

# Olfaction in the female sheep botfly

Simone Poddighe · Teun Dekker · Antonio Scala ·  
Anna Maria Angioy

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**Abstract** The nasal botfly *Oestrus ovis* (Diptera, Cyclorhapha: Oestridae) is a myiasis-causing insect species, which affects the health of sheep, goats and humans. Gravid females are viviparous and larviposit into the animal's nostrils. Host-searching and larvipositing flies are visually guided and influenced by climatic conditions, whereas olfaction seemed to play no role in this process. However, here, we show that the antennae of adult *O. ovis* female flies are relatively small but well developed and inhabited by several types of olfactory sensilla. Further, we show that the antennal lobes of this species receive input from antennal afferents and consist of a clearly defined glomerular organisation. We also give the first evidence of the fly's ability to detect several synthetic odour compounds. Our findings provide a morpho-functional basis for future investigations on olfactory-mediated behaviour of this insect pest.

**Keywords** *Oestrus ovis* · Antennal sensilla · Antennal lobes · Electroantennogram · Odour volatiles

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S. Poddighe (✉) · A. M. Angioy  
Department of Experimental Biology, University of Cagliari,  
I 09042 Monserrato, Cagliari, Italy  
e-mail: s.poddighe@unica.it

T. Dekker  
Department of Crop Science, Division of Chemical Ecology,  
Swedish University of Agricultural Sciences,  
P.O. Box 102, 230 53 Alnarp, Sweden

A. Scala  
Department of Animal Biology, Section of Parasitology and  
Parasitic Disease, University of Sassari,  
Via Vienna 2,  
07100 Sassari, Italy

## Introduction

The sheep's nasal botfly *Oestrus ovis* L. (Diptera, Cyclorhapha: Oestridae) is a myiasis-causing insect species frequently found in Mediterranean areas and in dry tropical countries (Touré 1994; Pampiglione et al. 1997; Scala et al. 2001; Papadopoulos et al. 2006). Fly larvae are obligatory parasites of sheep and goats and may severely affect the health of their host (Jacquet and Dorchies 2002).

Adult *O. ovis* flies do not feed during their short life (Dorchies and Alzieu 1997; Jacquet and Dorchies 2002). After mating, the viviparous female larviposits by striking first-instar larvae on the nostrils of the host. Larvae are provided with a number of spines and sensilla, allowing them to hold on to the host's muzzle and to search for a suitable site to develop, respectively (Colwell and Scholl 1995). They migrate from the nasal cavities to the ethmoid and the sinus cavities, where they develop to last-instar larvae. The latter move backward toward the nasal cavities and are sneezed by the animal on the ground where they pupate (Jacquet and Dorchies 2002).

Important losses in animal productions are associated with the larval development of the parasite (Shcherban 1973; Ilchmann et al. 1976). In countries with a high prevalence in sheep and goats, myiasis can also occur in humans, causing infections mainly on the eyes (Zumt 1965; Pampiglione et al. 1997; Delhaes et al. 2001). In spite of the many studies on chemoprophylaxis and zoonosis control strategies (Scala et al. 2002; Sánchez-Andrade et al. 2005), the nasal botfly *O. ovis* still represents a serious pest in many countries with hot and dry climate.

Most investigations focused on parasite's larval control, whereas the physiology and behaviour of non-parasitic adult flies have received little attention. Control through trapping of gravid females can work in the oestrid species

*Cephenemyia trompe* and *Hypoderma tarandi* (Anderson and Olkowski 1968; Anderson and Nilssen 1996) and in dipteran pests (Welch 1988), but mechanisms of host location and larviposition by gravid female *O. ovis* flies are largely unknown. A field study indicated that *O. ovis* larviposition behaviour was strongly influenced by climatic conditions and possibly affected by the sight of a moving host, whereas it seemed independent of olfaction (Cepeda-Palacios and Scholl 2000). On the other hand, the ability to sense host-related odours has been proven in the oestrid species *C. trompe* and *H. tarandi* (Tømmerås et al. 1993, 1996). It is well known that olfactory cues play a major role in the location of carrion or animal host as oviposition sites by egg-laying dipteran pest species (Eisemann 1988; Ashworth and Wall 1994). By recording antennal receptor neuron activity, specific responses to host odours were demonstrated in the female Australian sheep blowfly, *Lucilia cuprina* (Park and Cork 1999). Sensory and behavioural responses to host volatiles were described in the stable fly, *Stomoxys calcitrans* (Jeanbourquin and Guerin 2007).

Here, we describe the morphology of the primary olfactory structures, the antennae, and the first centre of olfactory processing, the antennal lobes (ALs), in the female nose botfly, *O. ovis*. We further investigated if female flies are able to detect odour stimuli. The study provides a basis for application of physiological investigation, allowing research on trapping of this insect pest.

