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Three-dimensional antennal lobe atlas of male and female moths, *Lobesia botrana* (Lepidoptera: Tortricidae) and glomerular representation of plant volatiles in females

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Summary

Spatiotemporal odour coding is thought to be linked closely with the specific glomerular anatomy of the primary olfactory centre. In most insects the number of the glomeruli within the antennal lobe is limited to fewer than 100, allowing their individual identification. In the grapevine moth, *Lobesia botrana*, a map of the antennal lobe glomeruli was reconstructed three-dimensionally, by comparing three different brains in males and females. The map of the antennal lobe of females served then as a basis to identify glomeruli containing dendritic arborisations of 14 physiologically characterised projection neurons. Projection neurons responding to the same plant compound did not always arborise in the same glomerulus and some neurons arborising in the same glomerulus responded to different compounds. Different zones of target glomeruli were, however, identified when pooling all neurons responding to one of two different compounds respectively (α-farnesene and nonatriene). All identified glomeruli of specifically responding projection neurons were situated close to the anterior surface of the antennal lobe. One broadly responding projection neuron arborised in a more posteriorly situated glomerulus. A local interneuron responding to only one compound was arborising densely in a neighbouring glomerulus and had sparse branches in all other glomeruli. These results are discussed with respect to plant odour processing and structure–function relations in antennal lobe neurons. The 3D AL atlas will, in the future, also be used to obtain a better understanding of coding mechanisms of grapevine odours in this pest insect.

Key words: olfaction, tortricid moth, plant volatiles, glomeruli, intracellular recording, anatomical reconstruction, confocal microscopy.

Introduction

In insects, olfaction plays an important role in their search for hosts, feeding sites or mating partners. Odours are detected by receptor neurons housed in sensilla mainly located on the antennae. Olfactory receptor neurons (ORNs) send projections directly to the antennal lobe (AL), the primary olfactory centre of the insect brain. As in vertebrates, the AL is formed of spherical structures called glomeruli, in which terminals from olfactory receptor neurons make synapses with second-order neurons (Boeckh and Tolbert, 1993; Anton and Homberg, 1999). The number of glomeruli is species-specific, several thousands in vertebrates compared with between 40 and 100 in insects (Rospars, 1988; Anton and Homberg, 1999). Investigations on the role of some AL glomeruli in odour processing revealed a functional organisation. Morphologically distinct glomeruli represent functionally distinct processing centres for incoming olfactory information. The macroglomerular complex (MGC), a structure of enlarged glomeruli present only in the male AL, processes specifically sex pheromone information (for reviews see Mustaparta, 1996; Anton and Homberg, 1999; Hansson and Christensen, 1999). Each ORN type responding to one pheromone component projects to one MGC glomerulus in several moth species (Hansson et al., 1992; Ochieng' et al., 1995; Todd et al., 1995; Berg et al., 1998). By contrast, the representation within glomeruli that process non-pheromone odours is less evident. Plant volatile information, in contrast to sex pheromone, is processed in so-called ordinary glomeruli that are present in both male and female moths (review in Anton and Homberg, 1999). The specificity of plant volatile receptor projections is only indirectly known *via* olfactory membrane receptor expression (Gao et al., 2000; Vosshall, 2000; Bhalerao et al., 2003) and optical imaging studies in moths (Galizia et al., 2000; Carlsson et al., 2002; Carlsson and Hansson, 2003; Meijerink

et al., 2003; Skiri et al., 2004), honeybees (Joerges et al., 1997; Galizia and Menzel, 2000; Galizia and Menzel, 2001) and flies (Fiala et al., 2002; Wang et al., 2003).

The integration of plant odour information in the moth AL has only been started to be investigated recently. In female *Manduca sexta*, a female-specific enlarged glomerulus was found to house arborisations of projection neurons (PNs) responding to the plant compound linalool only (King et al., 2000), and more specifically to one enantiomer of this compound (Reisenmann et al., 2004). In *Drosophila melanogaster*, electrophysiological characterisation revealed, conversely, broad tuning and complex responses in second order neurons (Wilson et al., 2004).

To understand better the general organization and the functional significance of AL glomeruli in odour processing, three-dimensional (3D) AL maps of a few insect species have been reconstructed (Rospars, 1983; Rospars and Chambille, 1989; Galizia et al., 1999; Laissue et al., 1999; Rospars and Hildebrand, 2000; Sadek et al., 2002; Berg et al., 2002; Smid et al., 2003; Greiner et al., 2004). Comparison of AL atlases from different species enables us to add new information on general organization principles and to explain specific adaptation to different biological constraints, such as body size or a specific odour environment.

The European grapevine moth, *Lobesia botrana* (Denis and Schiffmüller), is a major pest of vineyards throughout the world. Although grapevine is the main host plant, it is a polyphagous insect that can develop on different species (Bovey, 1966; Stoeva, 1982). *L. botrana* has been shown to be attracted by a non-host plant, tansy (Gabel et al., 1992). Gas-chromatography coupled with electroantennograms (EAGs) revealed that female antennae responded to some tansy volatile compounds, such as thujyl alcohol, α-thujone and β-thujone (Gabel et al., 1992). Wind tunnel experiments showed that mated females responded to host plant parts, but not virgin females and males (I. Masante-Roca, personal observation). Peripheral EAGs recordings (I. Masante-Roca, personal communication) and AL intracellular recordings experiments (Masante-Roca et al., 2002), using grapevine compounds as stimuli, revealed that both males and females respond to most of the tested plant odours (Masante-Roca et al., 2002). Moreover, no significant difference in threshold responses was found between virgin and mated males or females. However, AL neurons of mated females seemed to respond more frequently to the tested plant odours than those of mated males and unmated females (Masante-Roca et al., 2002).

In the present study, we established a three-dimensional (3D) atlas of the AL of male and female *L. botrana.* The map of the female AL serves further as a tool to identify the respective target glomeruli of physiologically characterised PNs, to reveal possible structure–function relationships. The 3D AL atlas will, in the future, also be used to obtain a better understanding of coding mechanisms of grapevine odours as a function of mating and environmental conditions in this pest insect.

Materials and methods

Insects

Larvae of *L. botrana* Denis and Schiffmüller, originating from a laboratory culture in Bordeaux, France, were reared on a semi-artificial diet under a 16 h:8 h L:D photoperiod and 22±1°C temperature (adapted from Stockel et al., 1989). Two or three-day-old male and female moths were used for anatomical investigations and mated females were used for intracellular recordings. Mated moths were obtained by pairing virgin males and females in a cage. Insects were observed and mating pairs were transferred to another cage and left until the next day. Mating was confirmed by checking the presence of a spermatophore in the female abdomen at the end of each experiment. Preparations for intracellular recordings were done according to standard methods for moths (Kanzaki et al., 1989) and adapted to our moth species (Masante-Roca et al., 2002). Briefly, an insect was mounted in a cut plastic pipette tip with the head protruding. The cuticle was removed from the front of the head and tissue overlaying the brain was removed. The preparation was superfused with saline solution at pH 6.9 (Christensen and Hildebrand, 1987) and the antenna was exposed to a continuous air stream.

Intracellular recording and stimulation

AL neurons were randomly penetrated by a glass microelectrode with the tip filled with 4% Lucifer Yellow (LY) CH (Sigma, St Louis, MO, USA) and backfilled with 2 mol l^{-1} LiCl. The antenna ipsilateral to the recording site was ventilated by a steady stream of charcoal-filtered air that passed through a glass tube at a constant flow of about 20 ml s^{-1}. The base of the ipsilateral antenna was fixed with wax in an upright position. Half of the antenna was protruding into the glass tube. When intracellular contact was established, the antenna was stimulated by pulsing a 5 ml s^{-1} airflow during 0.5 s through a Pasteur pipette containing a clean filter paper, or a filter paper with the solvent or a test compound inserted into the glass tube ~20 cm from the outlet. The bar underneath the registrations in Fig. 6 indicates the electrical switch of the stimulation device (Stimulus Controler CS 55, Syntech, Hilversum, The Netherlands). The stimuli were presented in a random order separated by inter-stimulus-intervals of at least 10 s. For each neuron, the number of stimuli that could be tested varied depending on the duration of the recording. If possible, each stimulus was tested more than once to verify the reproducibility of the responses as described in Masante-Roca et al. (2002).

