Chem. Senses 27: 231–244, 2002

Spatial Representation of Odours in the Antennal Lobe of the Moth *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract

Glomeruli within the antennal lobe (AL) of moths are convergence sites for a large number of olfactory receptor neurons (ORNs). The ORNs target single glomeruli. In the male-specific cluster of glomeruli, the macroglomerular complex (MGC), the input is chemotypic in that each glomerulus of the MGC receives information about a specific component of the conspecific female sex pheromone. Little is known about how neurons that detect other odorants arborize in and amongst glomeruli. The present study focuses on how sex pheromones and biologically relevant semiochemicals are represented in the ALs of both sexes of the moth *Spodoptera littoralis*. To assess this, we optically measured odour-evoked changes of calcium concentration in the ALs. Foci of calcium increase corresponded in size and shape with anatomical glomeruli. More than one glomerulus was normally activated by a specific non-pheromonal odorant and the same glomerulus was activated by several odorants. All odorants and pheromone components tested evoked unique patterns of glomerular activity that were highly reproducible at repeated stimulations within an individual. Odour-evoked patterns were similar between individuals for a given odorant, implicating a spatial olfactory code. In addition, we demonstrated that activity patterns evoked by host-plant related volatiles are similar between males and females.

Introduction

The antennal lobe (AL) is the primary integration centre for olfactory information in insects, analogous to the olfactory bulb in vertebrates. The AL consists of a species-fixed number of spheroidal structures called glomeruli, where olfactory receptor neurons (ORNs) synapse with higher-order neurons. Olfactory glomeruli are found in both vertebrates and invertebrates (Hildebrand and Shepherd, 1997; Strausfeld and Hildebrand, 1999). The functional role of glomeruli is not fully understood. However, evidence from an accumulating number of studies, using activity-dependent staining techniques, converges on the hypothesis that glomeruli are functional units representing different odorants or molecular features (Sharp *et al*., 1975; Kauer, 1988; Rodrigues, 1988; Cinelli *et al*., 1995; Friedrich and Korsching, 1997, 1998; Joerges *et al*., 1997; Distler *et al*., 1998; Galizia *et al*., 1999; Rubin and Katz, 1999; Uchida *et al*., 2000; Meister and Bonhoeffer, 2001). Functionally characterized ORNs have been found to project in a chemotypic manner to identified glomeruli in moths (Hansson *et al*., 1992, 1995; Ochieng' *et al*., 1995; Todd *et al*., 1995; Berg *et al*., 1998). Individual ORNs likely house a single olfactory receptor type and axons expressing a given receptor gene converge onto the same morphologically identified glomerulus (or in one case either of two glomeruli) in *Drosophila melanogaster* (Gao *et al*., 2000; Vosshall *et al*., 2000). Furthermore, the number of receptor gene types showing expression in olfactory organs in *D. melanogaster* equals the number of glomeruli (Vosshall, 2001). Thus, it is highly likely that each glomerulus represents a certain olfactory receptor.

Apart from highly specific sex-pheromone-detecting neurons, many insect ORNs are more broadly tuned to odorants (Todd and Baker, 1999). Generally, the molecular receptive range of an ORN comprises closely related compounds and a certain receptor is probably narrowly tuned to a molecular determinant that is common to many odorants (Araneda *et al*., 2000). The same odorant often activates ORNs with differing response spectra and a certain receptor neuron can detect different molecules. Thus, to resolve the quality of an odour, an across-fibre comparison may be required. If odours are detected by ORNs in an across-fibre fashion, this would also be reflected in the AL by a multi-glomerular activity pattern. In some insect species, however, ORNs highly selective to plant-related volatiles have been observed. In scarab beetles, for example, receptor neurons were found that responded only to a single plant odorant

and not to closely related compounds (Hansson *et al*., 1999; Larsson *et al*., 2001; Stensmyr *et al*., 2001).

The sensory neurons target one of ~60 glomeruli in moths (Rospars, 1983; Rospars and Hildebrand, 1992). The number of 'ordinary' glomeruli is equal in males and females. Additionally, in males, a cluster of enlarged glomeruli, called the macroglomerular complex (MGC), is targeted by axons of ORNs selectively tuned to conspecific sex-pheromone components or to interspecific behavioural antagonists (Hansson, 1997). Anterograde stainings of physiologically identified ORNs tuned to sex-pheromone components, have demonstrated that each glomerulus within the MGC receives input from a specific component (Hansson *et al*., 1992, 1995; Ochieng' *et al*., 1995; Todd *et al*., 1995; Berg *et al*., 1998). This chemotypic organization has also been displayed in output signals by tracing glomerular dendritic arborizations of projection neurons (PNs) (Hansson *et al*., 1991, 1994; Berg *et al*., 1998; Vickers *et al*., 1998). However, the pheromone sensitive PNs (or ORNs) do not always branch in a glomerulus as predicted by their physiological characteristics. In the cabbage looper moth, *Trichoplusia ni*, a mismatching of neuronal input and output of the MGC glomeruli was evident (Anton and Hansson, 1999). In the female sphinx moth, *Manduca sexta*, two sexually dimorphic glomeruli, 'the large female glomeruli' (LFG), have similar positions to the MGC in males. A recent study (King *et al*., 2000) showed that all PNs arborizing in the lateral LFG were excited by the plant odorant linalool.

Much less is known about the function of the sexually isomorphic glomeruli in moths. These glomeruli receive input from ORNs tuned to compounds other than those involved in long-distance mate attraction. The only study using anterograde staining of such ORNs was performed in female *T. ni* (Todd and Baker, 1996). However, this study did not reveal any consistent spatial separation of functionally identified neurons into single glomeruli. Neither could dendritic arborizations of physiologically characterized PNs be shown to originate in glomeruli at consistent positions (Anton and Hansson, 1994, 1995) (M. Sadek, personal communication).

In the cotton leaf worm, *Spodoptera littoralis*, axons of ORNs tuned to two different components of the sexual pheromone and a behavioural antagonist have been traced to separate glomeruli within the MGC (Ochieng' *et al*., 1995). The glomerulus of the MGC located closest to the AN is called the cumulus or glomerulus 'a' and is targeted by ORNs tuned to the major pheromone component (*Z*,*E*)-9,11-tetradecadienyl acetate (*Z*9, *E*11–14:OAc). Two satellite glomeruli, termed 'b' and 'c', receive axons from ORNs tuned to a behavioural antagonist, (*Z*)-9-tetradecanol (*Z*9–14:OH) and to the minor component (*Z*,*E*)-9,12-tetradecadienyl acetate (*Z*9, *E*12–14:OAc), respectively. Figure 1 shows the position of the glomeruli within the MGC and the physiological types of ORN innervation they receive.

ORNs in *S. littoralis* responding to other biologically relevant odours, such as plant-associated compounds, odorants induced by larval host-plant feeding or odorants emitted from larval frass show different degrees of specificity (Anderson *et al*., 1993, 1995; Jönsson and Anderson, 1999). Some neurons appear to be highly specific, responding only to a single or a few compounds, whereas others are more broadly tuned. Intracellular recordings from AL neurons have also revealed specificity ranging from neurons responding to a single compound to neurons responding to all compounds tested (Anton and Hansson, 1994, 1995).