## Materials and methods

### Insects

Larvae of *O. ovis* were collected from heads of freshly slaughtered sheep at the abattoir in Thiesi and Settimo San Pietro, Sardinia, Italy, in the periods June–July 2008 and 2009. They were identified to species and instar according to keys described by Zumpt (1965). For pupation, single third-instar larvae were placed on sand in 500-mL glass beakers covered with metal gauze. All components were previously sterilised in an autoclave. Insects were maintained at constant environmental conditions (24–25°C; 60% RH; L:D=12:12). The pupal stage lasted 20.80±1.02 days. For the experiments, 2–3-day-old adult females were used.

### Scanning electron microscopy

The antennae of eight specimens were excised, fixed in 70% ethanol at 4°C, sonicated in 70% ethanol for 30 s (Labasco Bandelin Sonorex TK 30; Bandelin Electronic, Berlin, Germany), washed for 1 min in chloroform and then three times for 5 min each in 70% ethanol, mounted with

tape on aluminium stubs and coated with gold/palladium (3:2) by using a JEOL JFC-1100 (Jeol Fine Coat Ion sputter, Tokyo, Japan) before examination in a LEO 435VP microscope (LEO Electron Microscopy Ltd., Cambridge, UK). Micrograph files at a size of 1,024×768 pixels were processed on a standard Windows 2000 platform using Image Pro Plus 3.0 for Windows.

### Histological techniques

Anti-synapsin staining of the insect brain was performed according to the protocol of Dekker et al. (2006). Whole heads of eight specimens were fixed for 24 h at room temperature in 4% paraformaldehyde in phosphate-buffered saline with added 0.25% Triton X (PBST, pH 7.2). Brains were dissected out, washed three times for 5 min in PBST and incubated in an anti-synapsin primary antibody (Hybridoma, University of Iowa, Iowa, IA, USA), diluted 1:10 in PBST (volume/volume). Brains were subsequently incubated in a goat anti-mouse secondary antibody coupled to Alexafluor 546 (Molecular Probes, Carlsbad, CA, USA), diluted 1:100 in PBST. Each incubation lasted 24 h at 4°C and was followed by three 5-min washes in PBST.

Anterograde neurobiotin staining of the antennal nerve was made according to the protocol of Dekker et al. (2006). A solution of 2% neurobiotin (Molecular Probes) in 0.25 M KCl has been used for antennal backfills. Single living insects ( $n=6$ ) were inserted into a 1-mL truncated pipette tip with the antennae extending out from the tip. The pipette tip was fixed on a microscope slide with dental wax, and antennal observation was made by a Leica MZ8 (Leica Microsystems GmbH, Wetzlar, Germany). A glass capillary (inner diameter of 0.84 mm) containing the neurobiotin solution was placed over the whole flagellar surface and kept overnight at 4°C. Whole heads were fixed according to the protocol described for anti-synapsin staining. Brains were extracted, washed three times for 5 min each in PBST and incubated in a solution of 10% anti-synapsin coupled to 4% fluorescein-avidin 488 in PBST. Brains were subsequently incubated in a solution of 1% secondary antibody coupled to Alexafluor 546 in PBST. Each incubation lasted 24 h at 4°C and was followed by three 5-min washes in PBST. Brains were mounted in a Vectashield Hard set using double spacer rings (Secure-Seal imaging spacers, Sigma Aldrich, St. Louis, MO, USA) to protect them from pressure by the coverslip.

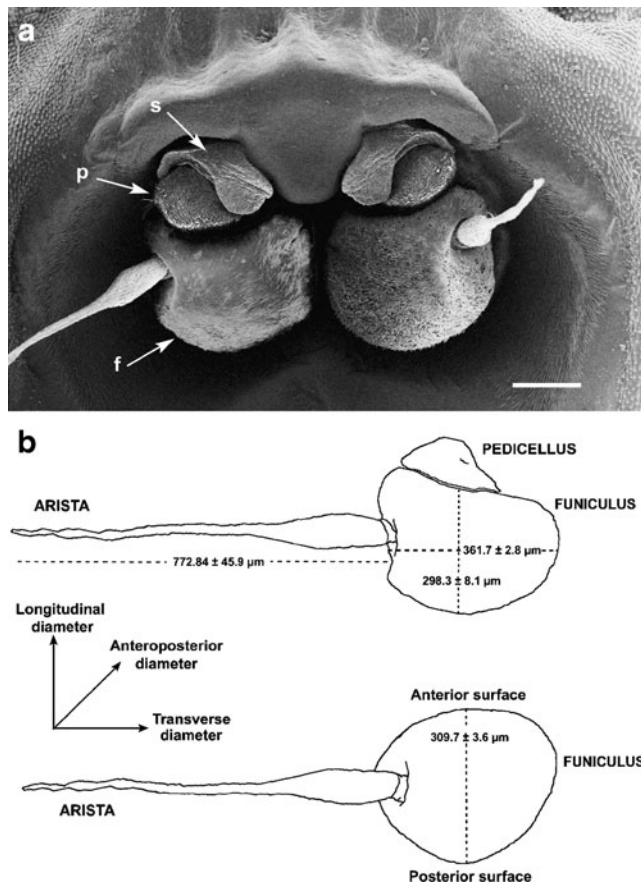
### Confocal microscopy

Preparations were viewed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) equipped with a ×40 oil-immersion objective lens. Structures labelled with Alexafluor 488 and fluorescein-avidin were excited with an argon