Six components present in grapevine berries [*EE*-α farnesene, (*E*)-2-hexenal, (+/–)-linalool, 1-hexanol, 4, 8-dimethyl-1, 3, (*E*)7,-nonatriene (nonatriene)] (Schreier et al., 1976), and in the non-host plant tansy (β-thujone) (Gabel et al., 1992) were used. The individual components were obtained from Bedoukian, Danbury, CT, USA (α-farnesene), Sigma [(*E*)-2-hexenal, (+/–)-linalool, 1-hexanol], W. Francke, University of Hamburg, Germany, *via* the Swedish Agricultural University, Alnarp, Sweden (nonatriene), and from the Pharmaceutical Faculty, Bratislava, Slovakia

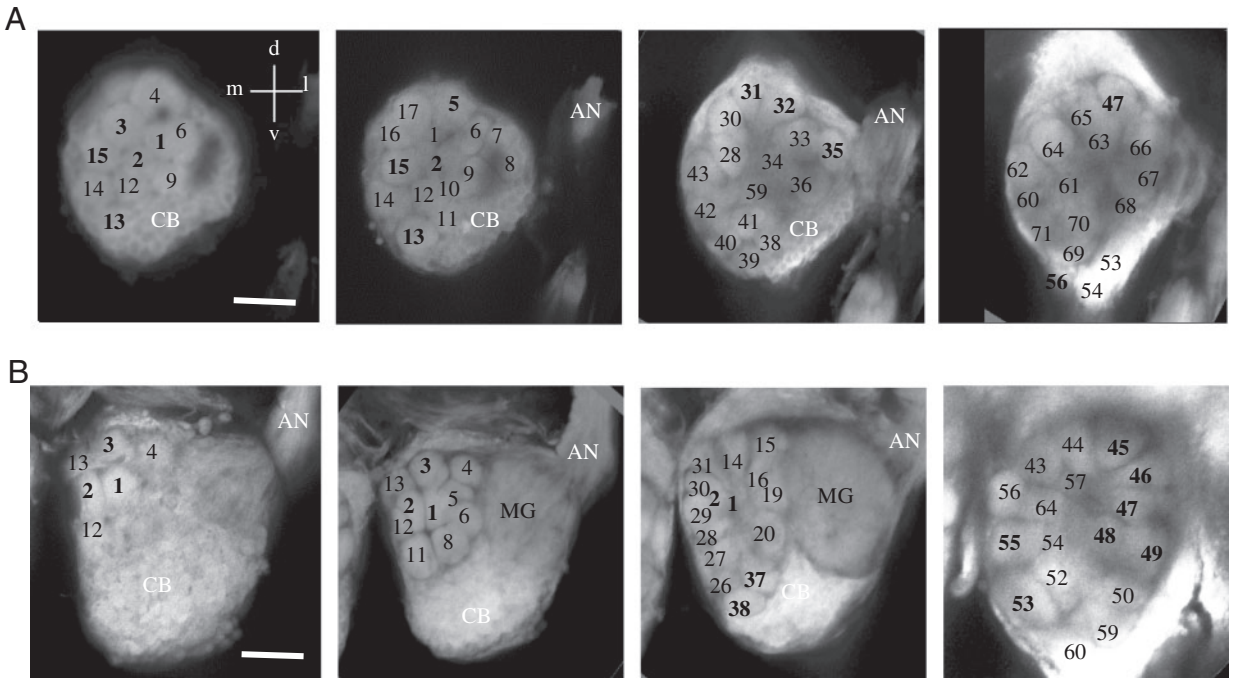


Fig. 1. Series of frontal confocal sections through the antennal lobe of a *Lobesia botrana* female (A) and male (B) from anterior (left) to posterior (right) (see also reconstruction in Figs 2–5), AN antennal nerve, CB cell body cluster, d dorsal, l lateral, m medial, MG macroglomerulus, v, ventral, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Note that especially posterior glomeruli are difficult to identify. Scale bar in A and B 30 μm.

(β-thujone). The compounds were diluted in hexane. To determine the response threshold of each recorded neuron, four different stimulus dilutions were used (0.01 μg μl^{-1}, 0.1 μg μl^{-1}, 1 μg μl^{-1}, 10 μg μl^{-1}). 10 μl of each respective dilution were applied on a piece of filter paper in a Pasteur pipette. A Pasteur pipette containing the solvent (hexane) was used as a blank stimulus.

Data analysis

The delay, the duration and patterns of excitatory and inhibitory parts of the responses were analysed manually in detail. Responses were quantified as described previously (Gadenne and Anton, 2000). Briefly, the net number of spikes (number of spikes during a 600 ms period after the stimulus minus the number of spikes counted during the preceding 600 ms representing spontaneous activity) produced in response to the blank stimulus was subtracted from the net number of spikes produced in response to an odour stimulus to quantify the response to a specific stimulus. A neuron was classified as responding to a stimulus when the odour response exceeded the blank response by at least 10%.

Histology and confocal microscopy

To investigate the neuroanatomical organization of the *L. botrana* AL, male and female brains were dissected and fixed in glutaraldehyde, washed in buffer, dehydrated and embedded in Fluoromount (Sigma). Optical sections were taken with a confocal microscope (Leica TCS NT; Leica Sollentuna, Sweden) and analysed on a Silicon Graphics computer work station using Imaris 2.7 Software (Bitplane, Zürich, Switzerland).

To localise the stained individual neurons after physiological recordings, standard staining methods were used (Anton and Hansson, 1994). The neurons were stained with 4% LY by passing ~0.5 nA of constant hyperpolarizing current through the LY-filled recording electrode for at least 5 min. After the recording, the brains were dissected, fixed in a buffered formaldehyde solution, rinsed in buffer, and examined as whole mounts in Vectashield mounting medium (Vector laboratories, Burlingame, CA, USA) to be observed in a laser scanning confocal microscope (Leica TCS SP2, Leica Rueil Malmaison, France), equipped with a krypton/argon laser. For overview scanning of the whole brain and for the detailed scanning of each AL, a Leica 20×0.7 dry objective was used. For the overview, the whole brain was scanned frontally with 2 μm step size whereas the step size was 1 μm for the detailed scanning of each AL. No additional staining was used, as auto-fluorescence made the glomerular outlines visible. For the drawing of the local interneuron (LN), a stack containing the completely stained neuron was printed out and redrawn manually using transparency paper as described in Masante-Roca et al. (2002).

Three-dimensional reconstruction

The right and left ALs of three different animals were entirely mapped manually tracing the outline of each individual glomerulus in every optical section using the Imaris 2.7 software, installed on a Silicon Graphics computer. The *z*-axis