In the present study, we examined spatial representation of odorants in the AL of male and female *S. littoralis* upon stimulation with biologically relevant stimuli. We optically measured changes in intracellular calcium concentration of *in vivo* preparations. The odorants used in the study evoked reproducible patterns of glomerular activity within an individual, unique to each odorant. Odour representations were generally consistent between individuals, implicating a spatial olfactory code. In addition, we have for the first

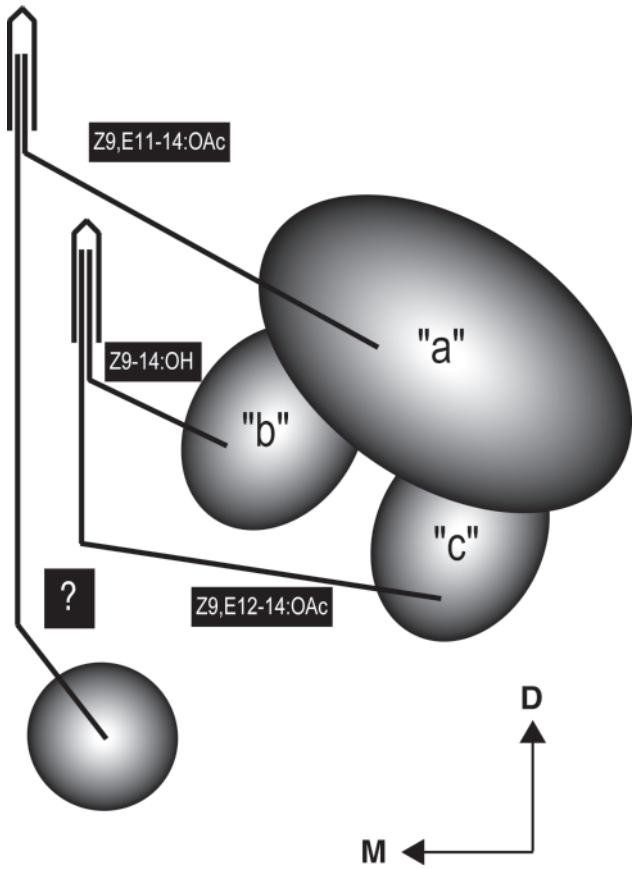


Figure 1 A schematic model illustrating the ORN projections in the MGC of a male *S. littoralis*. The three physiologically identified types of ORNs send axons to specific glomeruli within the MGC. The specificity of the neuron co-compartmentalized in a sensillum with the neuron tuned to *Z*9,*E*11–14:OAc is presently unknown, but it projects to an 'ordinary' glomerulus. Modified from Hansson (Hansson, 1997). M, medial; D, dorsal.

time demonstrated that both similarities and differences in glomerular responses exist between males and females.

Methods

Animals

Male and female, 1–5 days post-emergence moths were used in the study. The animals had been reared for several generations on a potato-based diet (Hinks and Byers, 1976). The pupae were separated according to sex and kept in plastic boxes at 70% relative humidity, 23°C and a 16 h:8 h light/dark cycle. Adult moths were given excess of water until the start of the experiment.

Morphology

Moth brains were dissected out and immunostained with synapsin (Klagges *et al*., 1996). The preparations were optically sectioned using a confocal microscope (Leica). Stacks of images were further processed on a Silicon Graphics workstation with Imaris 2.7 software (Bitplane AG, Switzerland) to obtain surface projections of the ALs. The final images were sharpened and contrast-enhanced in Adobe Photoshop.

Optical imaging preparation and staining

The staining procedure was similar to that previously described (Galizia *et al*., 1997, 1998). The animals were secured in plastic tubes with their head protruding and fixed with dental wax. The head capsule was cut open between the compound eyes, and muscles, glands and trachea were removed to expose the ALs. Great care was taken not to stretch or damage the antennal nerves. To minimize movement, the antennal muscles were cut off and the oesophagus was first stretched with a pair of forceps and then cut off. The neurolemma was left intact.

Subsequently, the animal was placed in a custom-made Plexiglass recording holder. A drop of dissolved calcium-sensitive dye was applied directly to the brain and a cover glass was fixed with wax over the cut window in the head. The preparation was then left in a dark and cooled (10–12°C) chamber for 1 h. After rinsing the brain, the animal was placed under the microscope (Olympus) where the brain was constantly perfused with fresh moth Ringer solution (Christensen and Hildebrand, 1987).

We used the calcium-sensitive probe Calcium-green-2-AM (Molecular Probes, Eugene, OR). The dye was dissolved in 20% Pluronic F-127 in dimethyl sulfoxide (Molecular Probes, Eugene, OR) and diluted in moth Ringer solution to a final concentration of ~30 μM.

Odour stimulation

A continuous charcoal-filtered and moistened air stream (30 ml/s) was ventilating the antenna ipsilateral to the recorded AL through a glass tube (7 mm internal diameter). The glass tube ended 10 mm from the antenna. Through a small hole in the glass tube, an empty Pasteur pipette was inserted blowing an air stream of ~15 ml/s. Odorants were applied to filter papers (5 × 15 mm) and inserted into Pasteur pipettes. All odorants were diluted in cyclohexane apart from the green leaf volatiles and (*Z*)-3-hexenylacetate, which were diluted in paraffin oil. Odorants were used in concentrations proved to elicit responses in extracellular recordings from ORNs (Anderson *et al*., 1993, 1995; Ljungberg *et al*., 1993; Jönsson and Anderson, 1999). Filter papers were loaded with a dose of 100 μg of an odorant. The compounds used are listed in Table 1. A puff of air (~15 ml/s) was blown through the odour-laden pipette for 1 s by a manually triggered puffer device (Syntech) and into the continuous stream of air. During stimulation, the airstream was switched from the empty to the odour-laden pipette, thereby minimizing mechanical stimulation. In some of the early recordings, odorant stimulations were made without switching from clean air to stimulus, i.e. odorant stimulation also led to an increased airflow. Interstimulus time was at least 1 min and all odorants were used once before a second series of stimulations with the same odorants.

Recordings

Recordings of series of 40 frames were made by an air-cooled CCD camera (Till Photonics) at 4 frames/s (200 ms exposure time) at 475 or 488 nm excitation. Filter settings were dichroic: 500 nm; emission LP 515 nm. Sequences were recorded through a 10× (NA 0.30; Olympus) or 20× (NA 0.50; Olympus) air objective.

Stimulation onset was at frame 12 and lasted 1 s. Images were binned 2× on chip (to 320 × 240 pixels) to increase signal-to-noise ratio. Execution of protocols and initial analyses of data were made using the software Till-vision (Till Photonics).

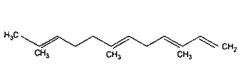
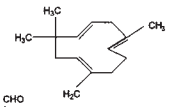
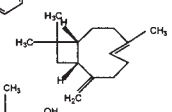
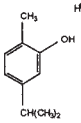
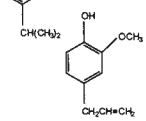
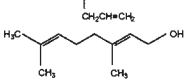
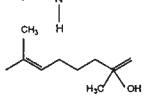
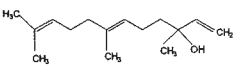
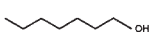
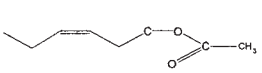
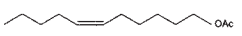
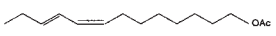
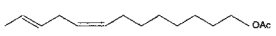
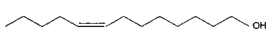
Background fluorescence (F) was defined as an average of frames 2–11, i.e. before onset of stimulation. F was subtracted from all frames to yield a dF and signals were expressed as dF/F, i.e. a relative change in fluorescence over background fluorescence. A sequence with pure air stimulation was first expressed as relative change in fluorescence (dF/F) and then subtracted from a sequence with odour stimulation in order to correct for bleaching. Air stimulus subtraction served an additional purpose, namely to subtract a possible mechanical component of the signal during pipette switching.

