The Journal of Experimental Biology 213, 4291-4303

doi:10.1242/jeb.047365

Coding and interaction of sex pheromone and plant volatile signals in the antennal lobe of the codling moth *Cydia pomonella*

Federica Trona[1,2,*], Gianfranco Anfora[1], Marie Bengtsson[2], Peter Witzgall[2] and Rickard Ignell[2]

[1]IASMA Research and Innovation Center, Fondazione E. Mach, via E. Mach 1, 38010 S. Michele a/A (TN), Italy and
[2]Chemical Ecology Group, SLU, Box 102, 23053 Alnarp, Sweden

*Author for correspondence (federica.trona@iasma.it)

Accepted 21 September 2010

SUMMARY

In the codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) plant volatiles attract males and females by upwind flight and synergise the male response to the female-produced sex pheromone, indicating a close relationship between the perception of social and environmental olfactory signals. We have studied the anatomical and functional organisation of the antennal lobe (AL), the primary olfactory centre, of *C. pomonella* with respect to the integration of sex pheromone and host-plant volatile information. A three-dimensional reconstruction of the glomerular structure of the AL revealed 50±2 and 49±2 glomeruli in males and females, respectively. These glomeruli are functional units involved in the coding of odour quality. The glomerular map of the AL was then integrated with electrophysiological recordings of the response of individual neurons in the AL of males and females to sex pheromone components and behaviourally active plant volatiles. By means of intracellular recordings and stainings, we physiologically characterised *ca.* 50 neurons in each sex, revealing complex patterns of activation and a wide variation in response dynamics to these test compounds. Stimulation with single chemicals and their two-component blends produced both synergistic and inhibitory interactions in projection neurons innervating ordinary glomeruli and the macroglomerular complex. Our results show that the sex pheromone and plant odours are processed in an across-fibre coding pattern. The lack of a clear segregation between the pheromone and general odour subsystems in the AL of the codling moth suggests a level of interaction that has not been reported from other insects.

Key words: Lepidoptera, olfaction, intracellular recording, glomeruli, blend interaction, synergism.

INTRODUCTION

Insects, like other animals, discriminate among an abundance of scents from plants, microorganisms and conspecifics those key compounds that facilitate the search of food and mates. Sex pheromones of moths have long served as a model of how odours are detected, processed and translated into appropriate behaviours, whereas host-plant recognition is a more recent research focus (De Bruyne and Baker, 2008; Riffell et al., 2009; Tasin et al., 2006). Insect herbivores generally release sex pheromones from suitable plant hosts and the response to the pheromone is modulated by plant odours in many species (Reddy and Guerrero, 2004). The neurobiological basis for this interaction is, however, not well understood and interactions between pheromones and plant volatile information occur within both the peripheral and the central nervous systems (Lei and Vickers, 2008; Namiki et al., 2008; Ochieng et al., 2002).

In moths, there is a clear anatomical separation between the pathways from olfactory receptor neurons (ORNs) that carry pheromone and environmental odorant information into the antennal lobes (ALs), the primary olfactory centres in the brain (Hansson and Anton, 2000). In male moths, pheromone information is relayed to the macroglomerular complex (MGC), a group of large, sexually dimorphic glomeruli, whereas environmental odour, including plant volatile, information is relayed to a larger number of sexually isomorphic, ordinary glomeruli (OGs) (Anton and Hansson, 1995; Mustaparta, 2002). Despite the apparent morphological separation of the two pathways, processing of pheromones and other olfactory information is not qualitatively different. Both pheromone and general odorants are encoded by the selective activation of specific subsets of ORNs and the glomeruli they innervate (Christensen and Hildebrand, 2002; Galizia and Rössler, 2010).

Electrophysiological recordings from projection neurons (PNs), the glomerular output neurons innervating the MGC, have shown that pheromone information may be processed through labelled lines (Christensen et al., 1991; Hansson et al., 1991). More commonly, the spatial output pattern does not overlap with the glomerular input representation, leading to the concept that pheromone information is processed in across-glomerular pathways, regulated by intercalated local interneurons (LNs) (Anton and Hansson, 1999; Christensen and Hildebrand, 2002).

Processing of plant odours is less thoroughly studied, but available evidence suggests a similar transformation of these signals in OGs (Carlsson et al., 2002; Christensen and Hildebrand, 2002; Masante-Roca et al., 2005; Sadek et al., 2002). The complexity of host-plant volatiles and the fact that these signals often are represented by a larger array of glomeruli complicates precise data analysis and interpretation (Ignell and Hansson, 2005). Furthermore, electrophysiological studies have shown that PNs in the MGC and in OGs may respond to both social and environmental odours, suggesting an interaction between these different signals at the output level (Anton and Hansson, 1994; Lei et al., 2004; Namiki et al., 2008).

The codling moth *Cydia pomonella*, a worldwide pest of pome fruit, has long been a model for the development of behaviour-modifying chemicals for sustainable insect control (Light et al., 2001; Knight et al., 2005; Roelofs et al., 1971; Wearing and

Hutchins, 1973; Witzgall et al., 2008; Witzgall et al., 2010). Studies of the behavioural and chemical ecology of codling moth emphasise the close evolutionary relationship in the detection and communication with sex pheromones and host-plant chemicals. Male moths are attracted to plant volatiles that mediate pre-mating flights around suitable host trees, well before the onset of female sex pheromone release (Coracini et al., 2004; Witzgall et al., 1999). Calling codling moth females release minute quantities of sex pheromone into orchard air that contains behaviourally active plant volatiles at concentrations that are several magnitudes higher (Bäckman et al., 1997; Witzgall et al., 2008). Some of these plant volatiles strongly synergise the male behavioural response to female-produced sex pheromone (Yang et al., 2004).

We present here three-dimensional atlases of codling moth ALs of both sexes that are a prerequisite for future studies of olfactory communication in this species. Similar brain maps have been produced for other tortricids, including the grapeberry moth *Lobesia botrana* (Masante-Roca et al., 2005) and the Oriental fruit moth *Grapholita molesta* (Varela et al., 2009). The morphology and physiology of AL interneurons in *C. pomonella* males and females was characterised by means of intracellular recording and staining techniques using a number of pheromone compounds and behaviourally active plant volatiles and their binary blends.

MATERIALS AND METHODS

Insects

Cydia pomonella (Linnaeus 1758) pupae were obtained from a laboratory rearing (Andermatt Biocontrol, Grossdietwil, Switzerland). Pupae were sexed and kept in a climate chamber under a 16 h:8 h light:dark photoperiod at 23±2°C and 70±5% relative humidity. Adults were fed with a 10% sucrose solution. For both intracellular recording and anatomical investigations, 2- to 3-day-old unmated males and females were used.

Test compounds

The test compounds (Fig. 1), 13 plant- and four pheromone compounds, have previously been shown to elicit electroantennographic and behavioural responses in *C. pomonella* (Ansebo et al., 2004; Witzgall et al., 2001). The purity of each test compound was assessed by gas chromatography: butyl hexanoate (97.8% chemical purity), (*E*)-β-farnesene (93.4%), (*E*,*E*)-α-farnesene (84.8%) and (*Z*)-3-hexenyl benzoate (96.2%, all from Bedoukian Research Inc., Danbury, CT, USA); acetic acid (99.7%, Fisher Scientific, Pittsburg, PA, USA); (±)-linalool (97.7%, ICN Biomedicals Inc., Irvine, CA, USA); β-caryophyllene (96.9%), ethyl (*E*,*Z*)-2,4-decadienoate (pear ester; 87.4%), farnesol (94.9%), (*Z*)-3-hexenol (80%) and methyl salicylate (83.7%, all from Sigma-Aldrich, Steinheim, Germany); hexyl 2-methyl-butanoate (89.5%, Anna-Karin Borg-Karlsson, Royal Institute of Technology, Stockholm, Sweden); and 4,8-dimethyl-1,3, (*E*)7-nonatriene (DMNT; 94.4%, Wittko Francke, University of Hamburg, Germany).

Pheromone compounds included the main pheromone component (*E*,*E*)-8,10-dodecadien-1-ol (*E*8,*E*10-12OH, codlemone; 99.5% purity, Bedoukian Research Inc.), its geometric isomer (*E*,*Z*)-8,10-dodecadien-1-ol (*E*8,*Z*10-12OH, *EZ*-codlemone; 99.5%, Rikard Unelius, University of Kalmar, Kalmar, Sweden) and the minor components (*E*,*E*)-8,10-dodecadienyl acetate (*E*8,*E*10-12Ac, codlemone acetate; 99.2%, Bedoukian Research Inc.) and dodecanol (12OH; 97.3%, Fluka, Buchs, Switzerland).

Each compound was dissolved in re-distilled hexane (Labscan, Malmo, Sweden), and 10 µl aliquots, containing 100 ng of pheromone or 10 µg of plant compounds, were applied onto filter paper (0.5×1 cm) that were inserted into Pasteur pipettes. For tests of two-component blends, two filter papers were placed in a pipette and the dose of each compound in the blend was the same as the dose of the single compounds. Filter papers formulated with solvent alone were used as blanks. Filter papers were prepared *ca*. 30 min before every experimental session.

Intracellular recordings

The standard method for intracellular recording in moths was used (Kanzaki et al., 1989). An insect was inserted from the wide end of a cut 1 ml plastic pipette tip, with the head protruding from the narrow end. The head was immobilised using dental wax (Surgident, Heraeus Kulzer Inc., South Bend, IN, USA) and, after removing all the scales, the head capsule was opened by incising the cuticle between the antenna and the eyes. The ALs were exposed by removing the mouthparts, muscle tissue and trachea, as well as the neural sheath. The brain was super-fused for the duration of the experiment with a ringer solution (pH 6.9; 150 mmol^{-1} NaCl, 3 mmol^{-1} $CaCl_2$, 3 mmol^{-1} KCl, 10 mmol^{-1} TES and 25 mmol^{-1} sucrose) delivered from a flow system.

The stimulated antenna was ipsilateral to the recording site. A charcoal-filtered humidified air stream (500 ml min^{-1}) passed through a glass tube, with the opening 1 cm from the antenna. A stimulus controller (SFC-2/b, Syntech, Kirchzarten, Germany) injected a 0.5 s puff (500 ml min^{-1}), through a Pasteur pipette containing a filter paper loaded with test compound, into this glass tube at 20 cm from the antenna. In order to avoid contamination in the odour delivery system, the glass tube was exchanged regularly. Moreover, the back-end of each Pasteur pipette was covered by a 1 ml pipette tip. Stimuli were presented in random order, including the control, after spontaneous activity.