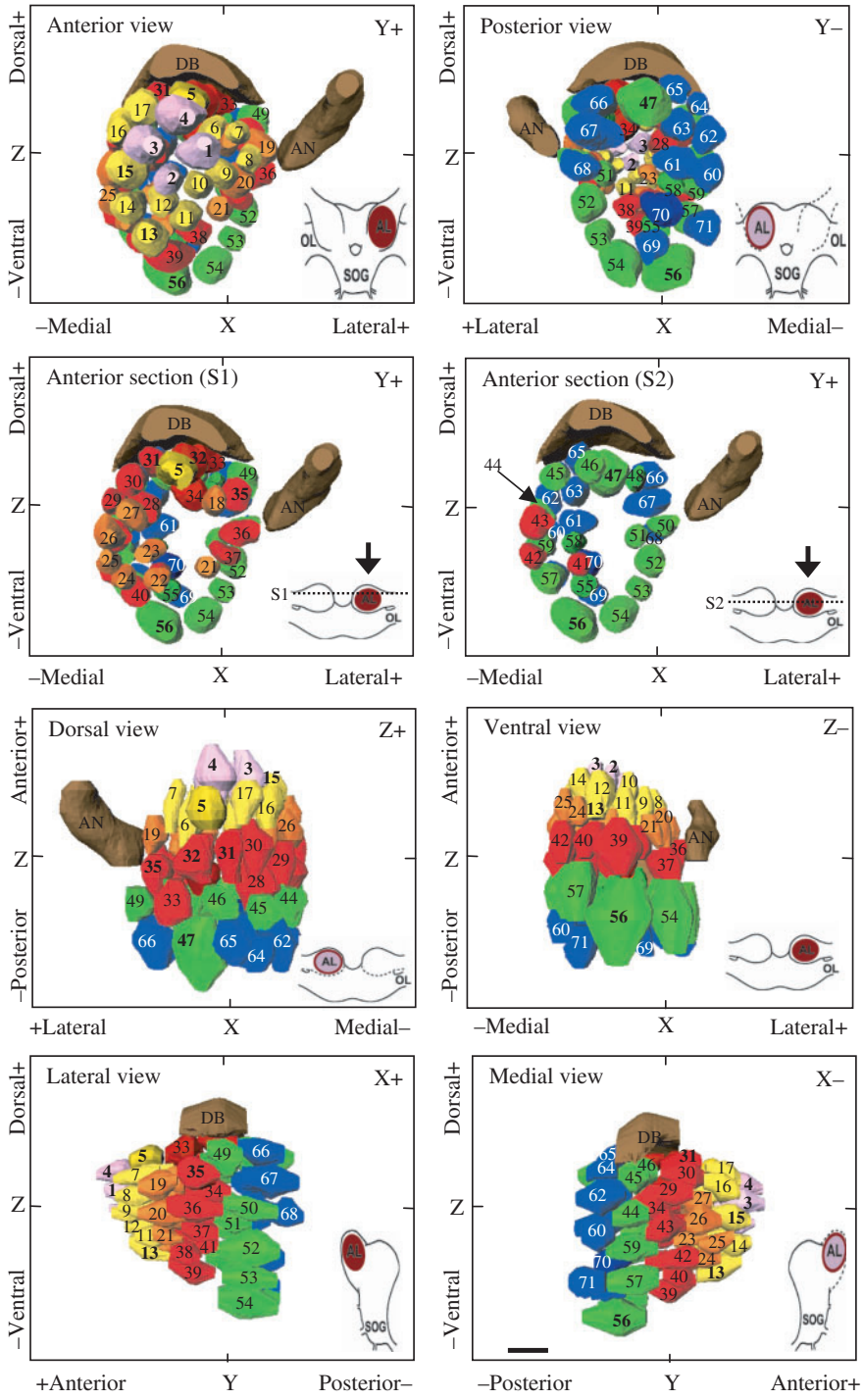


Fig. 2. Three-dimensional reconstruction of the left antennal lobe of a female *Lobesia botrana* (same brain as in Fig. 1A). Graphs in the first row show the projection onto the frontal orientation plane Y. Graphs in the second row show levels S1 and S2 in the Y+ orientation plane. Graphs in the third row show the projection onto the horizontal orientation plane Z. Graphs in the fourth row show the projection onto the sagittal orientation plane X. Brain sketches show the orientation of the brain and the relative position of the AL (marked red) for each view. In the lateral view, the AN reconstruction was omitted and in the dorsal view the reconstruction of the dorsal border of the AL was omitted to visualize the glomeruli hidden otherwise. AN antennal nerve, DB dorsal border of the AL, OL optic lobe, SOG suboesophageal ganglion, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Scale bar 20 µm.

dimension (thickness of sections) had to be corrected by a factor 1.6 because of the refractive index mismatch caused by air objectives. To facilitate usage of the 3D map, each identified glomerulus was numbered, from the most anterior to the most posterior. In addition, different colours were given to glomeruli at different anteroposterior levels (from pink to blue). The dorsal border of the AL, the anterior–ventral cell body cluster (not drawn to avoid hidden glomeruli), and the antennal nerve (AN), were used as additional references. The position and shape of all glomeruli within the complete maps of the three brains were compared on the computer screen by simultaneously three-dimensionally rotating the different reconstructions and by comparing sections at different levels throughout the AL. In addition to the MGC for males, the AN, the cell body cluster, and the dorsal border of the AL (the tissue forming the dorsal outline of the AL outside the glomeruli; DB), the three or four most anterior glomeruli and glomeruli at 'strategic' positions were localized in each AL reconstruction (male and female) and consequently served as landmark glomeruli. As next step, those glomeruli neighbouring the previously identified landmark glomeruli were matched. Finally also those glomeruli showing variations in shape, size or position could be matched due to their neighbouring positions. In the three investigated brains in males and in females, most glomeruli could be recognised by following this 3D matching process despite local variations.

To identify glomeruli containing dendritic arborisations of physiologically characterised PNs, ALs were partially reconstructed. These partial reconstructions were compared with the established 3D map using the same matching process as described above. All 3D reconstructions are presented in spatial two-dimensional graphs. The orientation planes are the sagittal plane X (perpendicular to *x*-axis, X+ as seen from lateral side, X– from medial side), frontal plane Y (perpendicular to *y*-axis, Y+ as seen from anterior side, Y– from posterior side) and horizontal plane Z (perpendicular to *z*-axis, Z+ as seen from dorsal side, Z– from ventral side). In addition, sections at the frontal plane were

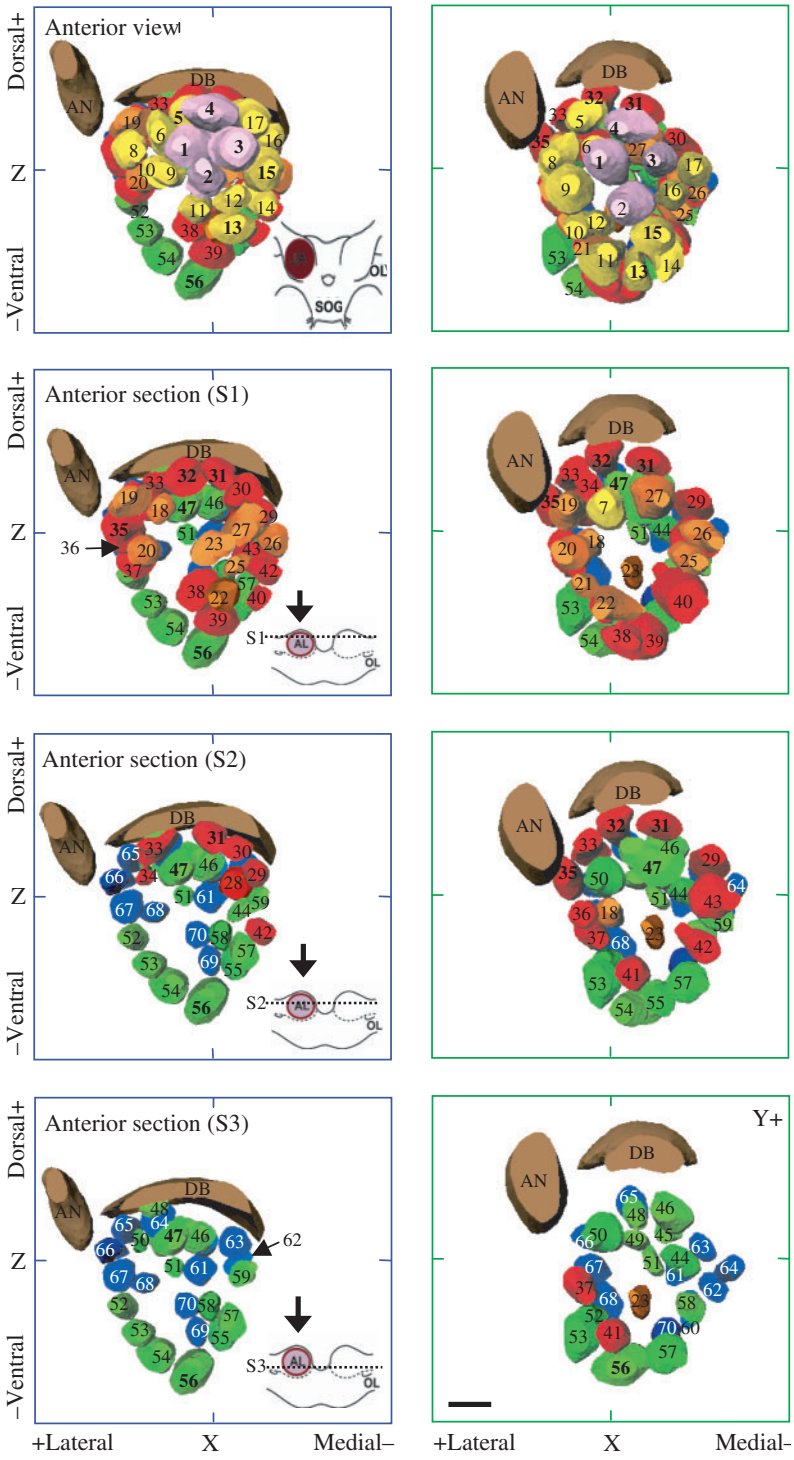


Fig. 3. Three-dimensional reconstruction of the right antennal lobes in two female *Lobesia botrana* individuals. Each vertical column marked with frames of the same colour corresponds to one brain, AL on the left is from the same brain as AL in figure 2. Graphs show the projection onto the frontal orientation plane Y+ viewed from the anterior side. Different sections through the reconstruction of the left lobe are shown to visualize the differently coloured layers of glomeruli. The anterior view shows mainly pink and yellow glomeruli, section S1 shows orange and some red glomeruli, section S2 the remaining red and most green glomeruli and section S3 shows the remaining green and blue glomeruli. Brain sketches indicate the orientation of the brain, the relative position of the AL (marked red) and the level of the sections (dotted lines). AN antennal nerve, DB dorsal border of the AL, OL optic lobe, SOG suboesophageal ganglion, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Scale bar 20 µm.

prepared by taking away the upper layers of glomeruli to visualize central glomeruli within the AL. Sketches of the moth brain showing sagittal, frontal and horizontal views, respectively, serve as additional orientation help.