For image presentation, an average of frames 14–18 (peak of activity) of a bleaching-corrected sequence was calculated and an average of frames 2–11 (prestimulation) was subtracted. The resulting image was subsequently filtered with a spatial average low-pass filter (13 × 13 pixels) and false-colour coded to its entire intensity range.

Data analyses

We measured the diameter of the normally circular or oval-shaped regions of increased activity horizontally and

Table 1 Chemical structures and biological significance of all odorants used in the experiment

Compound	Structure	Biological significance
α–farnesene		Herbivore-induced host plant (cotton) volatile (Paré and Tumlinson, 1998)
α–humulene		Host plant (cotton) odorant (Paré and Tumlinson, 1998)
benzaldehyde		Oviposition deterrent (Anderson et al., 1993)
β–caryophyllene		Host plant (cotton) odorant (Paré and Tumlinson, 1998)
carvacrol		Oviposition deterrent (Anderson et al., 1993)
eugenol		Oviposition deterrent (Anderson et al., 1993)
geraniol		General flower odorant
1-hexanol		General green leaf volatile
indol		Herbivore-induced host plant (cotton) volatile (Paré and Tumlinson, 1998)
(+/-)-linalool		Herbivore-induced host plant (cotton) volatile (Paré and Tumlinson, 1998)
nerolidol		Oviposition deterrent (Anderson et al., 1993)
1-octanol		General green leaf volatile
PAA		General flower odorant
(Z)3-hexenyl-acetate		Herbivore-induced host plant (cotton) volatile (Paré and Tumlinson, 1998)
Z7-12:OAc		Putative sex pheromone component (Ljungberg et al., 1993)
ZE9,11-14:OAc		Sex pheromone component (Kehat et al., 1976)
ZE9,12-14:OAc		Sex pheromone component (Kehat et al., 1976)
Z9-14:OH		Behavioural antagonist (Campion et al., 1980)

Purity and source of chemicals are reported elsewhere (Anderson *et al.*, 1993, 1995; Jönsson and Anderson, 1999).

vertically through the centres of gravity at 50% of maximal activity. The mean diameters of the activity foci were then compared with the mean diameter of glomeruli in histological preparations. These foci will hereafter be called glomeruli.

Measurements of calcium activity were made from 11–14

glomeruli in each animal. The intensity kinetics for the mean pixel value of the raw data of each glomerulus was calculated for all odorants. We defined the response of a glomerulus as the mean net response (d*F*/*F* odour stimulation – d*F*/*F* air control) during frames 14–18 (peak of activity) minus the mean net response during frames 6–10 (prestimulation). The response to an odorant was expressed as the responses of all selected glomeruli. The responses were normalized so that the most strongly activated glomerulus for each odorant was given the value 1. The mean pixel value in a glomerulus represents one dimension. Thus, each response to a stimulus was described as a single point in a multidimensional space of 11–14 dimensions. The response profiles for each odorant were then compared to all other odorants using Pearson correlation coefficients (Systat 5.2.1). A perfect match between two stimuli would yield the value 1 and perfectly complementary responses would yield the value –1. As concentrations of the odorants were not corrected for differing volatility, comparisons of absolute intensities were irrelevant. For example, two alcohols, 1-hexanol and 1-octanol, were both used at the same concentration. However, the vapour pressure of 1-hexanol is ~10× higher than of 1-octanol (Hass and Newton, 1975).

Results

Confocal surface reconstructions of the moth ALs revealed that 20–25 glomeruli are visible from a frontal view (Figure 2). This means that 30–40% of the population of glomeruli would be accessible for recording. The diameter of the normally circular or oval-shaped regions of increased activity were measured horizontally and vertically through the centres of gravity at 50% of maximal activity. Their size (56.5 ± 9.7 μm, mean ± SD; 46 measurements in five animals) correspond with the size of actual glomeruli measured from histological preparations (54.1 ± 4.19 μm, mean ± SD; M. Sadek, personal communication). In 15 males and 10 females, recordings were stable and most stimuli were tested at least once. Recordings from additional animals, in which only a few odorants were tested or too much movement made recordings unreliable, were not further analysed. Generally, only one lobe was recorded from each preparation. Images (Figure 5) from recordings of the right lobe were mirrored for easier comparison. The relative change in fluorescence, d*F*/*F*, of the most activated glomeruli was in the range 1–3 %. Time courses, i.e. d*F*/*F* plotted as functions of time, from three glomeruli are shown in Figure 3. Glomerulus 1 showed stronger increase in activity than glomeruli 2 and 3 when the animal was stimulated with the flower odour, geraniol. Signals reached the peak ~1 s after onset of stimulation and declined to background level after another 2–3 s.

Odorant-specific activity pattern

Stimulations led to odour-specific activity patterns in the ALs. Figure 4 shows responses to all 14 non-sex pheromonal odorants in a single male animal. The responses to some odorants were confined to a few glomeruli, whereas other odorants elicited more distributed activity patterns. Furthermore, activity maps were often overlapping in that the same glomerulus was activated by several different odorants.

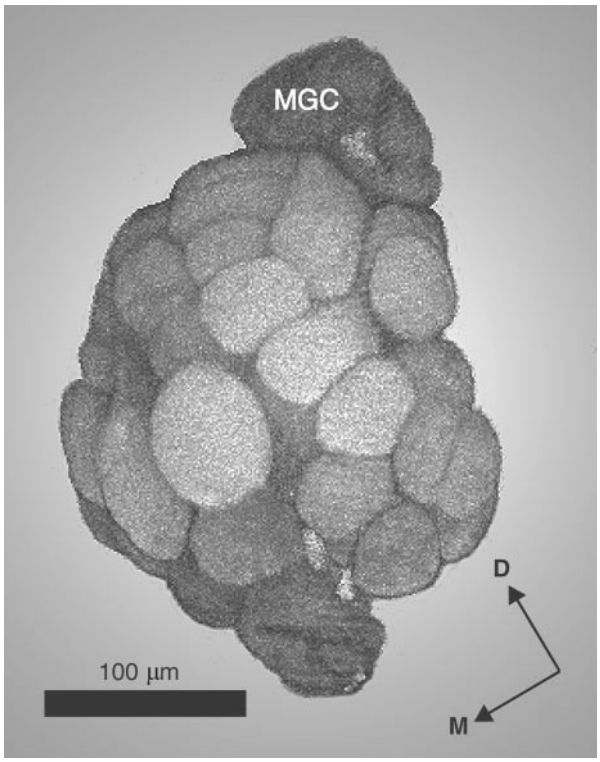
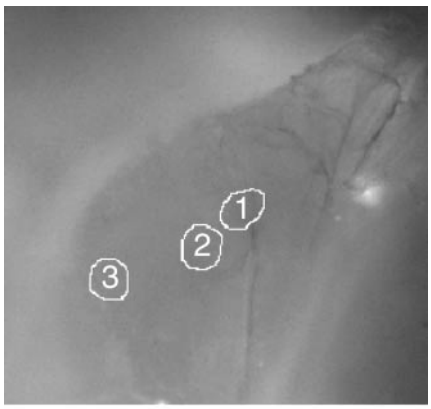


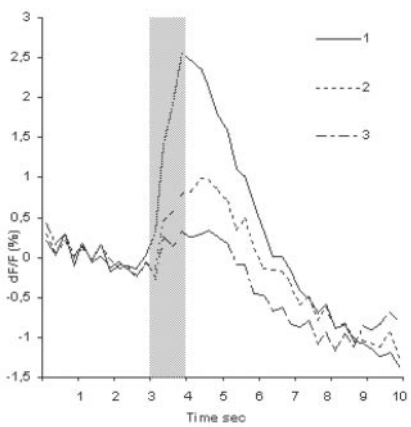
Figure 2 Surface reconstruction of a male AL. The view is frontal as in all Ca^{2+} recordings. M, medial; D, dorsal. The MGC is labeled.