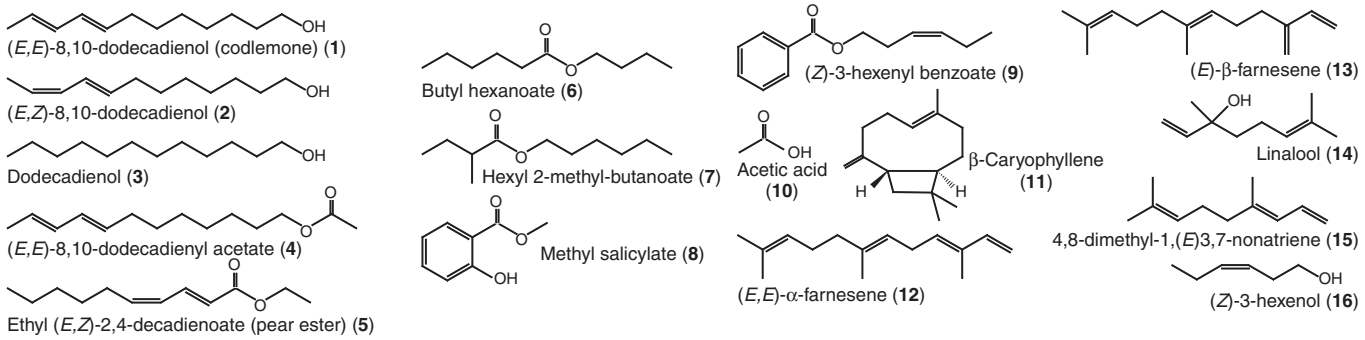

Fig. 1. Chemical structures of the test compounds, sex pheromone components (1–4) and plant-released volatiles (5–16).

A silver ground electrode was placed in contact with the ringer solution. Using a micromanipulator (MX300R, Newport, Irvine, CA, USA), the AL was randomly penetrated with a recording electrode – a glass electrode (0.5 mm i.d., Sutter Instrument, Novato, CA, USA) filled with 1% neurobiotin (Vector Laboratories, Burlingame, CA, USA) dissolved in 0.25 mol l^{-1} KCl – and then filled with 1 mmol l^{-1} KCl. The physiological responses were amplified, digitised at 10.7 kHz, filtered using a 100 Hz high-pass filter and a 5 kHz low-pass Bessel filter *via* an IDAC-4 USB interface (Syntech) and then visualised on a PC using AutoSpike 3.2 software (Syntech).

Data analysis

Intracellular recordings were stored and analysed using AutoSpike software. The net number of spikes per second (number of spikes 500 ms before stimulus onset subtracted from the number of spikes 500 ms after stimulus onset) in response to the blank were subtracted from the net number of spikes in response to stimuli. A neuron was considered to respond to a stimulus when the difference between the stimulus and the blank exceeded 20 spikes s^{-1}. Excitatory responses were classified as weak (difference between stimulus and blank 20–30 spikes s^{-1}), intermediate (30–60 spikes s^{-1}), strong (>60 spikes s^{-1}), delayed (when excitation started after the offset of the stimulation) or inhibitory (when cessation in spiking activity lasted during >300 ms after stimulation). A synergistic or suppressive response to a two-component blend was defined as a >50% increase or decrease in the number of spikes per second, respectively, compared to the sum of the responses to the single compounds. Response evolution was studied by plotting frequency histograms (spikes s^{-1}) during 100 ms bins. Data were evaluated only from those recording sessions during which the entire set of stimuli was tested.

Staining technique and neuroanatomy

Physiologically characterised neurons were stained using a depolarising current at 0.5–0.7 nA for 15 min. Thereafter, brains were fixed overnight at 4°C in 4% formaldehyde solution [diluted in phosphate buffered saline (PBS) solution with 0.25% Triton X-100, pH 7.2; PBS-TX], then dissected and washed in PBS-TX 3×10 min at room temperature. The brains were then dehydrated and re-hydrated through a graded series of ethanol (40, 50, 60, 70, 80, 90, 96 and 99.9%, each for 10 min), washed 3×10 min in PBS-TX and then incubated for 2 days at 4°C in a solution of 3% fluorescein Avidin (Invitrogen, Carlsbad, CA, USA), 3% α-synapsin (Hybridoma, University of Iowa, Iowa City, IA, USA) and 3% phalloidin Alexa Fluor 546 (Invitrogen) diluted in PBS-TX with 1% normal goat serum (NGS). After washing 3×10 min in PBS-TX, brains were incubated overnight at 4°C with a secondary antibody, 3% α-mouse Alexa Fluor 546 (Invitrogen) (diluted in PBS-TX, 1% NGS). After washing in PBS-TX for 3×10 min, brains were mounted on a microscopy slide, using spacer rings (Secure-Seal imaging spacer, Sigma-Aldrich), in Vectashield Hard Set mounting medium (Vector Laboratories). Stained neurons were examined using a laser scanning confocal microscope (Zeiss LSM 510, Carl Zeiss, Jena, Germany) with a 40×1.4 oil-immersion DIC objective. Structures labelled with Alexa Fluor 488 and fluorescein Avidin were excited with an argon laser at 488 nm and detected using a 505 nm long-pass filter; Alexa-Fluor-546-labeled structures were excited with a HeNe laser and detected using a 560 nm long-pass filter. Stacks of *X–Y* confocal images were scanned with a step size of 0.7 μm and the images were stored at a size of 1024×1024 pixels.

A three-dimensional (3-D) surface model of the AL glomeruli of *C. pomonella* females and males was constructed according to Ignell et al. (Ignell et al., 2005), after staining 10 brains of each sex with phalloidin and synapsin (see above). Moreover, the glomerular structure, as well as the innervation pattern of the ORNs, was studied after anterograde-neurobiotin backfills of the antennal nerve of males (*N*=5) and females (*N*=5), following the protocol of Ignell et al. (Ignell et al., 2005). Each AL was scanned with a series of optical sections collected as above. Images were analysed using AMIRA 3.0 software (Visage Imaging, Berlin, Germany). For each sex, three phalloidin-synapsin-stained and three anterograde neurobiotin backfill preparations were entirely mapped; each glomerulus was manually labelled, reconstructed and numbered from the most anterior to the most posterior glomerulus. Glomeruli of different individuals were compared and matched. As previously reported (Ignell et al., 2005), we identified three different classes of glomeruli: class 1 glomeruli, which were glomeruli of constant shape, size and position; class 2 glomeruli, which showed variation between individuals and which could be located based on the position of the landmark glomeruli, lateral clusters of cell bodies and the antennal nerve entrance; and class 3 glomeruli, which were poorly demarcated and were not identified in all preparations. By comparing the background stainings in the optical sections of physiologically characterised and stained neurons with the 3-D maps, target glomeruli of AL neurons were successfully localised in male and female ALs.

RESULTS

3-D atlas of the codling moth AL

The ALs of *C. pomonella* males and females were reconstructed in three dimensions, based on the comparison of phalloidin-synapsin stainings of the glomerular neuropil (Fig. 2) and anterograde neurobiotin backfills of the ORNs (data not shown). Each 3-D atlas was divided into three parts: anterior (Fig. 2A,D), central (Fig. 2B,E) and posterior (Fig. 2C,F).

Glomeruli borders were clearly delineated in the phalloidin-synapsin preparations, showing 50±2 and 49±2 olfactory glomeruli in the male and female AL, respectively, located in 1–3 layers around a central fibre core. This data was supported by reconstructions based on anterograde backfill preparations, showing 47±1 and 37±2 glomeruli in male and female ALs, respectively. The backfills showed fewer glomeruli, due to difficulties in distinguishing glomeruli in the posterior region of the AL. Interestingly, a comparison of phalloidin-synapsin with anterograde preparations demonstrated that a large glomerulus, OG 47 and OG 37, located in the ventro-posterior region of the male and female AL, respectively, did not receive innervation by antennal ORNs (data not shown).

Twenty-two and 24 glomeruli in the male and female AL, respectively, showed invariant positions, shape and size (class 1 glomeruli; Fig. 2) between individuals. Class 2 glomeruli (25 in males and 21 in females) differed in size and position between individual ALs. Class 3 glomeruli, which could not be identified in all preparations, were found both in the male AL (OG 28, 32 and 49) and in the female AL (OG 23, 36, 46 and 51). In both sexes, glomeruli were more densely packed in the anterior part of the AL compared with the central and posterior part.

The male AL was characterised by two large, irregularly shaped glomeruli at the entrance of the antennal nerve (AN) (glomeruli 27 and 48). Glomerulus 27 was divided into two lobes by a medio-lateral invagination, and is structurally analogous to the cumulus identified in several other moth species (Hansson and Anton, 2000). The large, elongated OG 48 was situated in close proximity to the cumulus, together with OGs 13, 20, 21, 22 and 37 in the anterior