Results

Three-dimensional atlas of the AL

In males and females, ordinary glomeruli arranged in several layers around a small central fibre core were identified in the left and right AL. In three females between 60 and 71 ordinary glomeruli were found (Figs 1A, 2, 3). The same number of glomeruli was found in the ALs on both sides for one individual (62), whereas two additional glomeruli were found on one side compared to the other in one female (60 and 62 glomeruli). In the third female, 66 and 71 glomeruli were found on the two sides respectively. In three male individuals, between 60 and 64 ordinary glomeruli and one enlarged glomerulus situated close to the entrance of the antennal nerve (identified anatomically as macroglomerular structure, MG) were found (Figs 1B, 4, 5). The number-code, with low numbers most anteriorly and high numbers most posteriorly serves as orientation guide for the reconstructions and the following use of the atlas. The colour code facilitates the depth perception of glomerular layers. Whereas glomeruli in pink, yellow, orange and red layers could be clearly identified between individual ALs, those situated most posteriorly (green and blue layers) were difficult to see and to match between different brains. Among the ordinary glomeruli, in both sexes, generally, the anterior glomeruli seem to be more densely packed than the posterior glomeruli. Most glomeruli are small, with a volume ranging from 2000 to 5000 µm^3. As they do not vary much in size, and as differences between male and female ALs were fairly big, we did not attempt to match ordinary glomeruli between sexes. We did, however, use the same numbering and colouring system progressing from anterior to posterior. Glomeruli of particular size and position were used as landmark glomeruli and are marked with bold numbers in the figures. Volume measurements were done on the AL illustrated in Figs 1 and 2 for females and on the AL illustrated in Figs 4 and 5 in males.

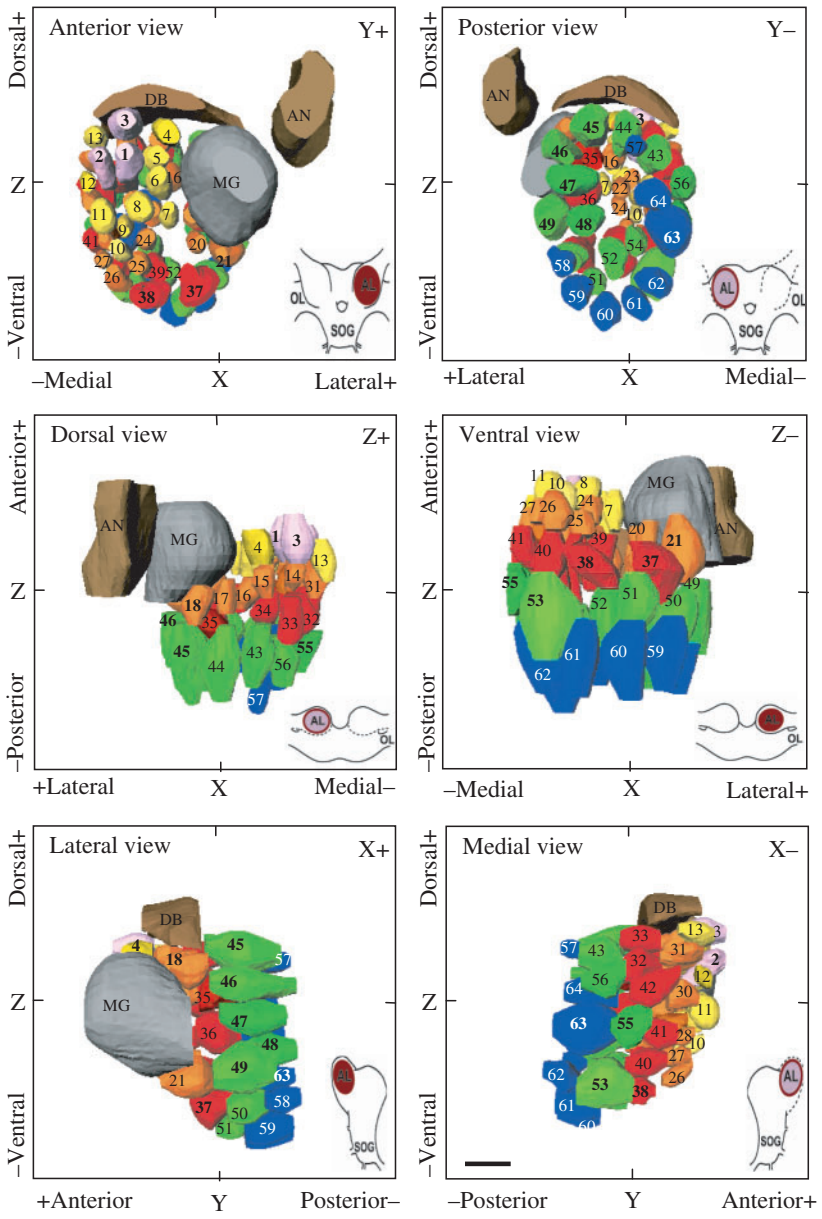


Fig. 4. Three-dimensional reconstruction of the left antennal lobe of a male *Lobesia botrana* in six different views (same brain as in Fig. 1B). Graphs in the first row show the projection onto the frontal orientation plane Y. Graphs in the second row show the projection onto the horizontal orientation plane Z. Graphs in the third row show the projection onto the sagittal orientation plane X. Brain sketches show the orientation of the brain and the relative position of the AL (marked red) for each view. In the lateral view, the AN reconstruction was omitted and in the dorsal view the reconstruction of the dorsal border of the AL was omitted to visualize the glomeruli hidden otherwise. AN antennal nerve, DB dorsal border of the AL, MG macroglomerulus, OL optic lobe, SOG suboesophageal ganglion, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Scale bar 30 µm.

In females, the most anterior, pink glomeruli (1–4) are relatively big (around 6000 µm^3) and very characteristic in their positions (Figs 2, 3). The next, yellow layer (5–17) is characterised by mostly very small glomeruli (2000 to 3500 µm^3) and somewhat bigger landmark glomeruli at a dorsal (5, 7600 µm^3), ventral (13, 4700 µm^3), and medial (15, 6000 µm^3) position. The orange layer (18–27) contains small glomeruli (between 3000 and 5000 µm^3) which can be best identified by their relative position between the yellow layer and the red layer (28–43), which itself contains mainly bigger glomeruli (3000 to 7000 µm^3) which partially can be easily identified due to their close position to the dorsal border of the AL (31–32) and the AN (35) (Figs 1–3). Within the green layer (44–59), glomeruli 47 and 56 serve as landmarks with their big size (6000 to 12 000 µm^3) and dorsal/ventral position respectively. Most of the glomeruli in the blue layer (61–71) are rather small (around 5000 µm^3) and difficult to identify, when comparing different brains. In the two female brains investigated, which had fewer glomeruli than the brain used as a map, 'missing' glomeruli belong to the orange, red, green and blue layers (Figs 1–3).

In males, the most obvious structure in the AL is the potential macroglomerulus (MG) (90 000 µm^3) situated at the entrance of the AN (Figs 1, 4, 5). The most anterior, pink layer of ordinary glomeruli consists only of three very small glomeruli (1–3, between 1500 and 3000 µm^3). Glomeruli in the yellow layer (4–13) are relatively small and arranged around the first three glomeruli (around 6000 µm^3). The orange layer (14–31) is characterised by many small (1500–3000 µm^3) and two bigger glomeruli posteromedially of the macroglomerulus (18, 21) (respectively 5000 and 4000 µm^3). The red layer (32–42) consists of a circle of glomeruli of different sizes. Glomerulus 38 is situated most ventrally and the neighbouring 37 (14,000 µm^3) is bigger than the other glomeruli in the same layer. Glomeruli in the green layer (43–56) are relatively big (between 5000 and 20,000 µm^3) and glomeruli 45–49 can be identified by their position close to the AN and posteriorly to the MG. The small glomerulus 55 (5000 µm^3) and the big glomerulus 53 (15,000 µm^3) are situated most medially. The glomeruli of the blue layer (57–63) form an open circle excluding the lateral side of the AL (Figs 1, 4, 5). The medial glomerulus 63 (14,000 µm^3) is bigger than the other glomeruli of that layer.