Figure 7 shows the overall glomerular pattern, expressed as absolute d*F*/*F* values, for all 14 non-sex pheromones in a male individual (same as 4). In this animal, measurements were made from 14 glomeruli. Noise levels were defined as the mean standard deviations of frames 2–11 (Sachse *et al*., 1999). Many odorants showed overlapping patterns, but each combination of activated glomeruli is unique to a certain odorant. For example, phenylacetaldehyde (PAA) evoked the strongest response in glomerulus 2, (+/–)-linalool in glomerulus 1, geraniol in glomerulus 9 and 1-hexanol in glomerulus 11. The two structurally similar sesquiterpenes, α-humulene and β-caryophyllene, evoked very similar patterns.

Normally, two or more stimulations were made with each odorant in each animal. Repeated stimulations with the same odorant showed highly reproducible activity patterns. Comparisons of overall glomerular activity patterns between two stimulations with the same odorant yielded a correlation index of 0.79 ± 0.13 (mean ± SD, 45 odorant pairs in four animals).

To compare the similarity of glomerular responses to different odorants, we calculated the correlation indices for all possible pairs of odorants (Table 2). We quantified the overall patterns of glomerular activity as the relative response in all glomeruli measured. For each odorant stimulation, the dF/F was normalized so that the strongest activated glomerulus was set to 1. Correlation analyses were made in four males and three females. PAA, for example, showed a low correlation with all other odorants and was generally most related in response to another aromatic compound with an attached aldehyde group, benzaldehyde, in both males and females. In both sexes there was also a high response similarity between 1-hexanol and (*Z*)-3-hexenylacetate. In males there was a high correlation between responses to α-humulene and β-caryophyllene (0.78–0.93, four animals). In contrast, the similarity in response to these two compounds was much lower in females (0.12–0.51, three animals).





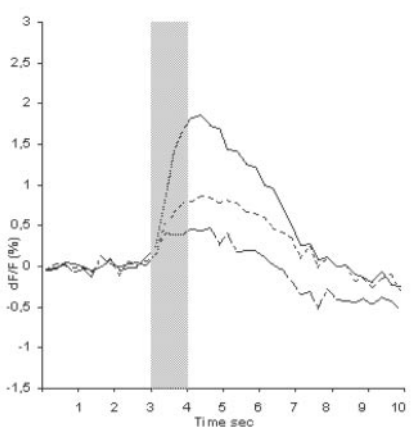


Figure 3 Time courses of the Ca^{2+} signals in three selected glomeruli at two repeated stimulations with 100 μg geraniol (57 min between). The shaded area shows the stimulus duration. The inset shows the positions of the three selected glomeruli.

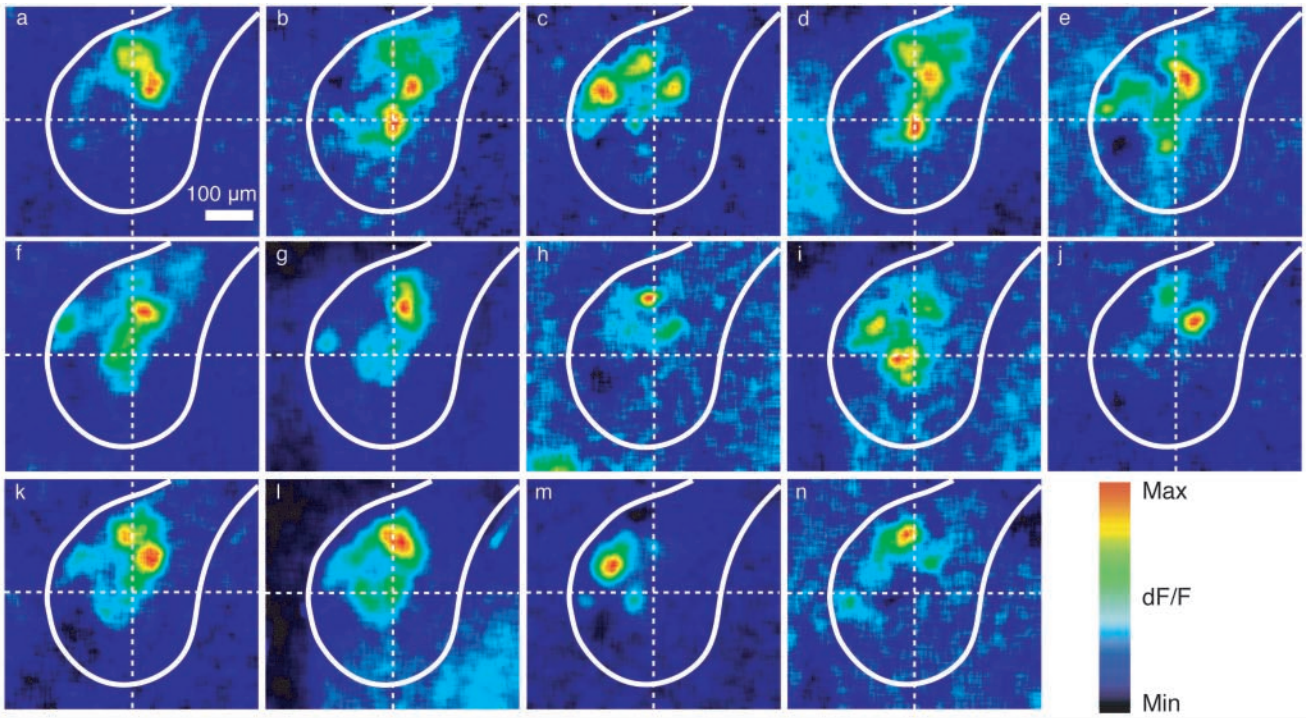
Consistency between individuals

Great care was taken to use equal views, angles of the preparations and focal plane between preparations, but direct comparisons between animals are difficult, mostly due to individual morphological differences. However, comparisons of the relative positions of foci of activity can be made. Figure 5 shows recordings from three males and two females and responses to geraniol, (+/–)-linalool and PAA (the same relative positions of the activity foci for these odorants were observed in all 25 animals). PAA activated a glomerulus in the dorsomedial area in all animals recorded. Both geraniol and (+/–)-linalool activated the central (frontal) area of the lobe, but the (+/–)-linalool-activated glomerulus was located more ventrally. Similar patterns were observed in males and females. Thus, the glomerular activity patterns for these odorants are not only preserved between individuals of the same sex, but also between sexes. However, the two sesquiterpenes, α-humulene and β-caryophyllene, did, as described above, elicit similar patterns only in male individuals. For a few odorants in the experiment,

Figure 4 False colour coded images of glomerular activity in a male individual to 14 non-sex pheromones. All odorants evoked distinct but generally overlapping patterns. The images show the relative change in activity (dF/F) during stimulation. Each image is scaled to its entire intensity range. The colour code is shown by the false colour bar and is valid for all images in this report. **(a)** α-Farnesene, **(b)** α-humulene, **(c)** benzaldehyde, **(d)** β-caryophyllene, **(e)** carvacrol, **(f)** eugenol, **(g)** geraniol, **(h)** 1-hexanol, **(i)** indol, **(j)** (+/–)-linalool, **(k)** nerolidol, **(l)** 1-octanol, **(m)** PAA, **(n)** (*Z*)-3-hexenylacetate.