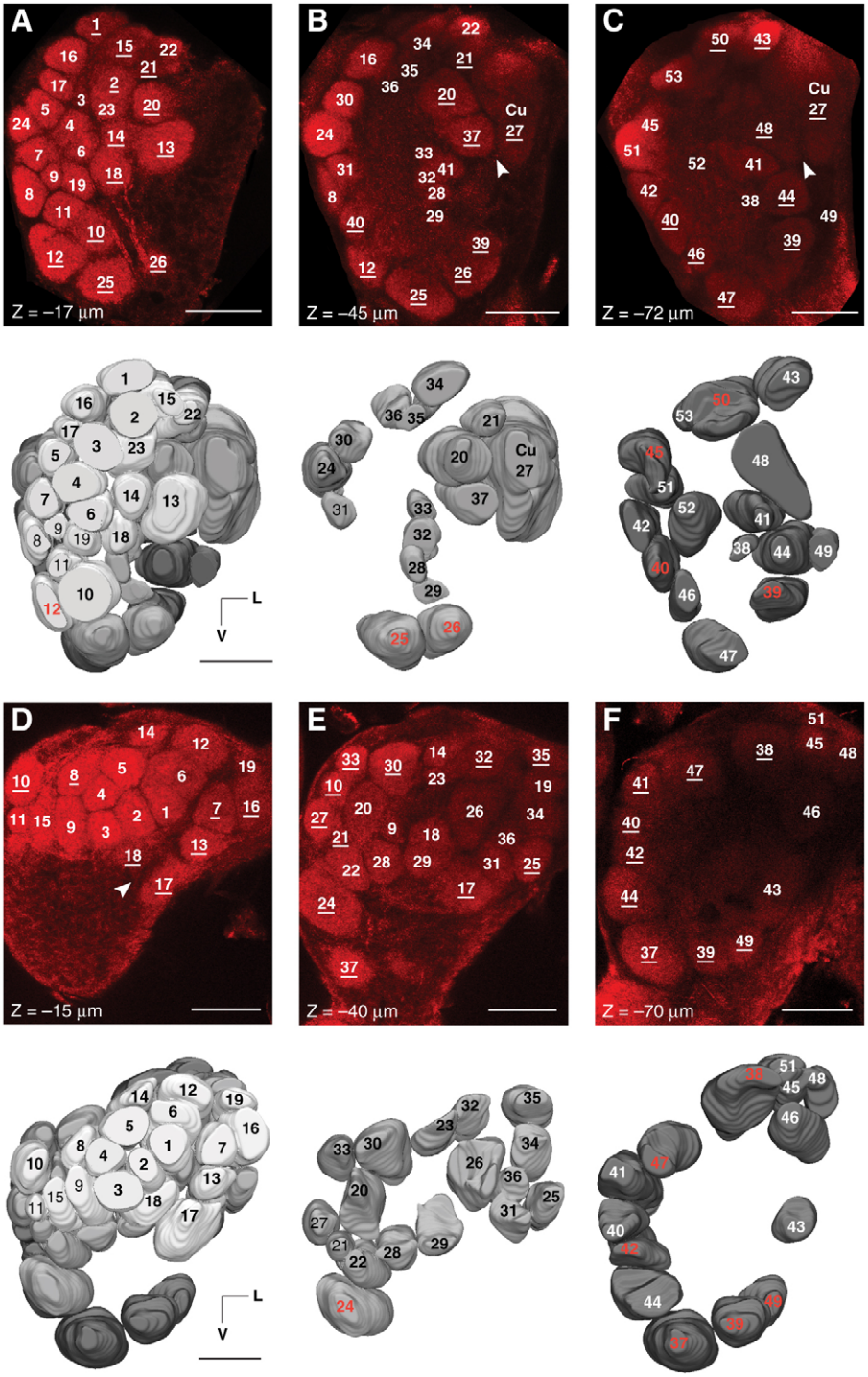

Fig. 2. Single confocal frontal sections and three-dimensional (3-D) reconstructions of *Cydia pomonella* male (A–C) and female (D–F) antennal lobes (ALs). Glomeruli are numbered from the most anterior to the most posterior. In the confocal sections (top panels), underlined numbers indicate landmark (class 1) glomeruli. The arrowheads show the gap between the cumulus (Cu 27) and the ordinary glomeruli (OGs) in the male AL, and between an array of glomeruli (7, 13, 16, 17 and 19) located close to the antennal nerve and other OGs in the female AL. The 3-D atlases (bottom panels) are divided into three parts: anterior (A,D), central (B,E) and posterior (C,F), coloured with different shades of grey to facilitate depth perception. Sexually isomorphic glomeruli are marked in red. L, lateral; V, ventral; Z, depth from the anterior side of the AL. Scale bars: 50 µm.

and central layers and OG 43 in the posterior layer of the AL. OGs were characterised by their spherical shape, but varied in size; the largest were OGs 10, 20, 25, 26, 44, 47, 48 and 50 (Fig. 2A–C).

In the female AL, a group of glomeruli (OGs 7, 13, 16, 17 and 19) was situated at the entrance of the AN; these were partially separated from the other glomeruli by a narrow gap (Fig. 2D, arrowhead). OG 16 was located at the base of the AN, similar to that of the cumulus in the male AL. In the anterior layer, OGs were distributed exclusively in the dorso-central part of the AL, whereas they were circularly arranged around the central fibre core in the posterior layer. Similar to the male AL, some OGs (17, 24, 26, 37, 38 and 44) were considerably larger than others (Fig. 2D–F).

Despite an obvious sexual dimorphism in glomerular organisation, we observed a number of sexually isomorphic glomeruli that displayed the same topographical position and size, including OGs 12/24, 25/37, 26/39, 39/49, 50/38, 40/42 and 45/47 in the male/female AL, respectively (Fig. 2). In addition, in both sexes, a small and densely packed glomerular array (OGs 8, 9, 19, 11 and 31 in males; OGs 9, 11, 15, 21 and 27 in females) characterised the medio-ventral part of the AL.

General physiological and morphological characteristics of AL neurons

A total of 56 and 60 neurons in the male and female AL, respectively, were challenged with the complete set of 17 single odorants and seven two-component blends. The response spectra of these neurons are shown in Tables 1 and 2.

The AL neurons showed several types of response dynamics, including excitatory (e.g. Fig. 3A) and delayed excitatory responses, as well as inhibitory responses, which lasted between 0.3 and 1.5 s after the onset of stimulation (e.g. Fig. 3F; acetic acid). In addition, the AL neurons exhibited different modes of termination kinetics, including phasic (e.g. Fig. 3A; codlemone acetate), tonic (e.g. Fig. 3A; pear ester) and phasic-tonic responses [e.g. Fig. 3C; (*Z*)-3-hexenol]. Furthermore, a single odorant was able to evoke different response dynamics in different neurons, and a single neuron often showed different response dynamics to different stimuli (Fig. 3A). The spontaneous activity ranged from 10 to 60 Hz and the spike amplitude varied between 5 and 50 mV.

The stained neurons were classified as either PNs or LNs, based on their morphological characteristics (Hansson and Anton, 2000). In total, 22 PNs and 5 LNs in male ALs (Table 1) and 27 PNs in female ALs (Table 2) were successfully stained. The cell bodies were observed in three clusters: the medial, lateral and antero-ventral cell clusters. All LNs had their cell bodies in the lateral cluster and

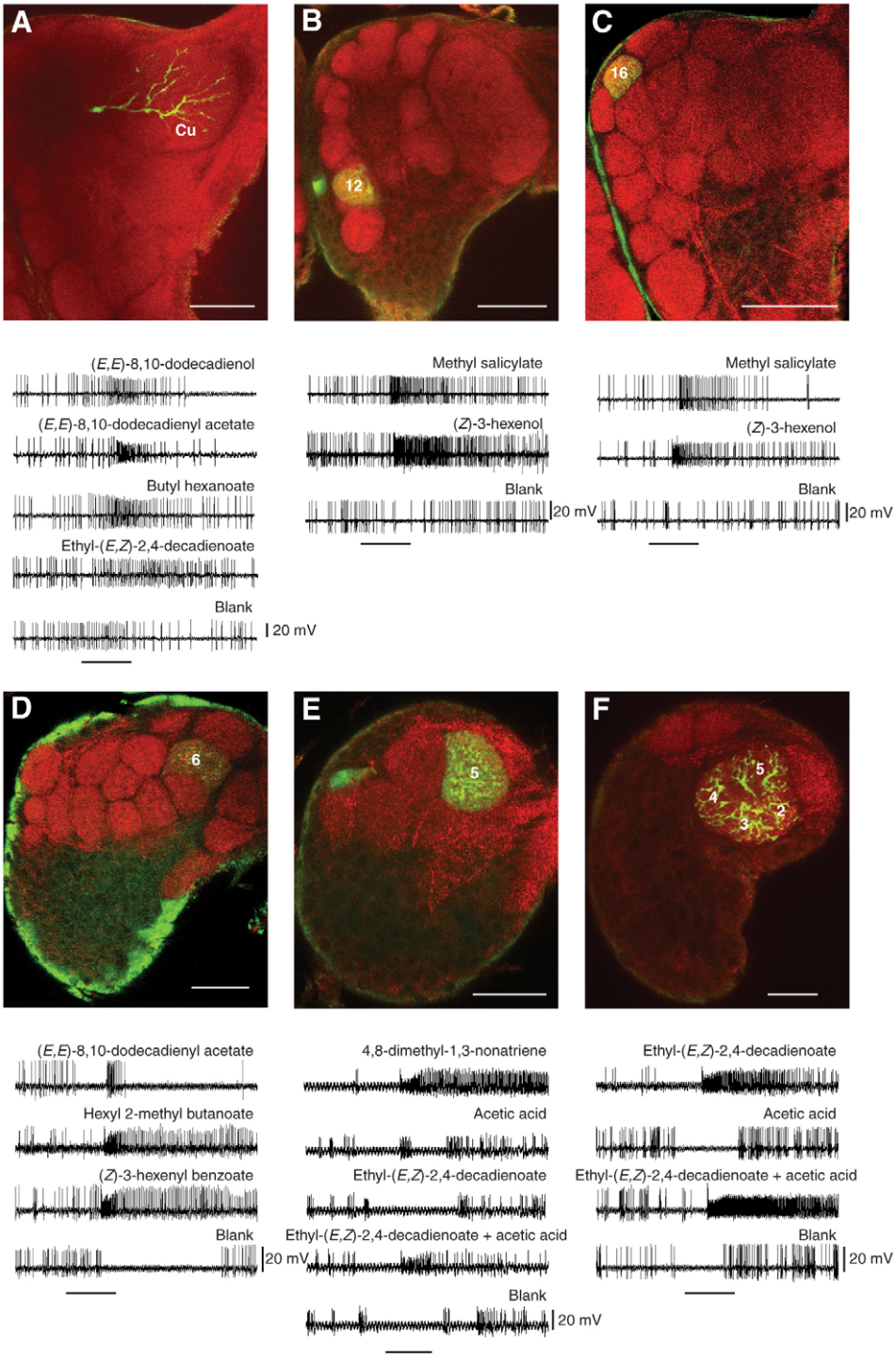

Fig. 3. Physiologically and morphologically characterised projection neurons (PNs) of *Cydia pomonella* male (A–C) and female (D–F) antennal lobes (ALs). Single confocal frontal sections showing: (A) PN 15 innervating the cumulus; (B) PN 6 and (C) PN 8 innervating OG 12 and OG 16, respectively, both responding to methyl salicylate and (*Z*)-3-hexenol; (D) PN 4 innervating OG 6 and responding to codlemone acetate and some plant volatiles (see Table 2); (E) PN 2 innervating OG 5, showing strong responses to some plant volatiles (see Table 2), and a synergistic response to the blend of pear ester and acetic acid; (F) multiglomerular PN 27 innervating OGs 2, 3, 4 and 5 and showing a synergistic response to a blend of pear ester and acetic acid. Cu, cumulus. The horizontal bars below the traces indicate the stimulus period (500 ms). Scale bars: 50 μm.