General physiology of AL neurons

In a total of 15 preparations containing stained neurons with arborisations in the AL, responses to at least one of the six tested plant odour components were found. Neuron responses were either excitatory (Fig. 6A), inhibitory or combined an initial excitation with a following inhibitory phase. Excitatory responses in the same neuron had different

delays after the onset of stimulation in some cases (Fig. 6A). Action potential amplitudes ranged from 10 to 40 mV. Nine PNs and one LN responded to only one of the six tested components, three PNs responded to two components at the same threshold, one PN responded to nonatriene at a lower threshold than to β-thujone and one PN responded to all six tested components (Table 1). Only the generalist neuron responded to E-2 hexenal, none of the specialist neurons was found to respond to this component. Most neurons had a response threshold at 0.1 μg of the applied odours. For hexanol, α-farnesene and β-thujone, a few neurons had a threshold of 1 μg and one neuron responded at a threshold of 10 μg to β-thujone (Table 1).

Anatomy of physiologically characterised AL neurons

Projection neurons

The cell bodies of the uniglomerular PNs were situated in a large anterior-ventral cell cluster (see Masante-Roca et al., 2002). The primary neurite leads to the innervated glomerulus and fine dendritic branches were observed throughout the entire glomerulus (Fig. 6B). The axon was in all cases only stained within the antennal lobe and therefore, projection areas within the protocerebrum could not be identified. In two preparations, two PNs (7a, b; 12 a, b) had been stained simultaneously and in these cases we identified both target glomeruli (glomeruli 1 and 9; 19 and 8, respectively) (Table 1).

PN 14 arborised in glomerulus 37 and responded to all tested odours (Fig. 6B). PNs responding specifically to a single compound arborised in clearly different glomeruli in different cases (Fig. 7A,B). Arborisations in the same, clearly identified glomerulus were found for neurons that responded to the same component (α-farnesene for neurons 1 and 2; β-thujone for 2 and 3; β-thujone for 4 and 5), but in all these cases, at least one of the neurons in each pair responded additionally to another component (Table 1). Two PNs responding specifically to different components arborised in the same, clearly identified glomerulus (Fig. 7C).

All glomeruli containing dendritic branches of PNs were situated in the anterior part of the AL. Penetrating with the electrode into posterior layers of the AL resulted in contacts with neurons which did not respond to the tested stimuli. None of the glomeruli in the green and blue layers received branches of a stained PN (Table 1). Only one specifically responding PN (neuron 13) and the generalist PN (neuron 14) arborised in glomeruli of the red layer (Table 1, Figs 6, 8). The vast majority of the stained PNs arborised in glomeruli of the pink and yellow layers (Table 1, Figs 7, 8).

Glomerulus 1 is involved in the processing of all tested components except E2-hexenal (Fig. 8). Glomeruli containing arborisations from PNs responding to α-farnesene occupy a relatively large S-shaped zone extending from the antero-dorsal to the posterocentral part of the AL (Fig. 8). Neurons responding to nonatriene arborise in a more posterolateral group of glomeruli, which does not overlap with the zone

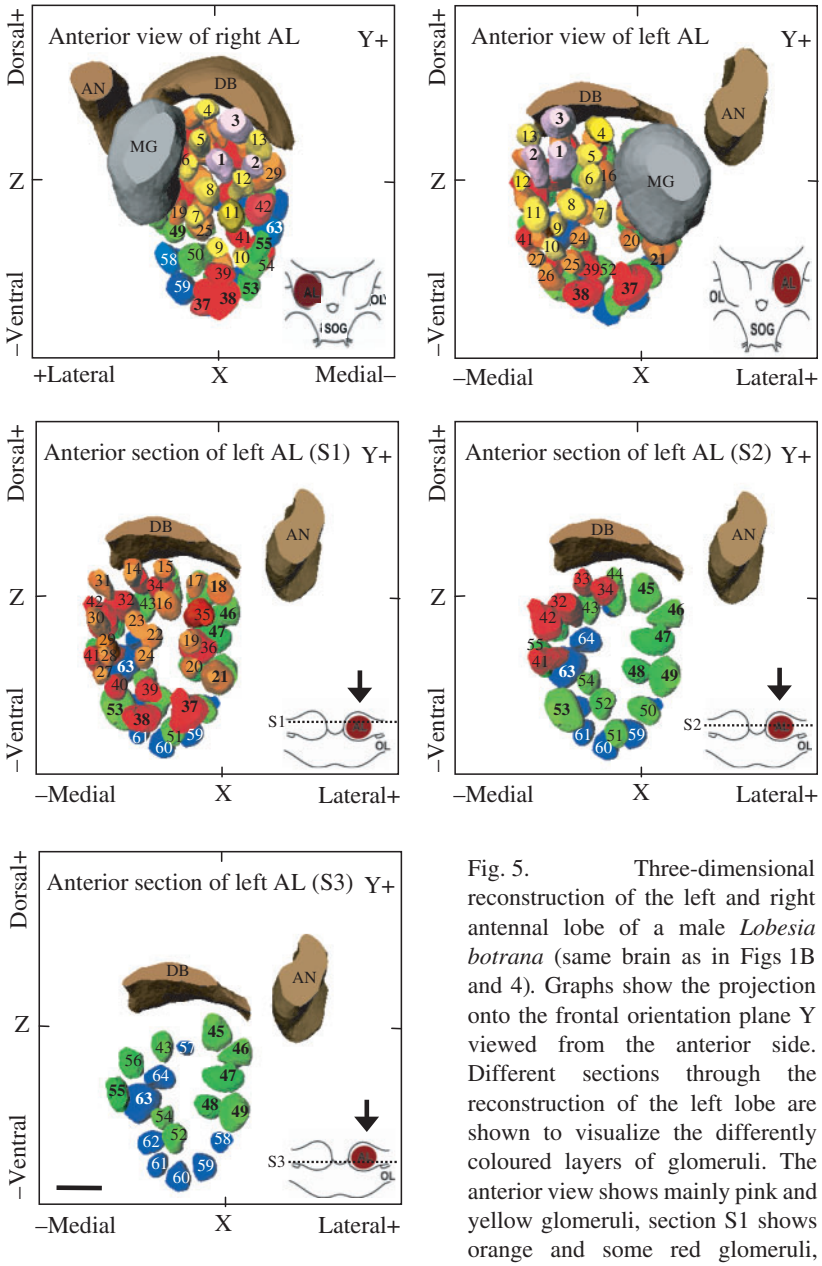


Fig. 5. Three-dimensional reconstruction of the left and right antennal lobe of a male *Lobesia botrana* (same brain as in Figs 1B and 4). Graphs show the projection onto the frontal orientation plane Y viewed from the anterior side. Different sections through the reconstruction of the left lobe are shown to visualize the differently coloured layers of glomeruli. The anterior view shows mainly pink and yellow glomeruli, section S1 shows orange and some red glomeruli, section S2 the remaining red and most green glomeruli and section S3 shows the remaining green and blue glomeruli. Brain sketches indicate the orientation of the brain, the relative position of the AL (marked red) and the level of the sections (dotted lines). AN antennal nerve, DB dorsal border of the AL, MG macroglomerulus, OL optic lobe, SOG suboesophageal ganglion, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Scale bar 30 μm.