Figure 5 Calcium responses in three males and two females to **(a)** geraniol, **(b)** (+/–)-linalool and **(c)** PAA. The arrows depict three glomeruli activated by one of the three odorants. In all animals, PAA evoked the strongest response in a glomerulus in the dorsomedial part of the lobe. Geraniol and (+/–)-linalool activated glomeruli in the centre (front) of the lobe. (+/–)-Linalool showed the strongest response in a glomerulus lateral to the glomerulus most strongly activated by geraniol.

Figure 6 False colour coded images of pheromone-evoked activity patterns in a male moth to **(a)** *Z*9,*E*11–14:OAc, **(b)** *Z*9,*E*12–14:OAc, **(c)** *Z*9–14:OH, **(d)** *Z*7–12:OAc. Recordings were made with 20× magnification and only the part of the lobe closest to the antennal nerve is shown.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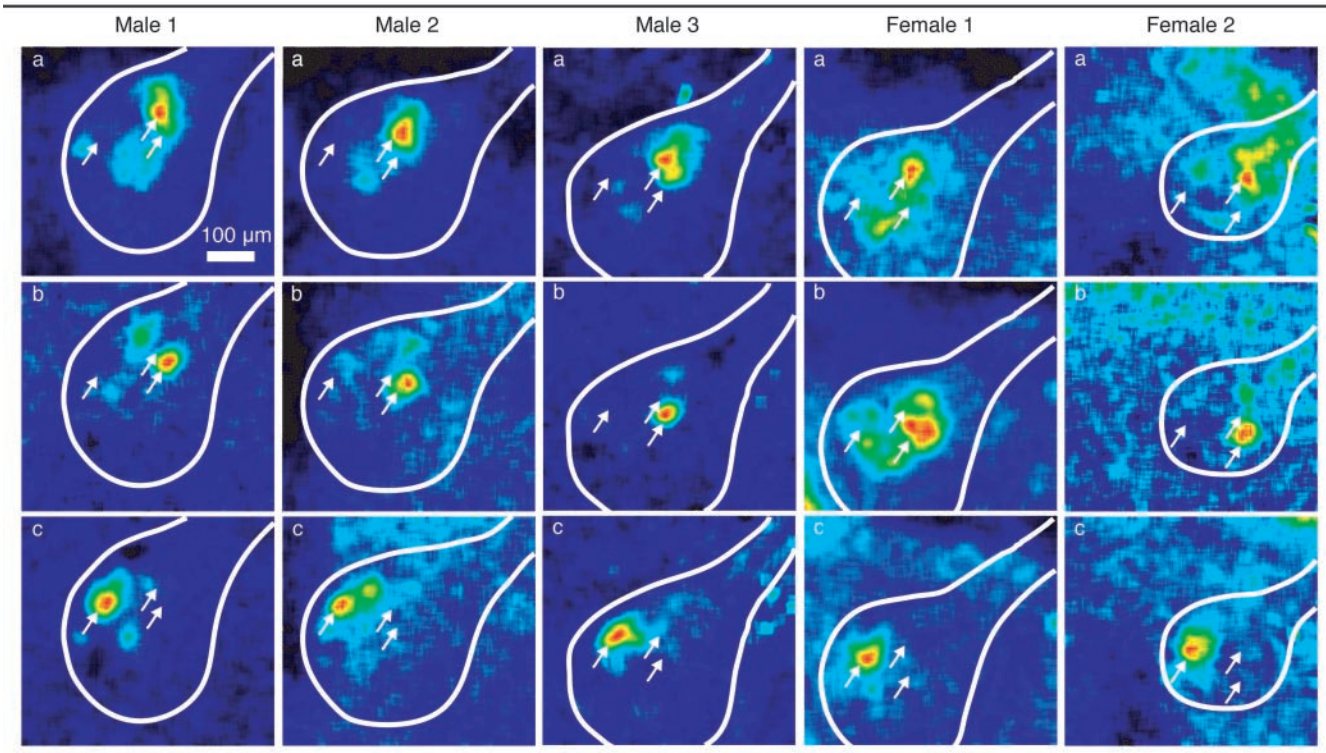
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Male 3
Female 1
Female 2
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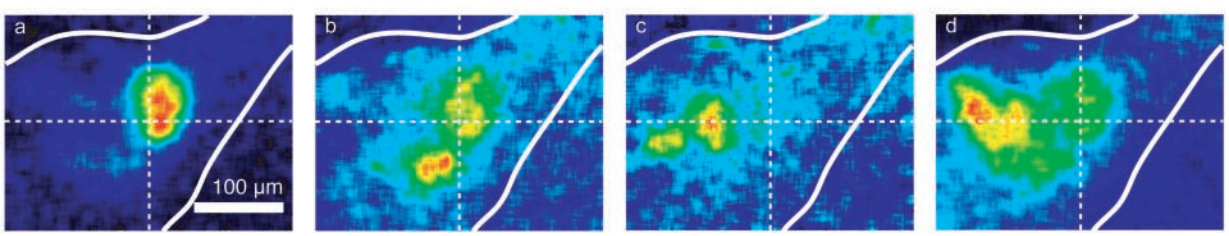
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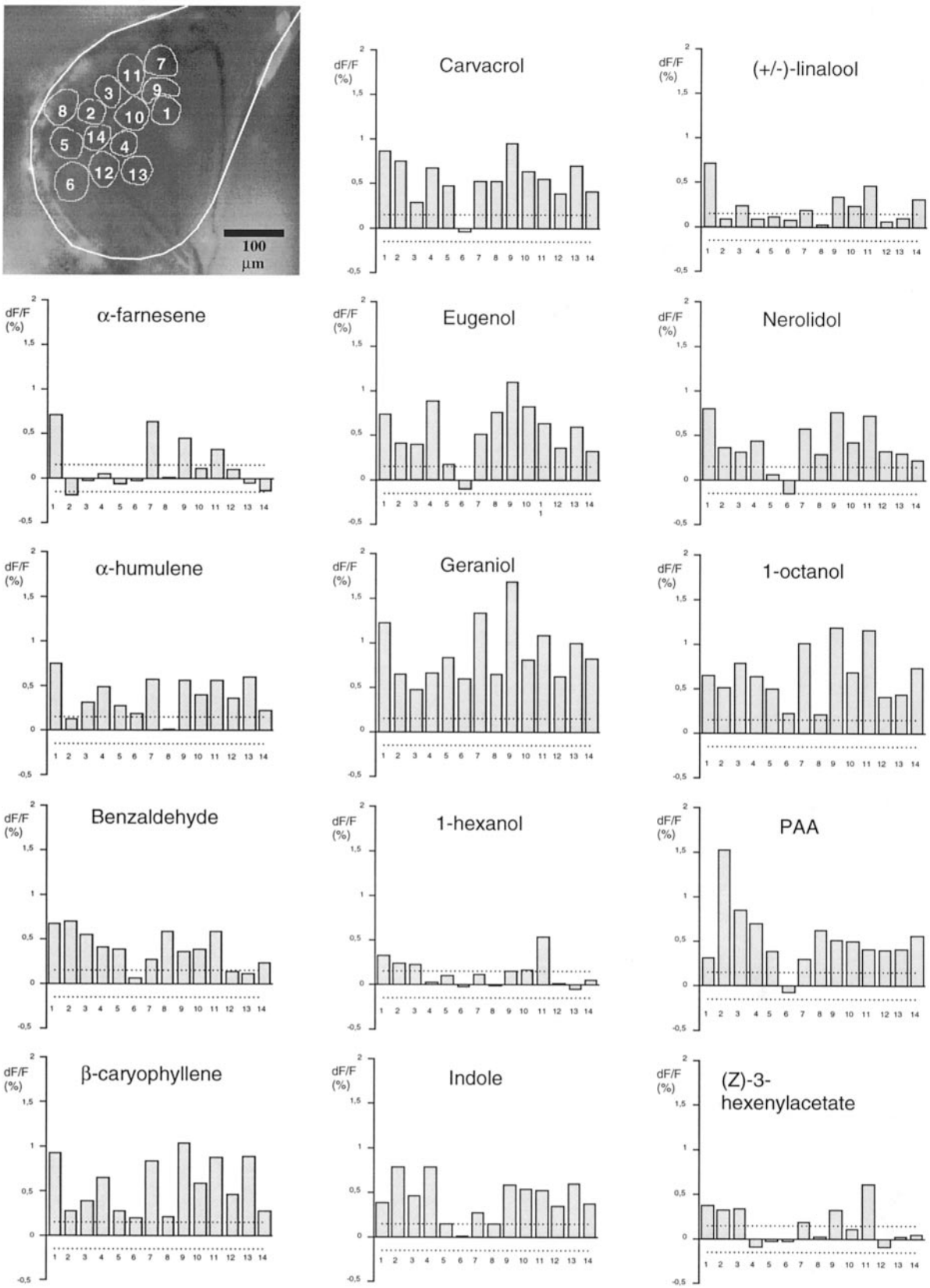