Table 1. The response of male *Cydia pomonella* antennal lobe (AL) interneurons to a suite of pheromone components, plant volatiles and binary blends

	PNs																				LNs							Non-stained neurons																				
Neuron no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Innervated glomerulus	1	2	11	Ga1	10	12	14	16	17	18	21	23	23	Cu	Cu	Ga2	36	36	36	43	48	49	all	all	all	all	all																					
CB	?	mc	av	mc	mc	mc	mc	?	lc	mc	lc	lc	av	lc	mc	lc	mc	mc	mc	mc	?	av	lc	lc	lc	lc	lc																					
Single compounds																																																
Pheromone components																																																
(*E*,*E*)-8,10-dodecadienol			•		•						⬤			●	•	•	○		○	•							○				•	•		•	•	•		•	•	•	●			⬤		●	•	•
(*E*,*Z*)-8,10-dodecadienol	○				•								○	•							•							●								●	●	•							⬤	○		
Dodecanol					•				○																○				•	○																		
(*E*,*E*)-8,10-dodecadienyl acetate													○	•	●	•			○	•		•					○														●			•	⬤	○	○	•
Esters																																																
Ethyl (*E*,*Z*)-2,4-decadienoate	♦	⬤			●				♦			•			•	●											○						○										•		•	○		•
Butyl hexanoate	♦	⬤							⬤		⬤	•	○	•	●		•	●	●	•	♦	•		•	○	○	○			○							♦			•	●	⬤	●	●	●	●	•	•
Hexyl 2-methyl-butanoate		⬤									●	●						●	●		○				○		○										○	•				⬤			•		•	●
Benzenoids																																																
Methyl salicylate		•		⬤		⬤		⬤	⬤	⬤		•	●			●	⬤	⬤	•	⬤	○				○		○						•										•					
(*Z*)-3-hexenyl benzoate	●	⬤											○			●				●					○		○															○	•	•		●		•
Acid																																																
Acetic acid		●											○							●	•	●		●	⬤	○	•												•							○		
Sesquiterpenes																																																
β-caryophyllene																										•							○													○		
(*E*,*E*)-α-farnesene	○												○							○	♦																						•			•	•	
(*E*)-β-farnesene	○	●							○				○											⬤	○		○															⬤				●	•	
Farnesol																																														•	•	
Monoterpenes																																																
Linalool	○	⬤										•				●		⬤	●						○	○																○						
Irregular terpenes																																																
4,8-dimethyl-1,3-nonatriene	○							○				●	○					⬤	⬤		•			●	○		○																	●		⬤	•	•
Alcohols																																																
(*Z*)-3-hexenol	○			⬤		⬤	•	●	⬤	⬤		●	⬤				♦	⬤	●		○			•			○			●																○		
Blends																																																
E-codlemone + dodecanol		+												+																																		
E-codlemone + *EZ*-codlemone																				–															–	–			–						–		–	
E-codlemone + pear ester						–		–															+																	+					–	–		
E-codlemone + acetic acid											+				+					–															–						+							
E-codlemone + α-farnesene															+					–								+								+												
Pear ester + acetic acid		–										+	+		–								+																–						+		+	
α-farnesene + β-farnesene																																																

The responses of 27 stained and 21 non-stained neurons are shown. Responses to single components were classified as weak (•), intermediate (●), strong (⬤), inhibitory (○), delayed (♦) or no response (no symbol). Responses to the binary blends were classified as synergistic (+), suppressive (–) or no interaction (no symbol).

av, antero-ventral cluster; CB, cell body position; Cu, cumulus (glomerulus 27); Ga1, glomerular array 1 (OGs 9, 19); Ga2, glomerular array 2 (OG 20 and Cu); lc, lateral cluster; mc, medial cluster; ?, cellular body could not be located.

Table 2. The response of female *Cydia pomonella* antennal lobe (AL) interneurons to a suite of pheromone components, plant volatiles and binary blends

	PNs																																																	
	Non-stained neurons																																																	
Neuron no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
Innervated glomerulus	1	5	5	6	6	7	9	10	12	12	14	16	17	17	18	20	22	22	26	29	31	32	38	43	48	51	Ga1																								
CB	lc	mc	av	mc	mc	lc	av	?	mc	mc	av	mc	?	mc	mc	lc	av	mc	lc	av	mc	mc	av	?	mc	mc	mc																							
Single compounds																																																		
Pheromone components																																																		
(*E*,*E*)-8,10-dodecadienol					●								•											○						•																				
(*E*,*Z*)-8,10-dodecadienol																																					•								•					•
Dodecanol																												•																		•				
(*E*,*E*)-8,10-dodecadienyl acetate				•								•												●														•								•			●	
Esters																																																		
Ethyl (*E*,*Z*)-2,4-decadienoate						●	○			○	○		•	●			○		●	•	•					○	●		•			○	○				•				●		○		○	•				●	
Butyl hexanoate		●		●					•	●	•		●	•						•			●	○	♦	○	●												●	♦		•	●				●	●	●	•
Hexyl 2-methyl-butanoate		●		●	●				•	●	•	•	●	●		•				●	•		●		♦		●						○								♦	•	•	♦			●	•		●
Benzenoids																																																		
Methyl salicylate		•					●			●			○		●			•			•	●	●			○		○					○		●				●		○			○	●			•		
(*Z*)-3-hexenyl benzoate		●		●						○	●	•		•					●						•	○		○				○								♦	♦				○		•	•	●	●
Acid																																																		
Acetic acid		•	●				●																									○	○	●												•				●
Sesquiterpenes																																																		
β-caryophyllene	○									○	○															●					●		○																	
(*E*,*E*)-α-farnesene		•				●													●		●		•		●	○	♦			○												•								●	●
(*E*)-β-farnesene		●		●	●	●	•				●								●														○										•	•	○		●	•	●	●
Farnesol						●										•			●	•		○				○																			○					•
Monoterpenes																																																		
Linalool		●		●			•	○	●	●	●										●	○	●				♦													●										
Irregular terpenes																																																		
4,8-dimethyl-1,3-nonatriene		●		●					•	●				•			○		●		•		●		♦	○	♦						○											♦						●
Alcohols																																																		
(*Z*)-3-hexenol							●		•	○	○	●			●		•			●	●		●			○		○				●	●			●		●						○	●	♦				○
Blends																																																		
E-codlemone + dodecanol	+				–		+																																											
E-codlemone + *EZ*-codlemone					–								–																																					
E-codlemone + pear ester							+						–	–																																			–	
E-codlemone + acetic acid					–		–																																											
E-codlemone + α-farnesene		+			–						+		–																																				–	
Pear ester + acetic acid		+											+	–							+						+															+								–
α-farnesene + β-farnesene										+					+																	+																		

The responses of 27 stained and 23 non-stained neurons are shown. Responses to single component were classified as weak (•), intermediate (●), strong (●), inhibitory (○), delayed (♦) or no response (no symbol). Responses to the binary blends were classified as synergistic (+), suppressive (–) or no interaction (no symbol).

av, antero-ventral cluster; CB, cell body position; Ga1: glomerular array 1 (OGs 2, 3, 4, 5); lc, lateral cluster; mc, medial cluster; ?, cellular body could not be located.

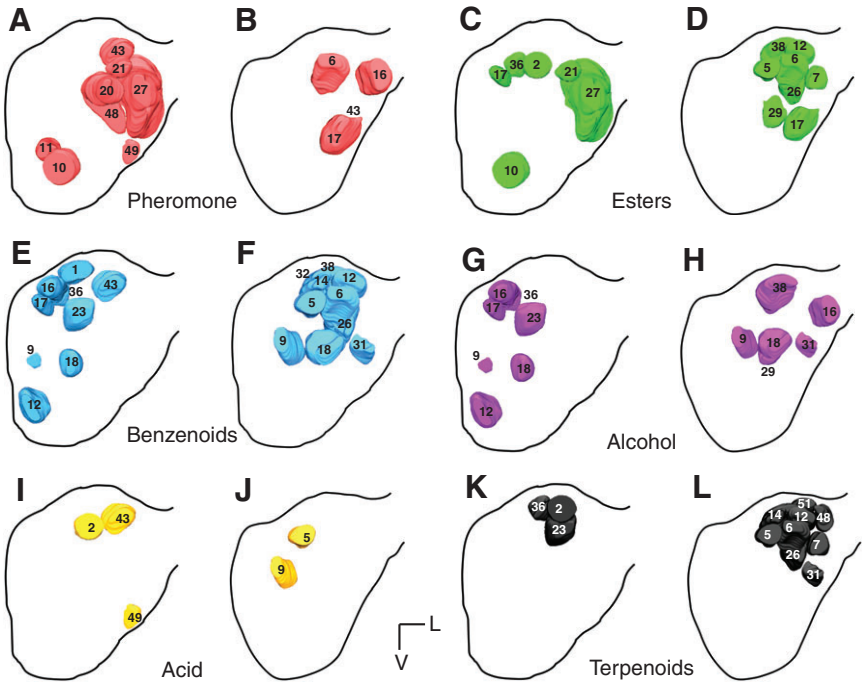

Fig. 4. Representation of the target glomeruli of pheromone sensitive projection neurons (PNs) in *Cydia pomonella* male (A) and female (B) antennal lobes (ALs). Target glomeruli of PNs in the male (C,E,G,I,K) and female (D,F,H,J,L) AL, respectively, showing intermediate to strong responses (>30 spikes s^{-1}) to compounds belonging to different chemical classes. Glomeruli numbers correspond to Fig. 2. L, lateral; V, ventral.

displayed homogeneous arborisations throughout the AL. Cell bodies of the PNs were primarily found in the medial cell cluster, but also in the lateral and antero-ventral clusters. Most of the PNs were uniglomerular, characterised by fine dendritic branches, which homogeneously innervated a single glomerulus (e.g. Fig. 3E). However, multiglomerular PNs were also found in both sexes (e.g. Fig. 3F). Few complete stainings of PNs were obtained, and their axonal projections were found to run through the medial antenno-protocerebral tract (see Galizia and Rössler, 2010) to the calyces of the mushroom body and the lateral horn of the protocerebrum (data not shown).

Male AL neurons

The response spectra of 27 stained and 21 non-stained AL neurons of male *C. pomonella* are shown in Table 1. These neurons varied widely in their specificity: 13 neurons showed an excitatory response to a single compound, with most of these responding to pheromone components; 15 neurons responded with excitation to two to three compounds; the remaining neurons were broadly tuned and responded to four to seven stimuli. Approximately one in three (18/48) neurons responded to both pheromone components and plant volatiles (Table 1).