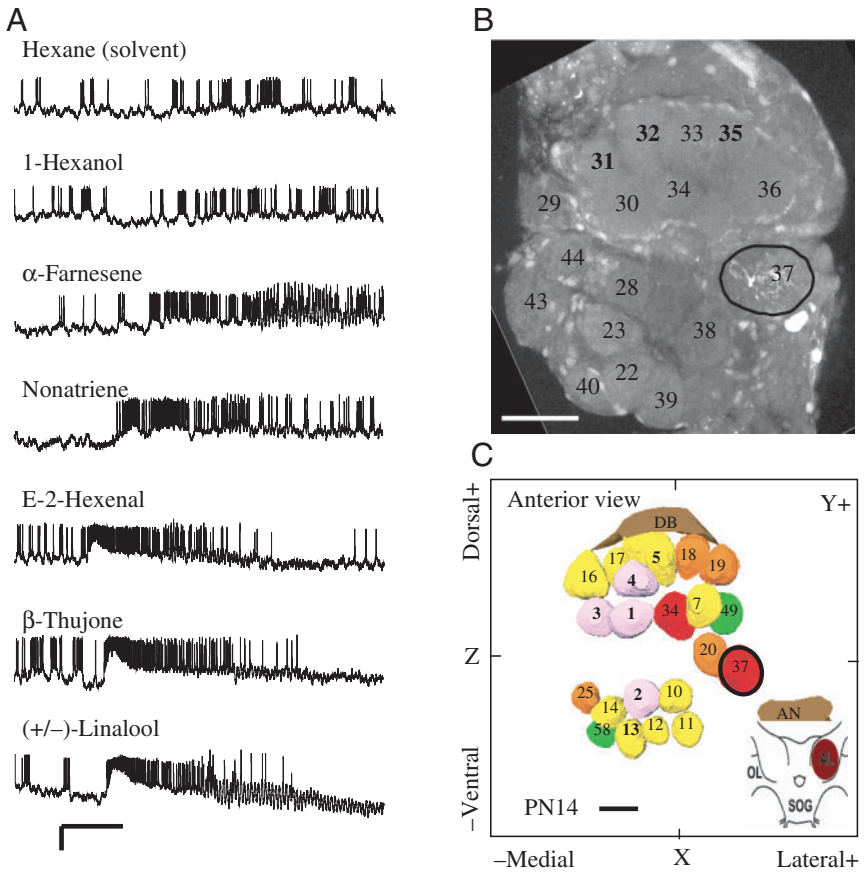


Fig. 6. Physiological and anatomical characteristics of broadly responding PNs in *Lobesia botrana*. (A) Example for a PN with excitatory responses observed for all tested compounds at 1 µg. Note the different delays in response to different compounds. This neuron is not included in the data set in Table 1, because the AL was damaged and the target glomerulus could not be identified. (B) Extended focus image of a stack of confocal sections through the intracellularly stained dendritic arborisations of the generalist PN 14 in glomerulus 37 (black outline). (C) Partial three-dimensional reconstruction of the AL containing the stained PN 14 in the anterior view Y+, indicating the innervated glomerulus 37 (black outline). Brain sketch indicates the orientation of the brain and the relative position of the AL (marked red). AN antennal nerve, DB dorsal border of the AL, OL optic lobe, SOG suboesophageal ganglion, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Horizontal scale bar in A, 500 ms, vertical scale bar in A, 40 mV. Scale bar in B 30 µm. Scale bar in C 30 µm.

occupied by the dendritic branches of α–farnesene responding PNs (Fig. 8). Four out of five PNs responding to β-thujone have overlapping dendritic arborisations with α-farnesene responding neurons in glomeruli 1 and 3 apart from one neuron targeting glomerulus 18 (Fig. 8).

Local interneuron

The stained LN arborised in all glomeruli of the AL. The type of arborisations varied, however, in different glomeruli (Fig. 9). One glomerulus contained very dense, fine branches (36), whereas all other glomeruli received sparse arborisations with bleb-like terminal specialisations. This neuron responded exclusively to α-farnesene.

Table 1. *Physiology and anatomical characteristics of the antennal lobe neurons studied in* L. botrana

Neuron number	(+/–)-Linalool	β-Thujone	α-Farnesene	E-2-Hexenal	1-Hexanol	Nonatriene	Neuron type	Glomeruli
1	NR	NR	1 µg	NR	NR	NR	PN	3
2	NR	1 µg	1 µg	NR	NR	NR	PN	3
3	NR	10 µg	NR	NR	NR	NR	PN	3
4	NR	1 µg	NR	NR	NR	0.1 µg	PN	1
5	0.1 µg	0.1 µg	NR	NR	NR	NR	PN	1
6	NR	0.1 µg	NR	NR	NR	NR	PN	18
7a,b	NR	NR	1 µg	NR	1 µg	NR	PN	1 and 9
8	NR	NR	0.1 µg	NR	NR	NR	PN	5
9	NR	NR	0.1 µg	NR	NR	NR	PN	9
10	NR	NR	0.1 µg	NR	NR	NR	PN	20
11	NR	NR	0.1 µg	NR	NR	NR	PN	21
12a,b	NR	NR	NR	NR	NR	0.1 µg	PN	19 and 8
13	NR	NR	NR	NR	NR	0.1 µg	PN	35
14	0.1 µg	0.1 µg	0.1 µg	0.1 µg	0.1 µg	0.1 µg	PN	37
15	NR	NR	0.1 µg	NR	NR	NR	LN	36

PN, projection neuron; LN, local interneuron; NR, no response. Threshold doses are given in response to each plant compound.

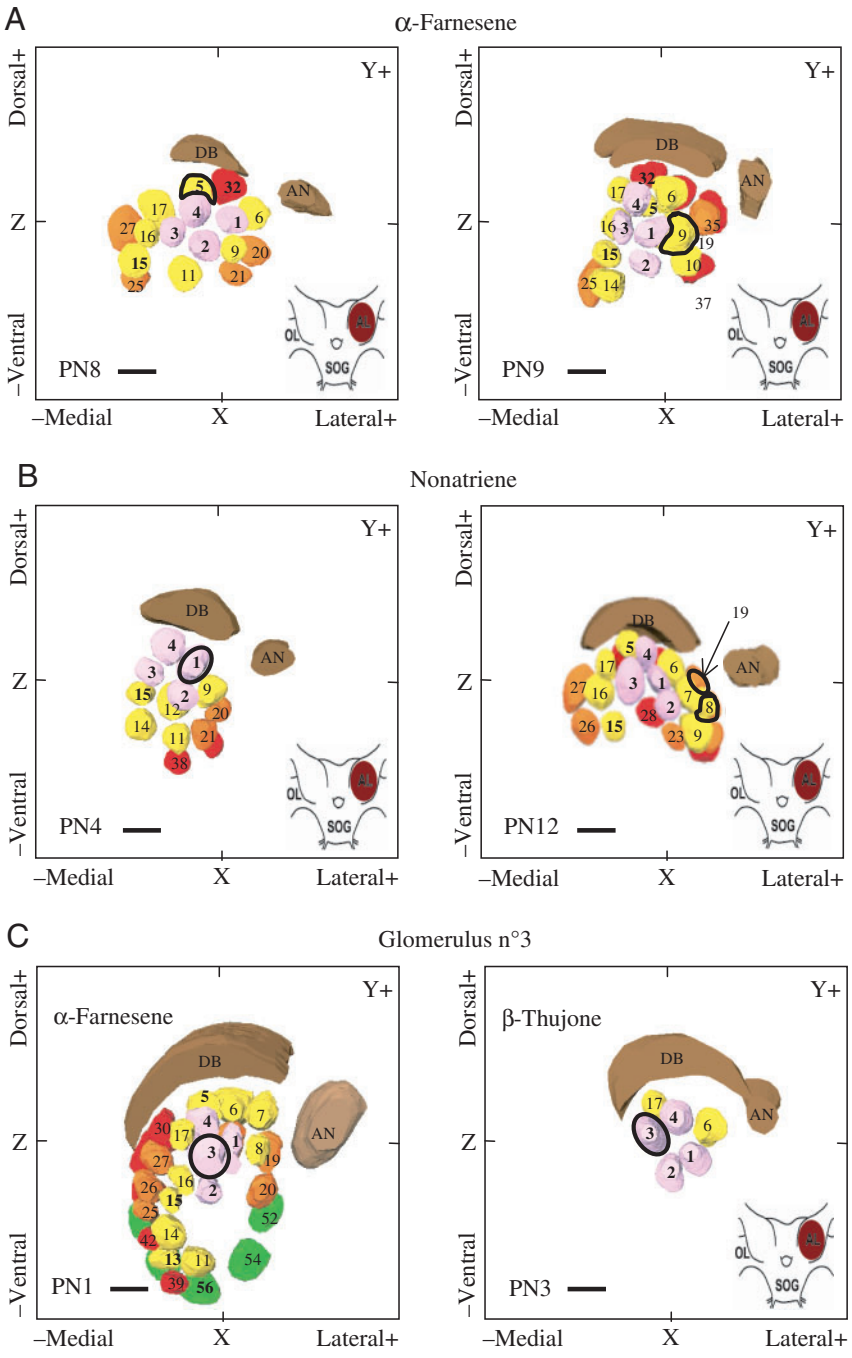


Fig. 7. Partial three-dimensional reconstructions of the ALs containing stained PNs in *Lobesia botrana* in the anterior view Y+, indicating the innervated glomeruli (black outline). (A) Two PNs responding exclusively to α-farnesene arborised in two clearly different glomeruli (9 and 5). (B) In two preparations with PNs responding to nonatriene at a lower threshold (PN4) or exclusively to nonatriene (PN12), one PN (PN4) arborised in glomerulus 1, and in the other preparation (PNs 12 a, b) a double staining was found (8, 19). (C) Two PNs arborising in the easily identifiable glomerulus 3, responded to α-farnesene and β-thujone respectively. Brain sketches indicate the orientation of the brain and the relative position of the AL (marked red). AN antennal nerve, DB dorsal border of the AL, OL optic lobe, SOG suboesophageal ganglion, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Scale bar in each reconstruction 20 μm.