Figure 7 Glomerular responses in 14 selected glomeruli (*x*-axes) in a male moth (same as in Figure 4). Intensities are expressed as relative change in fluorescence (d*F*/*F*). The hatched lines show the noise limits (mean standard deviations of frames 2–11). The inset shows the selected glomeruli (diameter 45–70 µm).

Table 2 Correlation matrix of glomerular response patterns to 14 non-sex pheromone compounds in males (upper table, mean four animals) and a females (lower table, mean three animals)

	Farn	Hum	Benz	Car	Carv	Eug	Ger	Hex	Ind	Lin	Ner	Oct	PAA	Z3hex
Farn	1.000													
Hum	0.553	1.000												
Benz	0.131	***–0.166***	1.000											
Car	0.608	**0.852**	***–0.221***	1.000										
Carv	0.331	0.306	***–0.047***	0.415	1.000									
Eug	0.260	0.294	0.337	0.255	0.460	1.000								
Ger	0.689	0.641	***–0.135***	0.772	0.619	0.321	1.000							
Hex	0.465	0.195	0.493	0.228	0.256	0.230	0.444	1.000						
Ind	0.028	0.235	0.125	0.254	0.588	0.293	0.349	0.418	1.000					
Lin	0.602	0.346	0.123	0.521	0.483	0.260	0.586	0.447	0.081	1.000				
Ner	0.704	0.642	***–0.076***	0.790	0.505	0.281	0.745	0.214	0.208	0.630	1.000			
Oct	0.573	0.405	***–0.040***	0.559	0.523	0.105	0.761	0.612	0.403	0.486	0.551	1.000		
PAA	***–0.141***	***–0.223***	0.699	***–0.310***	0.024	0.332	***–0.160***	0.416	0.421	***–0.144***	***–0.256***	***–0.039***	1.000	
Z3hex	0.280	0.112	0.303	0.185	0.465	0.160	0.369	0.694	0.390	0.466	0.289	0.541	0.187	1.000
Farn	1.000													
Hum	0.476	1.000												
Benz	0.006	***–0.283***	1.000											
Car	0.008	0.373	0.078	1.000										
Carv	***–0.332***	***–0.273***	0.399	0.158	1.000									
Eug	***–0.175***	***–0.314***	0.670	0.144	0.525	1.000								
Ger	0.274	0.415	***–0.373***	0.331	0.108	0.000	1.000							
Hex	0.322	0.140	0.602	0.194	0.332	0.266	0.151	1.000						
Ind	***–0.117***	***–0.032***	0.691	0.333	0.265	0.531	0.024	0.533	1.000					
Lin	0.642	0.499	***–0.177***	0.244	***–0.073***	0.005	0.779	0.393	0.036	1.000				
Ner	0.472	0.271	***–0.072***	0.131	0.048	***–0.302***	0.465	0.029	***–0.225***	0.480	1.000			
Oct	0.243	0.373	***–0.126***	0.483	0.017	0.129	0.645	0.395	0.319	0.607	0.250	1.000		
PAA	0.170	***–0.148***	0.700	***–0.023***	0.121	0.266	***–0.329***	0.543	0.618	***–0.135***	***–0.140***	0.185	1.000	
Z3hex	0.459	0.015	0.730	0.111	0.123	0.269	0.033	**0.821**	0.514	0.356	0.127	0.343	0.700	1.000

Patterns of high similarity (>0.80) are shown in bold, whereas patterns of low similarity (<0) are shown in bold italic. A perfect match would yield the value 1 and perfectly complementary responses would yield the value –1. Measurements were made from 11–14 glomeruli. Abbreviations: Farn, α-farnesene; Hum, α-humulene; Benz, benzaldehyde; Car, β-caryophyllene; Carv, carvacrol; Eug, eugenol; Ger, geraniol; Hex, 1-hexanol; Ind, indole; Lin, (+/–)-linalool; Ner, nerolidol; Oct, 1-octanol; PAA, phenylacetaldehyde; Z3hex, (*Z*)-3-hexenylacetate.

consistent and spatially stereotyped responses between individuals, regardless of sex, were less obvious. 1-hexanol, for example, elicited the strongest response in the same glomerulus as PAA in some animals, whereas in other animals the peak of activity was observed in a more centrally located glomerulus. The latter glomerulus was always also strongly activated by another primary alcohol, 1-octanol.

Pheromone-evoked activity

Pheromone components activated glomeruli in the region close to the entrance of the antennal nerve (Figure 6). This region was not or only weakly activated by non-sex pheromones. The major pheromone component *Z*9,*E*11–14:OAc activated a glomerulus close to the antennal nerve in all preparations that corresponds in position to the large identified MGC glomerulus called the cumulus or 'a' glomerulus (see Figure 1). Also, the position of the activity focus elicited by the minor pheromone component *Z*9,*E*12–14:OAc, i.e. ventrolateral to the former, corresponds with the position of the 'c' glomerulus. The behavioural antagonist *Z*9–14:OH activated a glomerulus medial to the former and which corresponds to the position of the glomerulus termed 'b'. The putative pheromone component *Z*7–12:OAc showed the strongest activity in an additional glomerulus, which was also activated by non-sex pheromones. Figure 8 shows the relative change in activity in four glomeruli in two different males. The major pheromone component *Z*9,*E*11–14:OAc elicited the strongest response in glomerulus 'a' and the minor component *Z*9,*E*12–14:OAc in glomerulus 'c'. The behavioural antagonist *Z*9–14:OH elicited the strongest response in glomerulus 'b', whereas *Z*7–12:OAc elicited high activity in both glomeruli 'b' and 'd'.