Of the 26 neurons that showed excitatory responses to sex pheromone compounds, seven were tuned to the main pheromone component, codlemone, to *EZ*-codlemone or to dodecanol (Table 1). The other pheromone-sensitive neurons were activated by more than one pheromone component, as well as by plant volatiles. Pheromone-sensitive neurons responded frequently to esters. Butyl hexanoate was the most active plant volatile in male AL neurons and elicited responses in approximately 50% of the recordings. Four neurons that responded to pheromone components were also activated by acetic acid. Several neurons in the male AL responded to plant volatiles only, and most of these were broadly tuned. However, some neurons were excited by one plant volatile compound, as well as inhibited by other compounds (Table 1). Particularly strong excitatory responses were observed with (*Z*)-3-hexenol and methyl salicylate. Interestingly, these two compounds often activated the same neurons. By contrast, sesquiterpenes, such as farnesol and β-caryophyllene, exhibited responses only in a few recordings.

Three PNs with arborisations restricted to the cumulus responded to codlemone, codlemone acetate and the esters (Fig. 3A). However, responses to sex pheromone components were also observed in PNs innervating ordinary glomeruli (Fig. 4A), including OGs 20, 21, 43, 48 and 49 located near the cumulus, and OGs 10 and 11 in the antero-ventral part of the AL. Apart from the PN innervating OG 11, all of these neurons also responded to plant volatiles.

We obtained stainings of uniglomerular PNs innervating the same OG that showed different response spectra (e.g. PNs 12 and 13 innervating OG 23; Table 1). Among the three PNs that innervated OG 36, PNs 18 and 19 responded, each with different response dynamics, to six plant compounds, whereas PN 17 responded to only three of these. Similarly, OGs 1 and 2 were innervated by PNs (PNs 1 and 2) that displayed different response dynamics in response to some common plant volatiles, including pear ester, butyl hexanoate and (*Z*)-3-hexenyl benzoate; PN 2 also responded to hexyl 2-methyl-butanoate, methyl salicylate, acetic acid, (*E*)-β-farnesene and linalool (Table 1). By contrast, other PNs that responded to the same compounds innervated different glomeruli, e.g. the PNs that innervated OGs 12 (Fig. 3B), 16 (Fig. 3C) and 18 all responded phasic-tonically to (*Z*)-3-hexenol and methyl salicylate, and the nearby OG 14 was innervated by a PN that showed a phasic response to (*Z*)-3-hexenol.

Besides uniglomerular PNs, we also stained two multiglomerular PNs, each arborising in two closely adjacent glomeruli: PN 16, which responded to the main pheromone component, codlemone acetate and some plant volatiles, innervated the cumulus and the adjacent OG 20; and PN 4, which responded specifically to (*Z*)-3-hexenol and methyl salicylate, innervated OGs 9 and 19.

On the basis of the identification of the target glomeruli of identified PNs showing intermediate to strong responses (above the threshold of 30 spikes s^{-1}) to different classes of plant compounds, we observed a tendency towards a chemotopic organisation in the AL (Fig. 4). PNs responding to esters and acetic acid mainly innervated glomeruli in the dorso-lateral part of the

AL (Fig. 4C,I). The distribution of these glomeruli also partially overlapped with those innervated by PNs responding to sex pheromone components (Fig. 4A). PNs responding to alcohols and benzenoids were frequently found innervating the same glomeruli in the dorso-medial and ventro-medial part of the AL (Fig. 4E,G). Terpenoids activated PNs innervating glomeruli in the dorsal part of the AL (Fig. 4K).

The morphologically identified LNs were characterised by different response profiles. Three of the five identified LNs (25, 26 and 27) displayed excitatory responses to a single compound and, at the same time, inhibition to several plant stimuli (Table 1). LN 24 was less specific and showed an excitatory response to several plant volatiles. Finally, LN 23 did not respond to any of the tested single stimuli but showed an excitatory response to the blends of codlemone with dodecanol and pear ester with acetic acid (Table 1).

Female AL neurons

The response spectra of 27 stained and 23 non-stained AL neurons of female *C. pomonella* are shown in Table 2. The excitatory response spectra of female AL neurons displayed some similarities with that of males: 15 neurons were excited by a single compound, 12 neurons responded to two to three compounds and the remaining neurons were activated by a broad range of odorants.

Responses to pheromone compounds were recorded in 13 neurons. Neurons 24, 28 and 30 showed an excitatory response to a single pheromone compound whereas the other pheromone-sensitive neurons also responded to plant volatiles. As observed in the males, butyl hexanoate was highly active, eliciting a response in 40% of the neurons, frequently in combination with hexyl 2-methyl-butanoate. In contrast to recordings from males, (*E*)-β-farnesene and (*E*,*E*)-α-farnesene elicited strong responses in female AL neurons, comparable with those observed with (*Z*)-3-hexenol and 4,8-dimethyl-1,3-(*E*)-7-nonatriene. The least active plant compound was β-caryophyllene, which only elicited a response in two neurons (neurons 26 and 31). As in males, inhibitory responses were repeatedly observed after stimulation with plant volatiles, but only in one neuron after stimulation with pheromone compounds.

The morphologically characterised PNs responding to sex pheromone compounds innervated a group of four OGs situated in the dorso-lateral part of the AL (Fig. 4B), including OG 16 at the entrance of the AN. PNs 4 (Fig. 3D) and 5 both innervated OG 6 and responded to codlemone acetate and codlemone, respectively, as well as to some plant volatiles. The main pheromone component also elicited a weak response in PN 13, which innervated OG 17. Codlemone acetate elicited a response in PNs innervating OGs 16 and 43.

Intracellularly stained uniglomerular PNs that innervated the same glomerulus displayed some differences in their response profiles and also in their temporal response dynamics. OG 17 was innervated by PNs 13 and 14, which responded phasically to esters; PN 13 also responded to the main pheromone component whereas PN 14 responded weakly to (*Z*)-3-hexenyl benzoate and to 4,8-dimethyl-1,3-(*E*)-7-nonatriene (Table 2). PNs 9 and 10, which both innervated OG 12, responded to some common plant odours but displayed different temporal dynamics. Although linalool strongly excited both neurons in the same manner, butyl hexanoate, hexyl 2-methyl-butanoate and 4,8-dimethyl-1,3-nonatriene elicited phasic responses in PN 9 and long-lasting phasic-tonic responses in PN 10. Moreover, PNs 9 and 10 responded to (*Z*)-3-hexenol with excitation and inhibition, respectively.

A chemotopic map, based on target glomeruli of PNs showing intermediate to strong responses to plant volatiles, was not as clearly delimited as in males (Fig. 4). Esters mainly elicited a response in PNs, innervating OGs on the lateral side of the AL, but strong responses occurred also in dorsally located glomeruli (Fig. 4D). As in the male AL, there was a partial overlap between the activation area of sex pheromones and the esters (Fig. 4B,D). Likewise, alcohols and benzenoids were processed in some common glomeruli (OGs 9, 18, 31 and 38; Fig. 4F,H) but in the female AL there was a partial overlap with esters and terpenoids. Terpenoids elicited responses in less-specific PNs, arborising in the dorsal-lateral part of the AL (Fig. 4L).

Blend interactions in male and female AL neurons

In the male AL (Table 1), the blend of two pheromone compounds, codlemone and dodecanol, produced a synergistic effect in PN 14, which innervated the cumulus. The main pheromone component, codlemone, also evoked increased responses in blends with acetic acid in PN 41 and in PNs 15 and 11, which innervated the cumulus and OG 21 adjacent to the cumulus, respectively. Furthermore, the response to codlemone was synergized by (*E*,*E*)-α-farnesene in PNs 15, 36 (Fig. 5A), and by pear ester in neuron 40 (Fig. 5B). Suppressed responses to blends were found in six recordings with codlemone plus *EZ*-codlemone (e.g Fig. 5C), in one recording when mixed with dodecanol and in six recordings in blends with any of the three non-pheromonal compounds tested (Table 1). None of these neurons innervated the cumulus, but PN 20, which showed suppressive responses to the blends of codlemone with *EZ*-codlemone, acetic acid or (*E*,*E*)-α-farnesene, arborised in OG 43 adjacent to the cumulus. Blend-specific responses, in which only the blend was active and not its single components, were recorded from PN 2 and neuron 28 with blends of codlemone and dodecanol or (*E*,*E*)-α-farnesene, and LN 23 with blends of pear ester and codlemone or acetic acid (Table 1).

Even in the female AL (Table 2), one neuron, PN 2, showed an increased response to codlemone plus α-farnesene. Suppressive interactions were observed with blends of codlemone and plant volatiles, and codlemone and minor pheromone components. As in the male AL, the blend of pear ester and acetic acid produced a synergistic effect in PNs 2 (Fig. 3E), 13 (Fig. 5D) and 21, and in the multiglomerular PN 27 (Fig. 3F). The same mixture evoked, however, suppressive responses in neurons 14 and 50. Some neurons responded to blends only, to codlemone and dodecanol (PNs 1 and 7), codlemone with pear ester or α-farnesene (PNs 7 and 11), pear ester and acetic acid (neuron 42), and to the blend of α-farnesene and β-farnesene (PNs 10, 15 and neuron 32; Table 2).

DISCUSSION

This first morphological and functional characterisation of the codling moth AL, on the basis of a reconstruction of its glomerular structure and intracellular recordings combined with stainings of its interneurons, reveals a highly integrated pattern of representations of social and environmental odours. Data on the integration of information from behaviourally relevant odours in the first olfactory centre of the brain complement investigations of the peripheral olfactory system (Ansebo et al., 2004; Bäckman et al., 2000; Bengtsson et al., 2001) and odour-mediated reproductive behaviour in codling moths (Light et al., 2001; Trona et al., 2010; Witzgall et al., 2001; Yang et al., 2004). An improved understanding of codling moth olfactory physiology is expected to lead to the further refinement of sustainable control methods that are based on behaviour-modifying chemicals for this worldwide pest of pome fruit (Weddle et al., 2009; Witzgall et al., 2008; Witzgall et al., 2010).