Discussion

The presented map of the AL glomeruli of the tortricid moth, *L. botrana*, is used as a tool to identify single glomeruli housing dendritic arborisations of AL output neurons, which have been characterised physiologically. The structure of the AL of this small tortricid moth was found to ressemble the glomerular organisation of other moth species, with a number of glomeruli in the same range as found in noctuid or sphingid species before (Rospars, 1983; Rospars and Hildebrand, 1992, 2000; Sadek et al., 2002; Berg et al., 2002; Greiner et al., 2004). The arrangement of the glomeruli in several layers around a small fibre core is, however, different from the single layer around a larger fibre core in most other species (see references above). The number of AL glomeruli ranged from 60 to 71 glomeruli in our three female brains and from 60 to 64 ordinary glomeruli in the male brains studied, but as the very posterior glomeruli were difficult to clearly identify for both sexes, this indication has to be taken with precaution. The reconstruction of the female AL with the highest number of identified glomeruli was shown in more detail, because glomeruli could be seen more clearly in that preparation than in others. Lower numbers of glomeruli in other preparations can be due to failure to identify the most posteriorly situated glomeruli individually because of rather uncertain boundaries between them. With the large number of closely situated small glomeruli a comparison between male and female ALs becomes a very difficult task. Quantitative analysis methods would be needed to achieve a meaningful solution of this problem, which was not within the scope of our study. Little variations in glomeruli numbers between individuals were also found in the noctuid moths *Helicoverpa assulta* and *Heliothis virescens* AL (Berg et al., 2002), and in the parasitic wasps *Cotesia glomerata* and *C. rubecula* (Smid et al., 2003). The number of glomeruli seems to be rather more dependent on the insect taxa than on the size of the insect. The parasitic wasps, *C. glomerata* and *C. rubecula*, have a high number of glomeruli, like other hymenoptera, e.g. the honey bee, which are arranged in clusters (Smid et al., 2003; Galizia et al., 1999). *D. melanogaster* and mosquitoes conversely have somewhat fewer glomeruli (Laissue et al., 1999; S. Anton, personal observation). Although the brain size of *L. botrana* is much smaller than that of other Lepidoptera studied so far, the number of glomeruli found is close to the number of glomeruli

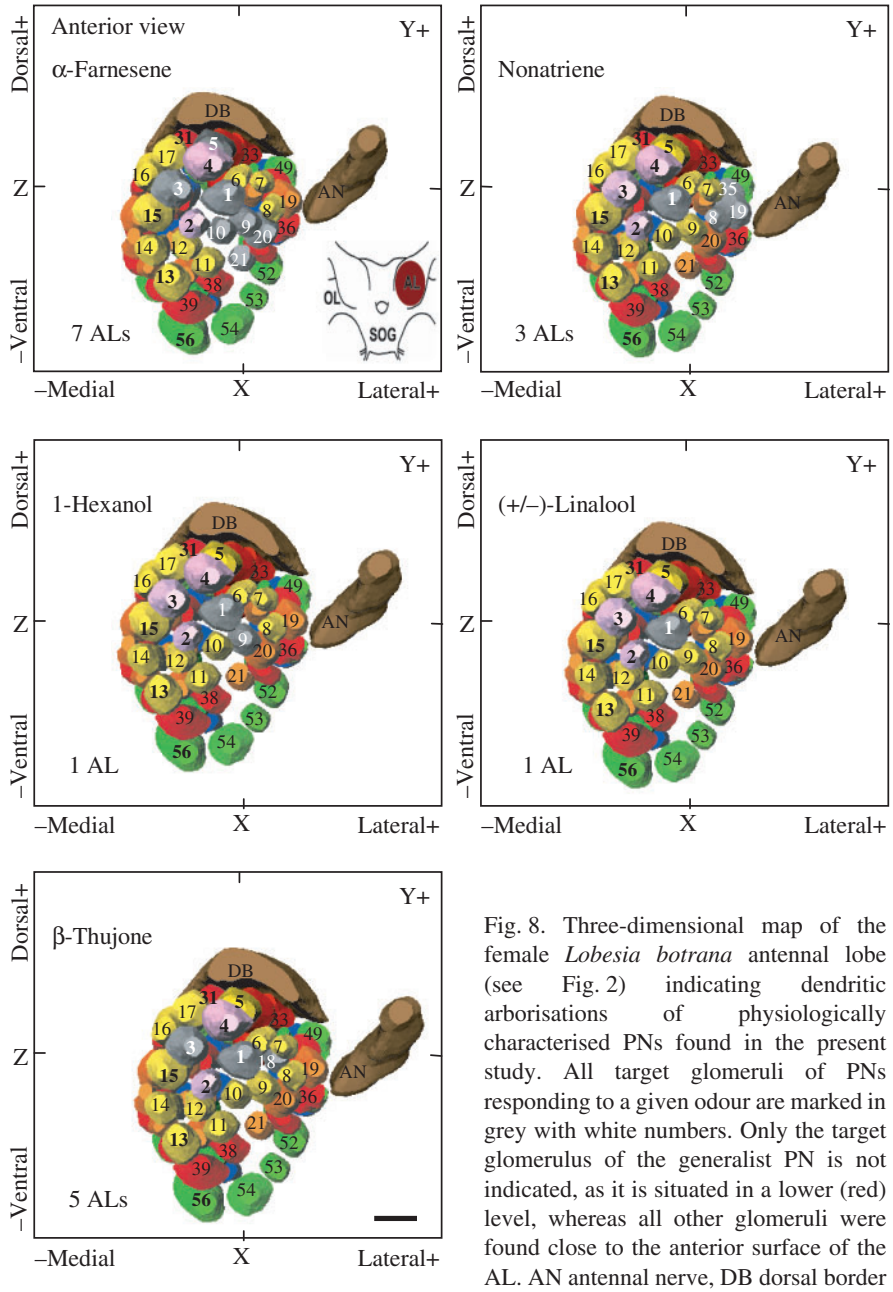


Fig. 8. Three-dimensional map of the female *Lobesia botrana* antennal lobe (see Fig. 2) indicating dendritic arborisations of physiologically characterised PNs found in the present study. All target glomeruli of PNs responding to a given odour are marked in grey with white numbers. Only the target glomerulus of the generalist PN is not indicated, as it is situated in a lower (red) level, whereas all other glomeruli were found close to the anterior surface of the AL. AN antennal nerve, DB dorsal border of the AL, OL optic lobe, SOG suboesophageal ganglion, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Scale bar 20 μm.

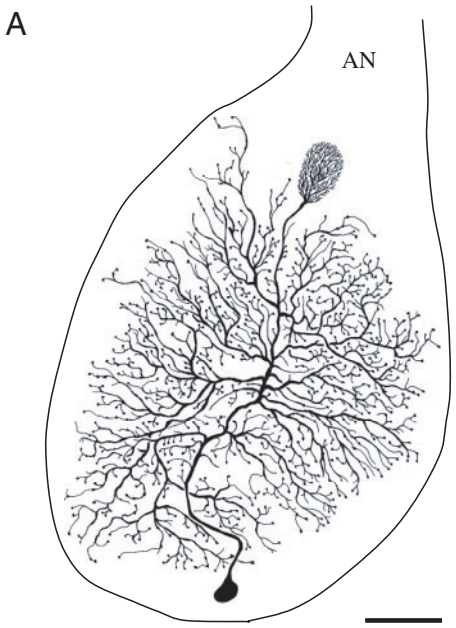
found in all other Lepidoptera species examined so far (Rospars, 1983; Rospars and Hildebrand, 2000; Sadek et al., 2002; Berg et al., 2002; Greiner et al., 2004). Thus, the number of glomeruli in Lepidoptera seems to be rather constant, independently of the size of the animals.

In Lepidoptera, the number of macroglomerular structures is thought to be correlated with the number of behaviourally active components forming the pheromone blend in the respective species (Hansson et al., 1992; Ochieng' et al., 1995; Todd et al., 1995; Berg et al., 1998). In *L. botrana* males, we found one large glomerulus close to the entrance of the antennal nerve. In this species, although one single pheromone component (E7,Z9–12:Ac) has first been identified as attractant for males (Roelofs et al., 1973; Buser et al., 1974), a blend of three compounds has more recently been found to be much more attractive (El Sayed et al., 1999). Therefore, we cannot exclude that other glomeruli are part of a more complex macroglomerular structure, which could only be identified using a combined physiological and anatomical approach.