Discussion

We have studied activity in neural populations in the antennal lobes of the cotton leaf worm *S. littoralis* by means of

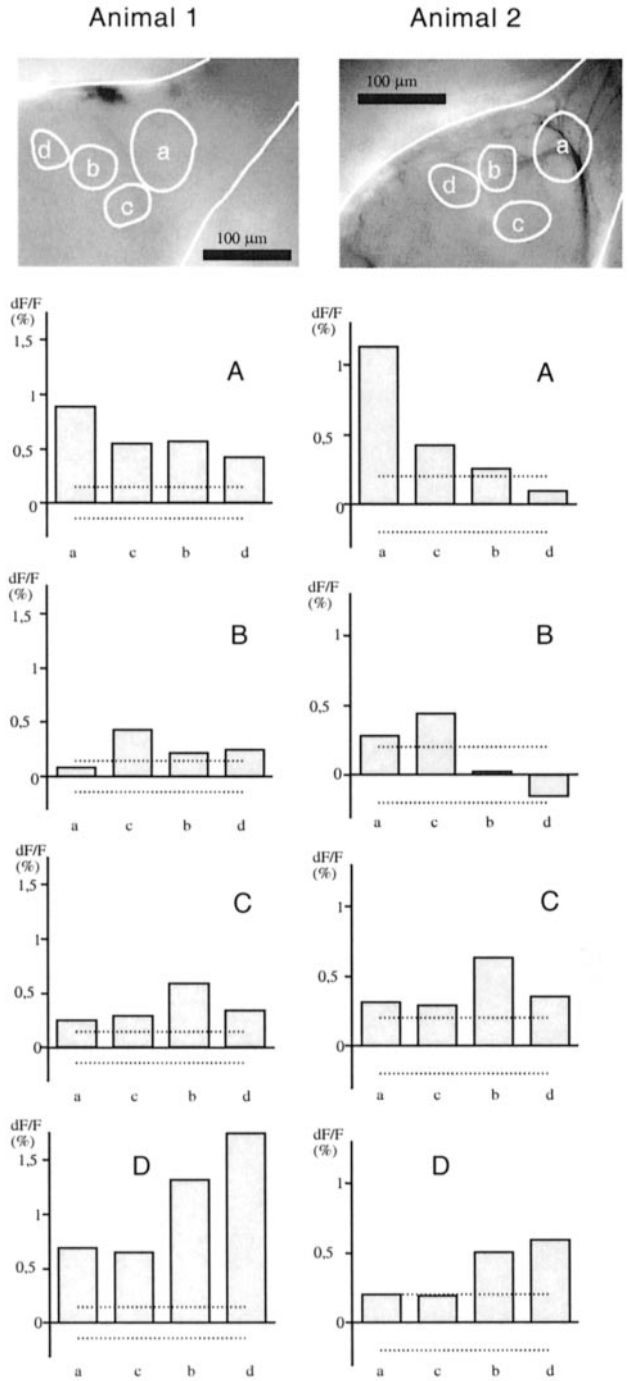

Figure 8 Calcium responses in four glomeruli to pheromone components in two different males (No. 1 is the same male as in Figure 6). Intensities are expressed as relative change in fluorescence (d*F*/*F*). The hatched lines show the noise limits (mean standard deviations of frames 2–11). The insets show the positions of the four glomeruli where measurements were made. **(a)** *Z*9,*E*11–14:OAc, **(b)** *Z*9,*E*12–14:OAc, **(c)** *Z*9–14:OH, **(d)** *Z*7–12:OAc.

calcium imaging. The results from this study contribute to the contemporary view that odorants are represented spatially in central olfactory neuropils. We have clearly showed that different odorants evoke distinct and reproducible activity patterns in the AL. These patterns are generally consistent between individuals. Furthermore, non-sex pheromones elicit similar activity patterns in males and females. Conspecific sex-pheromone components and an interspecific signal evoked responses that supported an earlier model of ORN projections in the MGC, thus facilitating the interpretation of responses evoked by other odorants.

The Ca^{2+} signals have been shown to originate in glomeruli in the honeybee (Galizia *et al.*, 1999). By superimposing morphological images on the physiological activity maps, the signals were shown to be confined within the borders of glomeruli. There is no reason to believe that the signals would originate from a different source in moths. The average size of the activity foci is close to the actual size of glomeruli measured in histological preparations. Furthermore, the normally circular or oval shape and the existence of a gravity centre in the activity spots indicate a spheroidal structure. As there are no other spheroidal structures in the ALs than glomeruli, we assume that the signals we measure are of glomerular origin.

Activity patterns elicited by plant-related odorants in males and females

The plant-related odorants used in this study have previously been shown to elicit responses at the peripheral level (Anderson *et al.*, 1993, 1995; Jönsson and Anderson, 1999). Consequently, we also found that the odorants used elicited patterns of elevated activity in the ALs. Several glomeruli were often activated when the animal was stimulated with non-pheromonal odorants. Furthermore, the same glomeruli responded to several different compounds. However, response amplitude was unevenly distributed in that one or a few glomeruli were more strongly activated than the rest. We can not exclude the possibility that more glomeruli, which were inaccessible to recording, may be activated in other parts of the AL.

All odorants tested evoked unique patterns of glomerular activity. A correlation analysis of response profiles of repeated stimulations of the same odorant revealed a high reproducibility. Activity patterns for some odorants were more similar than others. Two floral odorants, PAA and geraniol, elicited response patterns almost without overlap and showed low correlation in all animals (–0.32 ± 0.16, mean ± SD, seven animals). These two odorants can easily be discriminated by female *S. littoralis*, as has been shown in a differential conditioning task (Fan and Hansson, 2001). Responses to the two structurally similar sesquiterpenes, α-humulene and β-caryophyllene, were very similar in all male individuals. In both males and females a type of ORN tuned to α-humulene and β-caryophyllene showed equal responses to both compounds (Anderson *et al.*, 1995; Jönsson and Anderson, 1999; Carlsson and Hansson, unpublished observation). In contrast to males, the glomerular activity patterns elicited by the two compounds in

females showed low similarity. A previous study (Anderson *et al*., 1995) found a single ORN in a female that responded solely to α-humulene, thereby offering the animal a possible mechanism to discriminate between the compounds. Furthermore, intracellular recordings of AL neurons revealed that several interneurons responded solely to either of the compounds in females (M. Sadek, personal communication). The far weaker correlation between responses in females may indicate that only females possess an ability to discriminate between the compounds, or at least have a higher ability than males. The two sesquiterpenes are emitted from leaves of host plants, for example cotton, and may be important olfactory cues to guide a mated female to a suitable site for oviposition. It is not unlikely that the ratio between closely related compounds is important for recognition of a host plant, as has been demonstrated, for example in the Colorado potato beetle *Leptinotarsa decemlineata (*Visser and Avé, 1978). Intersexual differences are very interesting as they may reflect adaptations to sex-specific requirements.