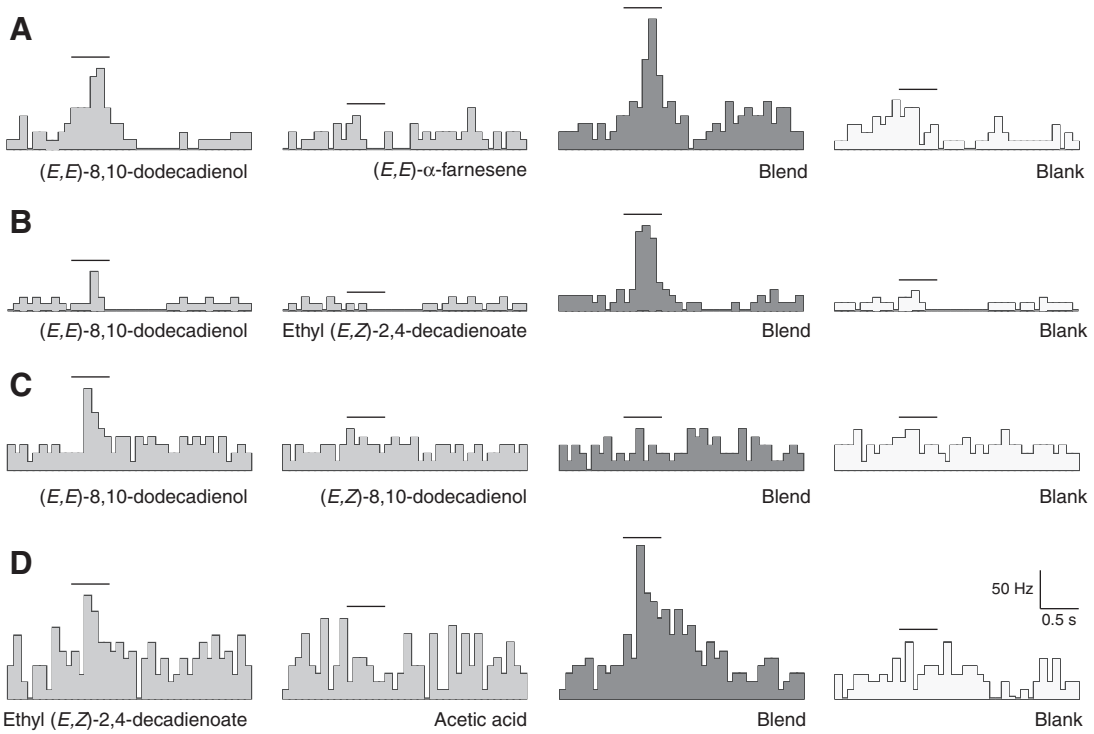

Fig. 5. Responses of neurons to two-compound blends in *Cydia pomonella* male (A,B,C) and female (D) antennal lobes (ALs). Spike frequency histograms (spikes s^{-1}) showing the firing change in 100 ms bins of: (A) PN 15, innervating the cumulus; (B) neuron 40; (C) neuron 47, showing a suppressive response to the blend of codlemone, (*E,E*)-8,10-dodecadienol, and *E,Z*-codlemone; and (D) PN 13, which innervates OG 17. The horizontal bars show the stimulus period (500 ms).

Anatomy of the antennal lobe

Olfactory glomeruli in the AL are functional units involved in the coding of odour quality. The ALs of codling moth males and females contain 50±2 and 49±2 glomeruli, respectively (Fig. 2). This finding compares to a closely related species, the Oriental fruit moth (Varela et al., 2009), where 50–53 and 48–49 glomeruli are present in females and males, respectively. The ALs of these species show some homologies, including, for example, a large glomerulus in the ventro-medial region of the AL of males and females (OGs 47 and 37). These large glomeruli do not receive afferent innervation from the antennae in either species and are probably analogous to the 'labial pit organ glomerulus' described in other moths (Kent et al., 1986; Rospars and Hildebrand, 2000).

The number of glomeruli found in the codling moth is slightly lower than in other moths; 50–55 glomeruli were found in the silk moth *Bombyx mori* (Koontz and Schneider, 1987), 60–71 in the grapevine moth (Masante-Roca et al., 2005) and 64–68 in the tobacco hornworm *Manduca sexta* (Rospars and Hildebrand, 1992), the European corn borer *Ostrinia nubilalis* (Karpati et al., 2008) and in several noctuid moths (e.g. Berg et al., 2002; Greiner et al., 2004; Sadek et al., 2002).

In the male AL, we observed two enlarged glomeruli at the entrance of the antennal nerve. One of these resembles the cumulus, which has been found in several other moths and is known to process sex pheromone information (Hansson and Anton, 2000). An array of seven glomeruli is closely associated with the cumulus, and intracellular recordings from stained PNs (Fig. 4A) suggest that five of these glomeruli are part of a macroglomerular complex known from other moths (Hansson et al., 1992).

The female AL did not contain large female glomeruli described from *M. sexta* (King et al., 2000), *Heliothis virescens* (Berg et al., 2002) and *O. nubilalis* (Karpati et al., 2008). However, as in females of *G. molesta* (Varela et al., 2009) and the cotton leafworm *Spodoptera littoralis* (Ochieng et al., 1995), an OG (OG 16), at the entrance of the AN, was in a similar position as the cumulus in males. Physiological characterisation of a PN innervating this glomerulus showed that it processes self-released sex pheromone in addition to plant volatiles (Fig. 4B).

Integration of behaviourally relevant chemical cues

The response specificity of AL neurons to the tested compounds varied widely. Approximately one quarter of the recordings showed specific responses to single compounds. In males, these highly tuned neurons responded mainly to the main pheromone compound whereas in females, more neurons responded specifically to plant compounds. Other neurons responded to a broader spectrum of stimuli, and there was a wide variation in response patterns between the tested neurons. A growing number of studies demonstrates that this functional heterogeneity in AL neuron responses provides a means of discriminating between odours (Christensen and Hildebrand, 2002). In particular, different temporal patterns of spiking activity facilitate the recognition of chemically related stimuli with overlapping spatial representation (Lei et al., 2004).

Pheromone-sensitive AL neurons were found in both sexes. In the male AL, several pheromone-sensitive neurons were activated by the minor components *EZ*-codlemone and codlemone acetate (Table 1). Both compounds are behavioural synergists when added in small amounts with codlemone, whereas larger amounts produce an antagonistic effect on male upwind flights (Witzgall et al., 2001). They are also the main pheromone components of several closely related species (Witzgall et al., 1996). Pheromone-sensitive PNs innervated the cumulus and five closely associated glomeruli,

suggesting that these glomeruli are part of an MGC, analogous to that found in other moths (Christensen and Hildebrand, 1987; Christensen et al., 1991; Hansson et al., 1991; Karpati et al., 2008). Responses to sex pheromone components were also observed in PNs innervating two OGs apart from the MGC (Fig. 4A), and we conclude that sex pheromone information in codling moth males is integrated in an across-glomerular pattern, similar to that found in the turnip moth *Agrotis segetum* and *S. littoralis* (Anton and Hansson, 1995; Hansson et al., 1994).

Auto-detection of female sex pheromone is known from several moths (e.g. Den Otter et al., 1996; Hansson et al., 1989; Schneider et al., 1998), including *C. pomonella* (Ansebo et al., 2004), and the central processing of sex pheromone information in females has previously been studied in *S. littoralis* (Anton and Hansson, 1995; Ochieng et al., 1995; Sadek et al., 2002) and *H. virescens* (Hillier et al., 2006). In codling moth females, pheromone components elicited a response in PNs innervating a group of OGs in the lateral region of the AL, close to the entrance of the AN; these PNs also responded to a number of plant volatiles (Table 2, Fig. 3D, Fig. 4B). Analogous glomeruli were also found in *H. virescens* and *S. littoralis* females (Hillier et al., 2006; Ochieng et al., 1995), suggesting that female moths have glomeruli, in a location corresponding to that of the male MGC, dedicated to the processing of sex pheromones.

To investigate the interaction between sex pheromones and plant volatiles, we tested a large number of biologically relevant plant compounds that belong to different chemical classes. In both sexes, we observed that a majority of the pheromone-sensitive AL neurons also responded to plant volatiles; for example, pheromone-sensitive PNs innervating the cumulus responded to some plant volatiles, particularly esters, and PNs innervating ordinary OGs also responded to sex pheromones (Fig. 3A, Fig. 4A). This interaction of signals, which has been previously found for *A. segetum* (Hansson et al., 1994) and *S. littoralis* (Sadek et al., 2002), may be explained by the interaction of intercalated LNs that transform the signals from the social and environmental input pathways (Christensen and Hildebrand, 2002; Galizia and Rossler, 2010; Ignell and Hansson, 2005). Alternatively, mixed responses may be explained by events at the peripheral level, where, e.g. in the case of *C. pomonella*, a subpopulation of ORNs is known to respond to both the main pheromone component and pear ester (Ansebo et al., 2005; De Cristofaro et al., 2004). Although we cannot exclude the possibility that the doses of plant volatiles and sex pheromone tested elicited unspecific responses in the ORNs, preliminary data suggest that this interaction of signals can be observed even using lower doses of odours (F.T., unpublished).

Among the plant volatiles that were tested, esters, the green leaf volatile (*Z*)3-hexenol and methyl salicylate evoked the highest activity in male and female AL neurons. These compounds all elicit a strong response from antennae (Ansebo et al., 2004) and they also play a behavioural role. Pear ester attracts males and females and has been shown to mediate the location of food sources and oviposition sites (Knight and Light, 2001; Landolt et al., 2007; Light et al., 2001). Butyl hexanoate, a component of ripe apple headspace, attracts mated females (Bengtsson et al., 2001; Hern and Dorn, 2004), whereas (*Z*)3-hexenol enhances the attraction of males toward sex pheromone (Light et al., 1993; Yang et al., 2004). In contrast to males, the sesquiterpenes (*E*,*E*)-α-farnesene and (*E*)-β-farnesene elicited strong responses in female AL neurons (Tables 1, 2). (*E*,*E*)-α-farnesene, a main component of apple headspace (Bengtsson et al., 2001), is detected by very sensitive ORNs (Bäckman et al., 2000) and has been shown to contribute to the attraction of females (Hern and Dorn, 1999) and males, in combination with (*E*)-β-farnesene (Coracini et al., 2004).