The response characteristics of neurons were similar to those of neurons studied earlier in *L. botrana* (Masante-Roca et al., 2002). The thresholds of AL neurons for the tested plant compounds are in the same range or lower as those found earlier in the same species and in other species for both peripheral receptor neurons and AL neurons (Anderson et al., 1995; Anton and Hansson, 1994, 1995; King et al., 2000; Masante-Roca et al., 2002; Sadek et al., 2002; Greiner et al., 2002). Larger specificity of the AL neurons found in the present investigation compared to an earlier study (Masante-Roca et al., 2002) is perhaps partially due to the more limited number of compounds tested and to the introduction of lower stimulus concentrations. Conversely, particularly behaviourally active components have been chosen, which might be represented by more specific neurons compared with more generally occurring plant compounds. The specificity of the investigated neurons enables us to test the hypothesis of odour representation in specific glomeruli at the output level more easily than in a previous studies where most output neurons had rather broad response spectra (Sadek et al., 2002).

A number of methodological problems have to be taken into consideration when interpreting our results. The identification of glomeruli in *L. botrana* is not unambiguous, because most glomeruli are small, tightly packed and many have a similar size. Glomeruli in the anterior layers of the AL could, however, be much more easily identified than glomeruli in the posterior part of the AL.

The LN with different types of arborisations in different glomeruli might give a hint on the functional role of this neuron type. Such multiglomerular LNs with dense arborisations in one single glomerulus and diffuse branching in the other AL glomeruli were found in different insects. In moths, this type of neuron has been found very rarely (Christensen et al., 1993). In honeybees this neuron type is commonly found (Fonta et al.,



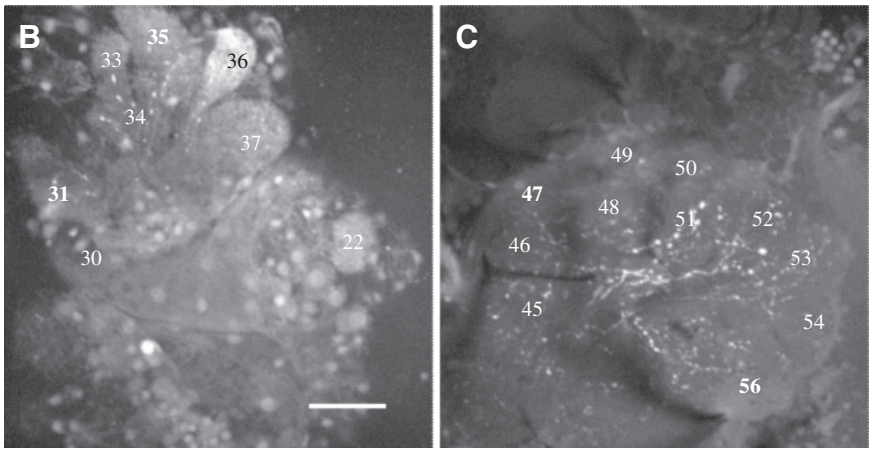


Fig. 9. Anatomy of a local interneuron, which responded only to α-farnesene. (A) The reconstruction of the entire neuron in an approximately frontal view shows that arborisations are much more dense in one glomerulus (36) than in the remaining glomeruli (see also B). (B) Single approximately frontal confocal section showing dense arborisations in glomerulus 36. (C) Single approximately frontal confocal section through the central part of the AL showing sparse, branching with varicosities in many glomeruli. AN antennal nerve, numbers indicate ordinary glomeruli, bold numbers indicate landmark glomeruli. Scale bars 30 μm.

1993; Sun et al., 1993; Galizia and Kimmerle, 2004) and from the detailed analysis of the arborisations, the authors conclude that the sparse arborisations are output areas (varicosities on branches) and that the densely innervated single glomerulus comprises input and output regions, characterised by smooth and varicose branches of the LN. In the beetle, *Pachnoda marginata*, LNs with the same features were found (Larsson, 2001). The dense and very fine arborisations in one single glomerulus in *L. botrana* could mean that the neuron receives mainly input in this glomerulus. The bleb-like varicosities on the other branches of the LN, which innervate most if not all other glomeruli might indicate that these are sites of information output. This type of LN might therefore be involved in distributing specific information widely over the AL, e.g. to sharpen a specific signal by lateral inhibition.

The representation patterns of odour components at the input and output level of AL glomeruli have only been started to be investigated. In *D. melanogaster*, the number of glomeruli is approximately the same as the number of olfactory receptor molecules and, apart from a few exceptions, each membrane receptor is expressed in only one or two glomeruli (Gao et al., 2000; Vosshall, 2000; Bhalerao et al., 2003). Olfactory receptor molecules, however, vary widely in their breadth of tuning (Hallem et al., 2004). Studies on a number of moth species (Galizia et al., 2000; Carlsson et al., 2002; Carlsson and Hansson, 2003; Meijerink et al., 2003; Hansson et al., 2003; Skiri et al., 2004) and on honeybees (Joerges et al., 1997; Galizia and Menzel, 2000, 2001), using optical imaging techniques, suggest that ORNs responding to a specific plant compound are represented in one or few identifiable glomeruli. Our data on AL output neurons in *L. botrana* support the hypothesis that complex interglomerular interactions shape the signal emitted by PNs: odours are represented in an array of PNs arborising in different glomeruli and a single glomerulus harbours arborisations of PNs responding to different odour components. In addition, different delays for the responses to different odours in the same PN indicate that we deal with a highly integrated signal at that level. Similar structure–function relationships were found in the noctuid moth, *S. littoralis*, even though data were not as strong as in the present study, because most neurons responded to many different compounds (Sadek et al., 2002). A recent electrophysiological study on second order AL neurons in *D. melanogaster* revealed also broad tuning and complex responses indicating lateral interactions between glomeruli within the AL (Wilson et al., 2004). These findings seem to contradict calcium imaging studies performed on the same species and on honeybees (Wang et al., 2003; Sachse and Galizia, 2002, 2003), where input and output patterns matched rather well. These differences can be explained by methodological problems, as the exact origin of the measured calcium signal is not yet completely understood (Wilson et al., 2004).

A number of recent studies suggest that, in fact, different input–output relationships might exist in parallel in the AL and what was interpreted as differences in olfactory processing between different species in the past, might be dependent mainly on the different experimental approaches and different subsystems studied. In specific systems like the pheromone system, a perfect match seems to be present in some species (*M. sexta*, Hansson et al., 2003; *H. virescens*, Vickers et al., 1998), whereas only some overlap between input and output is found in other species (*Trichoplusia ni*, Anton and Hansson, 1999). In female *M. sexta*, a female-specific enlarged

glomerulus, possibly representing also a specialised system, was found to house arborisations of PNs responding to the plant compound linalool only (King et al., 2000), and more specifically to one enantiomer of this compound (Reisenmann et al., 2004). PNs arborising in adjacent glomeruli responded less specifically to the linalool enantiomers or a racemic blend (Reisenmann et al., 2004).

Of the nine PNs and one LN that responded to only one of the six tested compounds, six responded to α-farnesene. The majority of these PNs arborised in the anterior central part of the AL. α-farnesene has been shown to be a potent attractant in the codling moth, *Cydia pomonella*, another tortricid moth (Sutherland and Hutchins, 1972; Hern and Dorn, 1999; Bengtsson et al., 2001; Coracini et al., 2004). Our previous study showed that AL neurons in both males and females of *L. botrana* responded to α-farnesene (Masante-Roca et al., 2002). Although this compound is common in many plants, we cannot exclude that it plays a specific role in host plant attraction in the European grapevine moth.

In conclusion, by analyzing structure–function relationships in AL output neurons we found specifically responding neurons, which arborised in an array of different glomeruli, indicating complex interglomerular interactions influencing the information leaving the AL. The 3D AL atlas and PN data for mated females will in the future be used to obtain a better understanding of coding mechanisms of grapevine odours in this pest insect as a function of mating and environmental conditions.

List of abbreviations

AL	antennal lobe
AN	antennal nerve
CB	cell body cluster
d	dorsal
DB	dorsal border of the antennal lobe
l	lateral
LN	local interneuron
LY	Lucifer Yellow
m	medial
MG	macroglomerulus
MGC	macroglomerular complex
OL	optic lobe
ORN	olfactory receptor neuron
PN	projection neuron
SOG	suboesophageal ganglion
v	ventral

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