cardiac response method compared to measuring behavioural responses through wing vibration observations. Differences might also reside in the stimulating systems adopted. Interestingly, a highly sensitive female response to its own pheromone was also demonstrated, a phenomenon consistent with the fact that females possess ORNs (Ljungberg *et al.*, 1993; Anderson *et al.*, 1995; Jönsson and Anderson, 1999) and antennal lobe neurons (Anton and Hansson, 1995) selectively sensitive to sex-pheromone stimulation. This observation highlights the need for future behavioural investigations, as the significance of the autodetection of pheromone so far is unknown. However, a higher response threshold to pheromone was found in the female. The sensitivity of sex-pheromone-specific ORNs is equivalent in the two sexes, the receptor threshold being in the range 10^{-8}–10^{-7} g of stimulus loaded onto filter paper, while the number of pheromone sensilla is lower in the female (Ljungberg *et al.*, 1993). The male pheromone-detecting system thus displays a higher rate of convergence of ORNs onto AL neurons, which can explain the lower male response threshold in heart recordings. The finding that the female response threshold to the pheromone blend is much lower than to single components indicates the synergistic effect of the two components. On the other hand, the male response thresholds to the blend and to the major pheromone component were equivalent, even though the presence of the complete blend is known to be imperative for attraction to occur. In spite of these sex-related differences in information processing, males and females shared the characteristic of having a cardiac response threshold much lower than that of ORNs. This finding highlights the strong convergence and sensitivity amplification that takes place within the moth olfactory system.

The recordings also showed that plant-associated odours were detected at very low concentrations, suggesting that the pheromone-detecting system is not unique in its sensitivity. The detection threshold for geraniol was <390 molecules applied to the stimulus filter paper and, consequently, <10 molecules hitting the antenna. *S. littoralis* moths have specific ORNs tuned to each of the three compounds tested (Anderson *et al.*, 1993, 1995; Jönsson and Anderson, 1999) and some antennal lobe neurons respond with high specificity to them (Anton and Hansson, 1995). The response threshold of antennal ORNs specific to plant-produced odours is in the same range as in ORNs tuned to pheromone components. In *S. littoralis* females, it is ~10^{-9} g (Jönsson and Anderson, 1999). The lower sensitivity to linalool correlates well with the fact that ORNs tuned to this compound are significantly less sensitive compared to those tuned to geraniol (Anderson *et al.*, 1993) and indole (Jönsson and Anderson, 1999). Receptor neurons detecting linalool are present in higher numbers in male antennae, while being quite rare in the female. The consequent difference in sensory input after linalool stimulation may thus account for the lower cardiac reactivity detected in females.

Our experimental results show that moths display an olfactory sensitivity even more pronounced than that suggested from theoretical calculations. In the male pheromone-detecting system around five molecules and in the plant-odour-detecting system ~10 molecules potentially hitting the antenna during 1 s are enough to trigger a heartbeat frequency change. This result demonstrates that the moth olfactory system has evolved a sensitivity beyond any other chemosensory detection systems known today. No direct behavioural response has been registered at the low concentrations applied in the present study. The significance of the heart rate change can most likely be found in the formation of awareness regarding the presence of a certain stimulus and a readiness to respond behaviourally.

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