The central processing of environmental signals has received less attention than the coding of sex pheromones, partially because a behavioural role has been assigned to plant volatiles in only a few species. In codling moth males and females, few glomeruli processed information about single plant volatiles. Instead, most glomeruli participated in the integration of a number of plant odours, including compounds belonging to different chemical classes (Tables 1, 2). This is in line with what has been found in *L. botrana* (Masante-Roca et al., 2005), and *S. littoralis* (Anton and Hansson, 1995; Carlsson et al., 2002; Sadek et al., 2002). We successfully obtained intracellular stainings of uniglomerular PNs, from different preparations, innervating the same glomerulus. In the male AL, PNs 12 and 13, which both innervated OG 23, showed a tonic response to (*Z*)-3-hexenol and methyl salicylate, whereas they differed in the rest of their response spectrum (Table 1A). Similarly, among the three PNs that innervated OG 36, PNs 18 and 19 responded, each with different response dynamics, to butyl hexanoate, hexenyl 2-methyl-butanoate, methyl salicylate, linalool, 4,8-dimethyl-1,3-nonatriene and (*Z*)-3-hexenol, whereas PN 17 responded to only three of these stimuli (Table 1A). Even with partially overlapping activity patterns, we observed a tendency for a chemotopic pattern in the AL of both sexes (Fig. 4). A topographical organisation of the responses to general odours has previously been proposed for *Drosophila melanogaster* on the basis of ORN responses and the glomeruli they innervate (Couto et al., 2005), and by means of optical imaging in honeybee and moths (Carlson et al., 2002; Hansson et al., 2003; Joerges et al., 1997). Our results support the idea that plant odour discrimination is based on a combinatorial spatial and temporal olfactory code (Christensen and Hildebrand, 2002; Riffell et al., 2009).

Blend interactions

Blend interactions among pheromone components have been extensively studied in the moth AL (Anton and Hansson, 1994; Christensen et al., 1991; Hansson et al., 1994; Lei and Vickers, 2008; Vickers et al., 1998), whereas less is known about coding of blends of pheromones and general odours (Namiki et al., 2008). We examined the responses of male and female AL interneurons to binary blends of sex pheromone components, plant odours and their combination (Tables 1, 2), as both synergistic and suppressive, as well as blend-specific, responses can transmit signals of blend interaction to higher brain centres and thus explain behavioural effects of odour blends.

Addition of *EZ*-codlemone to the main pheromone component codlemone has a synergistic or antagonistic effect on male attraction, depending on the blend ratio (Witzgall et al., 2001). A 1:1 blend of *EZ*-codlemone and codlemone elicited several suppressive interactions in male AL neurons. It remains to be determined whether these neurons are sensitive to the blend ratio and how their activity correlates with the behavioural output. A puzzling observation is that codling moth males discriminate between codlemone alone and 1:1 blends of codlemone and *EZ*-codlemone only during the initiation of the upwind flight response and not during upwind flight (Trona et al., 2010).

Stimulation with a blend of codlemone and dodecanol synergised the response of PNs responding to codlemone alone, as well as of neurons that did not respond to individual compounds, in both males and females (Table 1). Pheromone blend-specific neurons have been described in several moth species (Hansson and Anton, 2000; Vickers et al., 1998; Wu et al., 1996). A synergistic blend interaction

of codlemone and dodecanol in the AL confirms a behavioural role of the saturated alcohol, which is an ingredient of commercial mating disruption formulations (Witzgall et al., 2001; Witzgall et al., 2008).

Plant volatiles may enhance the attraction of codling moth males to codlemone (Knight et al., 2005; Yang et al., 2004). This is in line with our finding that blends of codlemone and plant volatiles elicited increased response, blend-specific responses or inhibition in AL neurons. Similarly, in *B. mori*, simultaneous exposure to the main pheromone component and to a plant odour increased the responses of the pheromone-sensitive PNs, innervating the MGC (Namiki et al., 2008).

Mixture interactions between general odours are known from other moth species (Carlsson et al., 2007; Namiki et al., 2008; Piñero et al., 2008), and we found consistent effects in male and female AL neurons to the mixture of pear ester and acetic acid, which has been shown to be behaviourally synergistic in both sexes (Landolt et al., 2007).

Conclusions

The response of AL neurons to a wide range of biologically relevant odours demonstrates complex activation patterns in the processing of olfactory cues in the codling moth. Information on social and general odours is seemingly integrated in across-glomerular coding patterns, suggesting a more complex level of interaction between the two olfactory subsystems than previously observed in other species. Future studies will focus on how odour concentration affects this interaction between sex pheromones and plant volatile information in the AL. An integrated analysis of blend interaction will involve both AL coding and behavioural studies.

LIST OF ABBREVIATIONS

AL	antennal lobe
AN	antennal nerve
LN	local interneuron
MGC	macroglomerular complex
OG	ordinary glomerulus
ORN	olfactory receptor neuron
PN	projection neuron

ACKNOWLEDGEMENTS

We thank Zsolt Karpati for technical assistance and helpful discussions, and Sylvia Anton for constructive criticism and comments on the manuscript.

REFERENCES

Ansebo, L., Coracini, A., Bengtsson, M., Liblikas, I., Ramìrez, M., Borg-Karlson, A. K., Tasin, M. and Witzgall, P. (2004). Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. *J. Appl. Entomol.* **128**, 488-493.

Ansebo, L., Ignell, R., Löfqvist, J. and Hansson, B. (2005). Responses to sex pheromone and plant odours by olfactory receptor neurons housed in sensilla auricillica of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *J. Insect Physiol.* **51**, 1066-1074.

Anton, S. and Hansson, B. S. (1994). Central processing of sex pheromone, host odour, and oviposition deterrent information by interneurons in the antennal lobe of female *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J. Comp. Neurol.* **350**, 199-214.

Anton, S. and Hansson, B. S. (1995). Sex pheromone and plant-associated odour processing in the antennal lobe interneurons of male *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J. Comp. Physiol. A* **176**, 773-789.

Anton, S. and Hansson, B. S. (1999). Physiological mismatching between neurons innervating olfactory glomeruli in a moth. *Proc. R. Soc. Lond. B Biol. Sci.* **266**, 1813-1820.

Bäckman, A. C., Bengtsson, M. and Witzgall, P. (1997). Pheromone release by individual females of codling moth, *Cydia pomonella. J. Chem. Ecol.* **23**, 807-815.

Bäckman, A. C., Anderson, P., Bengtsson, M., Löfqvist, J., Unelius, C. R. and Witzgall, P. (2000). Antennal response of codling moth males, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), to the geometric isomers of codlemone and codlemone acetate. *J. Comp. Physiol. A* **186**, 513-519.

Bengtsson, M., Bäckman, A. C., Liblikas, I., Ramirez, M. I., Borg-Karson, A. K., Ansebo, L., Anderson, P., Löfqvist, J. and Witzgall, P. (2001). Plant odor analysis of Apple: antennal response of codling moth females to apple volatiles during phonological development. *J. Agric. Food Chem.* **49**, 3736-3741.

Berg, B. G., Galizia, C. G., Brandt, R. and Mustaparta, H. (2002). Digital atlases of the antennal lobe in two species of tobacco budworm moths, the oriental *Helicoverpa assulta* (male) and the American *Heliothis virescens* (male and female). *J. Comp. Neurol.* **446**, 123-134.

Carlsson, M. A., Galizia, C. G. and Hansson, B. S. (2002). Spatial representation of odours in the antennal lobe of the moth *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Chem. Senses* **27**, 231-244.

Carlsson, M. A., Chong, K. Y., Daniels, W., Hansson, B. S. and Pearce, T. C. (2007). Component Information is preserved in glomerular responses to binary odor mixtures in the moth *Spodoptera littoralis. Chem. Senses* **32**, 433-443.

Christensen, T. A. and Hildebrand, J. G. (1987). Male-specific, sex pheromone-selective projection neurons in the antennal lobes of the moth *Manduca sexta. J. Comp. Physiol. A* **160**, 553-569.

Christensen, T. A. and Hildebrand, J. G. (2002). Pheromonal and host-odor processing in the insect antennal lobe: how different? *Curr. Opin. Neurobiol.* **12**, 393-399.

Christensen, T. A., Mustaparta, H. and Hildebrand, J. G. (1991). Chemical communication in heliothine moths. II. Central processing of intra and interspecific olfactory messages in the male corn earworm moth *Helicoverpa zea. J. Comp. Physiol. A* **169**, 259-274.

Coracini, M., Bengtsson, M., Liblikas, I. and Witzgall, P. (2004). Attraction of codling moth males to apple volatiles. *Entomol. Exp. Appl.* **110**, 1-10.

Couto, A., Alenius, M. and Dickson, B. J. (2005). Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol.* **15**, 1535-1547.

De Bruyne, M. and Baker, T. C. (2008). Odor detection in insects: volatile codes. *J. Chem. Ecol.* **34**, 882-897.

De Cristofaro, A., Ioriatti, C., Pasqualini, E., Anfora, G., Germinara, G. S., Villa, M. and Rotundo, G. (2004). Electrophysiological responses of *Cydia pomonella* (L.) to codlemone and pear ester ethyl (*E*,*Z*)-2,4-decadienoate: peripheral interactions in their perception and evidences for cells responding to both compounds. *Bull. Insectology* **57**, 137-144.

Den Otter, C. J., De Cristofaro, A., Voskamp, K. E. and Rotundo, G. (1996). Electrophysiological and behavioural responses of chestnut moths, *Cydia fagiglandana* and *C. splendana* (Lep., Tortricidae), to sex attractants and odours of host plant. *J. Appl. Entomol.* **120**, 413-421.

Galizia, C. G. and Rössler, W. (2010). Parallel olfactory system in insects: anatomy and function. *Annu. Rev. Entomol.* **55**, 399-420.

Greiner, B., Gadenne, C. and Anton, S. (2004). Three-dimensional antennal lobe atlas of the male moth, *Agrotis ipsilon*: a tool to study structure-function correlation. *J. Comp. Neurol.* **475**, 205-210.

Hansson, B. S. and Anton, S. (2000). Function and morphology of the antennal lobe: new developments. *Annu. Rev. Entomol.* **45**, 203-231.

Hansson, B. S., Löfqvist, J. and Van der Pers, J. N. C. (1989). Comparison of male and female olfactory cell response to pheromone compounds and plant volatiles in the turnip moth, *Agrotis segetum. Physiol. Entomol.* **14**, 147-155.

Hansson, B. S., Christensen, T. A. and Hildebrand, J. G. (1991). Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth *Manduca sexta. J. Comp. Neurol.* **312**, 264-278.

Hansson, B. S., Ljungberg, H., Hallberg, E. and Lofstedt, C. (1992). Functional specialization of olfactory glomeruli in a moth. *Science* **256**, 1313-1315.

Hansson, B. S., Anton, S. and Christensen, T. A. (1994). Structure and functions of the antennal lobe neurons in the male turnip moth, *Agrotis segetum* (Lepidoptera: Noctuidae). *J. Comp. Physiol. A* **175**, 547-562.

Hansson, B. S., Carlsson, M. A. and Kalinova, B. (2003). Olfactory activation patterns in the antennal lobe of the sphinx moth, *Manduca sexta. J. Comp. Physiol. A* **189**, 301-308.