Pheromone evoked activity patterns in males

As with the moth *Heliothis virescens* (Galizia *et al*., 2000), pheromone-evoked calcium responses in the male-specific MGC in *S. littoralis* corroborated previous studies of AL projections of physiologically characterized single ORNs (Ochieng' *et al*., 1995; Berg *et al*., 1998). In contrast to *H. virescens*, there does not seem to be a consistent correspondence between input and output of the MGC in *S. littoralis* (Anton and Hansson, 1995; Vickers *et al*., 1998). The major component, *Z*9,*E*11–14:OAc, activated an area close to the entrance of the AN, which is not activated by non-pheromones. This glomerulus is most likely identical to the 'cumulus' or 'a' glomerulus, which has been shown to be the convergence site for ORNs tuned to the major component. Also, the minor component *Z*9,*E*12–14:OAc and the behavioural antagonist *Z*9–14:OH activated areas that corresponded in location to glomeruli 'c' and 'b', respectively. The putative pheromone component *Z*7–12: OAc activated the 'b' glomerulus and an additional glomerulus located further dorsally. It is unclear if this second glomerulus responding to *Z*7–12:OAc belongs to the cluster of glomeruli comprising the MGC. Projection neurons responding exclusively to *Z*7–12:OAc have been found to arborize in ordinary glomeruli (Anton and Hansson, 1995). The glomeruli within the MGC were not (or only weakly) activated by odorants other than sex pheromones.

Interindividual comparisons

Direct comparisons between animals would be facilitated if we could see the outlines of glomeruli and if we could compare this anatomy with a glomerular atlas of the AL. However, visual inspection of the relative positions of the most strongly activated glomeruli in *S. littoralis* clearly showed that the patterns for a number of odorants were conserved between individuals of both sexes. For instance, PAA always elicited high activity in the dorsomedial part of both lobes. Another floral odour, geraniol, activated glomeruli closer to the entrance of the antennal nerve in all animals tested and a third odour, (+/–)-linalool, evoked the highest activity ventral to the glomeruli activated by geraniol. Most of the odorants tested elicited activity patterns that were similar in all animals, which may indicate a spatial olfactory code as has been suggested for the honeybee (Galizia *et al*., 1999). However, similar patterns do not constitute evidence for involvement of homologous glomeruli. A glomerular map of the AL in *S. littoralis* is currently under construction (M. Sadek, in preparation) and future experiments may prove that a homology does indeed exist. For a few odorants, however, we did not observe patterns that were spatially stereotyped between all animals. For example, the response to 1-hexanol varied between individuals in that the most strongly activated glomerulus in a number of animals was the same, as was that most strongly activated by 1-octanol, whereas in some animals the peak of activity was seen in the same glomerulus that was always strongly activated by PAA. Thus, there may be some individual differences in response patterns. It has been reported (Galizia *et al*., 1999) that intraspecific variability does indeed exist and is not due to variations in glomerular positions between animals.

Glomerular activity patterns and receptor specificity

Pheromone components are detected by extremely specific ORNs, whereas most non-pheromonal odorants are detected by less specific receptor types with different but overlapping response spectra. These less specific ORNs may indeed be narrowly tuned with respect to a certain molecular feature, but the number of odorants sharing this determinant can potentially be large. An olfactory system relying simply on a system in which each input channel is devoted to a specific odorant would leave the animal with severe restrictions. Only a limited number of odorants may be detected and the animal would have difficulties coping with novel olfactory-related situations. On the other hand, such a one compound/receptor type system would be advantageous for processing highly predictable signals such as those involved in intraspecific communication. In vertebrates, most odours are detected by a combination of ORNs (Buck, 1996). ORNs in the olfactory epithelium are broadly tuned (Duchamp-Viret *et al*., 1999, 2000) and the best characterized receptor, I7 from the rat, responds to an entire family of molecules, but with a molecular determinant in common (Araneda *et al*., 2000). This fact is also reflected in the main olfactory bulb, where all odorants are represented by different combinations of activated glomeruli (Rubin and Katz, 1999; Uchida *et al*., 2000; Meister and Bonhoeffer, 2001). However, it was recently demonstrated (Leinders-Zufall *et al*., 2000) that sensory neurons in the vomeronasal organ

in mice are highly selectively tuned to pheromones. Similar mechanisms thus seem to exist in insects and vertebrates.

If receptors have broad and overlapping response profiles, an odorant would be detected by several different receptor types and each receptor type would detect a number of different odorants. Consequently, if all receptor neurons representing a certain receptor type terminate in the same glomerulus and the stimulating odorant is detected by several types of receptors, an array of glomeruli would be activated. The number of activated glomeruli depends on the specificity of the ORNs. The glomeruli of the moth MGC receive highly specialized afferents and it is very likely that each of the MGC glomeruli represents a specific receptor type. It is still not known if all ORNs housing a certain receptor type terminate in the same ordinary glomerulus (or glomeruli) in moths, as has recently been demonstrated in *D. melanogaster* (Vosshall *et al*., 2000) and earlier in vertebrates (Mombaerts, 1996; Mombaerts *et al*., 1996). It can not be excluded that several types of ORNs innervate the same glomerulus and that one type of ORN innervates several glomeruli. However, it is highly unlikely that the glomeruli receive identical input. As a major contributor to the calcium signals is presynaptic influx of Ca^{2+} in the ORNs (Galizia *et al*., 1998), the odour-evoked patterns in the AL would reflect the array of receptors activated at the antennal level. The fact that the activity patterns evoked by sex-pheromone components in the males correspond with single ORN projections further indicates that a substantial part of the signal is of afferent origin, as prediction of the dendritic arborizations of interneurons based on their physiology often fails (Anton and Hansson, 1995). It is, however, important to note that application of the Ca^{2+}-sensitive dye directly on the brain tissue results in a non-selective dye uptake. Therefore, $[Ca^{2+}]$ changes in the AL interneurons may also contribute to a composite signal. Interneurons confined to the AL generally synapse within most glomeruli and may explain why we often observed some weak activity in most parts of the lobe.

Combinations of responding glomeruli in the ALs may constitute a spatial olfactory code that underlies final odour perceptions as results from recordings in the honeybee AL suggest (Galizia *et al*., 1999). However, both slow temporal patterns in individual PNs and synchrony of PN ensembles have been shown to be odorant-specific (Laurent and Davidowitz, 1994; Laurent *et al*., 1996). Thus, spatial as well as temporal features of an olfactory response carry information about the odour identity. We can not exclude the possibility that these mechanisms work in parallel and are required for fine odour discrimination.

Calcium recordings have an obvious drawback; namely, they tell us little about how odour information is computed and integrated in the ALs. However, these recordings provide us with information about many other interesting aspects of olfaction—for example about ORN specificity and the topographic organization of glomeruli—and allow us to make interindividual and intersexual comparisons. To solve questions regarding olfactory information processing in the lobes, we intend to integrate calcium recordings with single-cell recordings of AL interneurons in future experiments.

This study builds on previous behavioural and physiological work in *S. littoralis*. Our results confirm the model of functional organization in the MGC in this species. Biologically relevant host-plant-related odorants are represented in an across-glomerular fashion. These patterns of glomerular activity are roughly conserved between individuals. In addition, we show for the first time that representations of non-pheromonal odorants are generally similar between males and females, despite obvious differences in olfactory-related ecological requirements.

Acknowledgements

We would like to thank Mr M. Stensmyr and Mrs K. Johnsson for help with AL reconstruction and immunostaining. This research was supported by grants from the Swedish Natural Science Research Council (NFR) to B.S.H.

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Accepted November 28, 2001