Hern, A. and Dorn, S. (1999). Sexual dimorphism in the olfactory orientation of adult *Cydia pomonella* in response to α-farnesene. *Entomol. Exp. Appl.* **92**, 63-72.

Hern, A. and Dorn, S. (2004). A female-specific attractant for the codling moth, *Cydia pomonella*, from apple fruit volatiles. *Naturwissenschaften* **91**, 77-80.

Hillier, N. K., Kleineidam, C. and Vickers, N. J. (2006). Physiology and glomerular projections of olfactory receptor neurons on the antenna of female *Heliothis virescens* (Lepidoptera Noctuidae) responsive to behaviourally relevant odors. *J. Comp. Physiol. A* **192**, 199-219.

Ignell, R. and Hansson, B. S. (2005). Insect olfactory neuroethology-an electrophysiological perspective. In *Methods in Insect Sensory Neuroscience* (ed. T. A. Christensen), pp. 319-347. Boca Raton, FL: CRC Press.

Ignell, R., Dekker, T., Ghanidia, M. and Hansson, B. S. (2005). The neural architecture of the mosquito deutocerebrum. *J. Comp. Neurol.* **493**, 207-240.

Joerges, J., Kuttner, A., Galizia, C. G. and Menzel, R. (1997). Representations of odours and odour mixtures visualized in the honeybee brain. *Nature* **387**, 282-288.

Kanzaki, R., Arbas, E. A., Strausfeld, N. L. and Hildebrand, J. G. (1989). Physiology and morphology of projection neurons in the antennal lobe of the male moth *Manduca sexta. J. Comp. Physiol. A* **165**, 427-453.

Karpati, Z., Dekker, T. and Hansson, B. S. (2008). Reversed functional topology in the antennal lobe of the male European corn borer. *J. Exp. Biol.* **211**, 2841-2848.

Kent, K. S., Harrow, I. D., Quartaro, P. and Hildebrand, J. G. (1986). An accessory olfactory pathway in Lepidoptera: the labial pit organ and its central projection in *Manduca sexta* and certain other sphinx moths and silk moths. *Cell Tissue Res.* **245**, 237-245.

King, J. R., Christensen, T. A. and Hildebrand, J. G. (2000). Response characteristics of an identified, sexually dimorphic olfactory glomerulus. *J. Neurosci.* **20**, 2391-2399.

Knight, A. L. and Light, D. M. (2001). Attractants from Bartlett pear for codling moth, *Cydia pomonella* (L.), larvae. *Naturwissenschaften* **88**, 339-342.

Knight, A. L., Hilton, R. and Light, D. M. (2005). Monitoring codling moth (Lepidoptera: Tortricidae) in apple with blends of ethyl (E,Z)-2,4-decadienoate and codlemone. *Environ. Entomol.* **34**, 598-603.

Koontz, M. A. and Schneider, D. (1987). Sexual dimorphism in neuronal projections from the antennae of silk moths (*Bombyx mori, Antherea polyphemus*) and the gypsy moth (*Lymantria dispar*). *Cell Tissue Res.* **245**, 237-245.

Landolt, P. J., Suckling, D. M. and Judd, G. J. R. (2007). Positive interaction of a feeding attractant and a host kairomone for trapping the codling moth, *Cydia pomonella* (L.). *J. Chem. Ecol.* **33**, 2236-2244.

Lei, H. and Vickers, N. (2008). Central processing of natural odor mixtures in insects. *J. Chem. Ecol.* **34**, 915-927.

Lei, H., Christensen, T. A. and Hildebrand, J. G. (2004). Spatial and temporal organization of ensemble representations for different odor classes in the moth antennal lobe. *J. Neurosci.* **24**, 11108-11119.

Light, D. M., Flath, R. A., Buttery, R. G., Zalom, F. G., Rice, R. E., Dickens, J. C. and Jang, E. B. (1993). Host-plant green-leaf volatiles synergize the synthetic sex pheromones of the corn earworm and codling moth (Lepidoptera). *Chemoecology* **4**, 145-152.

Light, D. M., Knight, A. L., Henrick, C. A., Rajapaska, D., Lingren, B., Dickens, J. C., Reynolds, K. M., Buttery, R. G., Merrill, G., Roitman, J. et al. (2001). A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). *Naturwissenschaften* **88**, 333-338.

Masante-Roca, I., Gadenne, C. and Anton, S. (2005). Three-dimensional antennal lobe atlas of male and female moths, *Lobesia botrana* (Lepidoptera: Tortricidae) and glomerular representation of plant volatiles in females. *J. Exp. Biol.* **208**, 1147-1159.

Mustaparta, H. (2002). Encoding of plant odour information in insects: peripheral and central mechanisms. *Entomol. Exp. Appl.* **104**, 1-13.

Namiki, S., Iwabuchi, S. and Kanzaki, R. (2008). Representation of a mixture of pheromone and host plant odor by antennal lobe projection neurons of the silkmoth *Bombyx mori*. *J. Comp. Physiol. A* **194**, 501-515.

Ochieng, S. A., Anderson, P. and Hansson, B. S. (1995). Antennal lobe projection patterns of olfactory projection neurons involved in sex pheromone detection in *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Tissue Cell* **27**, 221-232.

Ochieng, S. A., Park, K. C. and Baker, T. C. (2002). Host plant volatiles synergize responses of sex pheromone-specific olfactory receptor neurons in male *Helicoverpa zea*. *J. Comp. Physiol. A* **188**, 325-333.

Piñero, J. C., Galizia, C. G. and Dorn, S. (2008). Synergistic behavioural responses of female oriental fruit moths (Lepidoptera: Tortricidae) to synthetic host plant-derived mixtures are mirrored by odor-evoked calcium activity in their antennal lobes. *J. Insect Physiol.* **54**, 333-343.

Reddy, G. V. P. and Guerrero, A. (2004). Interactions of insect pheromones and plant semiochemicals. *Trends Plant Sci.* **9**, 253-261.

Riffel, J. A., Lei, H., Christensen, T. A. and Hildebrand, J. G. (2009). Characterization and coding of behaviourally significant odor mixtures. *Curr. Biol.* **19**, 335-340.

Roelofs, W. L., Comeau, A., Hill, A. and Milicevic, G. (1971). Sex attractant of the codling moth: characterization with electroantennogram technique. *Science* **174**, 297-299.

Rospars, J. P. and Hildebrand, J. G. (1992). Anatomical identification of glomeruli in the antennal lobes of the male sphinx moth *Manduca sexta*. *Cell Tissue Res.* **270**, 205-227.

Rospars, J. P. and Hildebrand, J. G. (2000). Sexually dimorphic and isomorphic glomeruli in the antennal lobes of the sphinx moth *Manduca sexta*. *Chem. Senses* **25**, 119-129.

Sadek, M. M., Hansson, B. S., Rospars, J. B. and Anton, S. (2002). Glomerular representation of plant volatiles and sex pheromone components in the antennal lobe of the female *Spodoptera littoralis*. *J. Exp. Biol.* **205**, 1363-1376.

Schneider, D., Schultz, S., Priesner, E., Ziesmann, J. and Francke, W. (1998). Autodetection and chemistry of female and male pheromone in both sexes of the tiger moth *Panaxia quadripunctaria*. *J. Comp. Physiol. A* **182**, 153-161.

Tasin, M., Bäckman, A.-C., Bengtsson, M., Ioriatti, C. and Witzgall, P. (2006). Essential host plant cues in the grapevine moth. *Naturwissenschaften* **93**, 141-144.

Trona, F., Casado, D., Coracini, M., Bengtsson, M., Ioriatti, C. and Witzgall, P. (2010). Flight tunnel response of codling moth *Cydia pomonella* to blends of codlemone, codlemone antagonists and pear ester. *Physiol. Entomol.* **35**, 249-253.

Varela, N., Couton, L., Gemeno, C., Avilla, J., Rospars, J. P. and Anton, S. (2009). Three-dimensional antennal lobe atlas of the oriental fruit moth, *Cydia molesta* (Busck) (Lepidoptera: Tortricidae): comparison of male and female glomerular organization. *Cell Tissue Res.* **337**, 513-526.

Vickers, N. J., Christensen, T. A. and Hildebrand, J. G. (1998). Combinatorial odor discrimination in the brain: attractive and antagonist odor blends are represented in distinct combinations of uniquely identifiable glomeruli. *J. Comp. Neurol.* **400**, 35-56.

Wearing, C. H. and Hutchins, R. F. N. (1973). α-farnesene, a naturally occurring oviposition stimulant for the codling moth, *Laspeyresia pomonella*. *J. Insect Physiol.* **19**, 1251-1256.

Weddle, P. W., Welter, S. C. and Thomson, D. (2009). History of IPM in California pears-50 years of pesticide use and the transition to biologically intensive IPM. *Pest Manag. Sci.* **65**, 1287-1292.

Witzgall, P., Chambon, J.-P., Bengtsson, M., Unelius, C. R., Appelgren, M., Makranczy, G., Muraleedharan, N., Reed, D. W., Hellrigl, K., Buser, H.-R. et al. (1996). Sex pheromones and attractants in the Eucosmini and Grapholitini (Lepidoptera, Tortricidae). *Chemoecology* **7**, 13-23.

Witzgall, P., Bäckman, A.-C., Svensson, M., Koch, U. T., Rama, F., El-Sayed, A., Brauchli, J., Arn, H., Bengtsson, M. and Löfqvist, J. (1999). Behavioral observations of codling moth, *Cydia pomonella*, in orchards permeated with synthetic pheromone. *BioControl* **44**, 211-237.

Witzgall, P., Bengtsson, M., Rauscher, S., Liblikas, I., Bäckman, A. C., Coracini, M., Anderson, P. and Löfqvist, J. (2001). Identification of further sex pheromone synergists in the codling moth, *Cydia pomonella*. *Entomol. Exp. Appl.* **101**, 131-141.

Witzgall, P., Stelinski, L., Gut, L. and Thomson, D. (2008). Codling moth management and chemical ecology. *Annu. Rev. Entomol.* **53**, 503-522.

Witzgall, P., Kirsch, P. and Cork, A. (2010). Sex pheromones and their impact on pest management. *J. Chem. Ecol.* **36**, 80-100.

Wu, W., Anton, S., Lofstedt, C. and Hansson, B. S. (1996). Discrimination among pheromone component blends by interneurons in male antennal lobes of two populations of the turnip moth, *Agrotis segetum*. *Proc. Natl. Acad. Sci. USA* **93**, 8022-8027.

Yang, Z., Bengtsson, M. and Witzgall, P. (2004). Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. *J. Chem. Ecol.* **30**, 619-629.