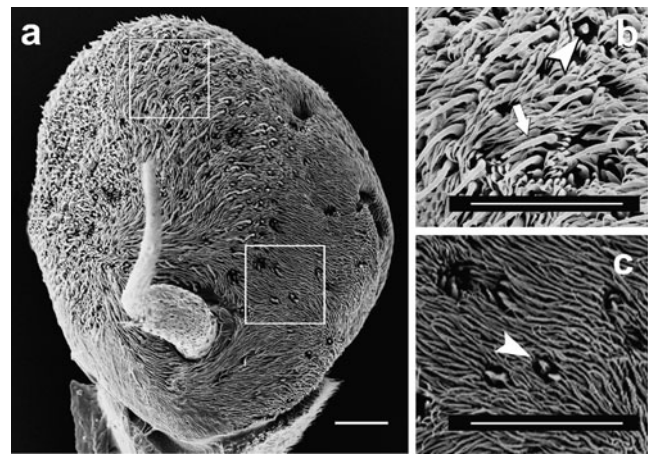
laser at 488 nm, and fluorescence was detected by filtering the emitted light in the range of 505–515 nm. Alexa 546-labelled structures were excited with a HeNe laser at 543 nm, and fluorescence was detected using a 560-nm long pass filter. Stacks of 50–200 images of each preparation were scanned and stored at 1,024×1,024 pixels. Image stacks of 14 specimens were examined. The ALs were analysed for size, morphology, number of glomeruli and possible differences between them. Image processing and 3D analysis were performed on a standard Windows XP platform using AMIRA 3.0 for Windows (Indeed-Visual Concepts GmbH, Berlin, Germany) according to Ignell et al. (2005).

Electroantennogram recordings

Live 2–3-day-old female flies were fixed supine with wax on a plastic holder and viewed with an Olympus BX51WI light microscope (Olympus, Tokyo, Japan). The recording electrode, a glass micropipette filled with NaCl 0.15 M and containing a silver wire, was gently positioned on the funiculus tip. A silver-wire indifferent electrode was put in

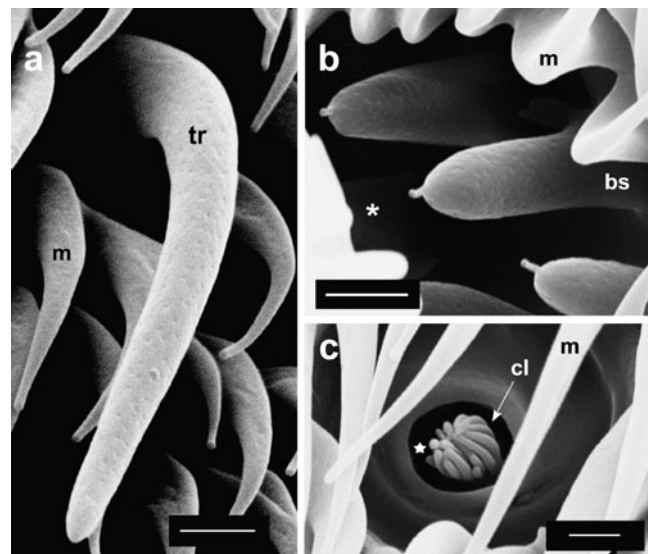


**Fig. 1** Antennae of a female *O. ovis* fly. **a** Frontal view of a pair of antennae in situ. *Arrows* point to the scapus (*s*), the pedicellus (*p*) and the flagellum (*f*). *Scale bar*=100 μm. **b** Diagrammatic reconstruction of the flagellum (*above*: frontal plane; *below*: dorsal plane). Numbers are mean values (±SE) of flagellar diameters (*n*=8)



**Fig. 2** Antennal sensilla of a female *O. ovis* fly. **a** Lateral view of a right antenna showing a different distribution of sensilla on the anterior funicular surface (*left side*) with respect to the posterior one (*right side*). **b** High-magnification photograph of the anterior funicular surface in the *left insert* in **a**. The *arrow* points to an elongated sensillum type. The *arrowhead* marks a smaller sensillum type lying in a pit. **c** High-magnification photograph of the posterior funicular surface in the *right insert* in **a**. The *arrowhead* points to two small-type sensilla located in a single pit, resembling the smaller sensillum type in **b**. *Scale bars*=50 μm

contact with the hemolymph through a small opening made below an eye. The electroantennogram (EAG) recording setup consisted of a preamplifier (Universal AC/DC probe ×10), a data acquisition interface (IDAC 4) and a data capture software (GC/EAD32 3.0) from Syntech (Hilversum, the Netherlands).



**Fig. 3** Sensilla on the funiculus of a female *O. ovis* fly. **a** A multiporous trichodeum sensillum (*tr*) extends above the level of microtrichia (*m*). *Scale bar*=2 μm. **b** Three multiporous basiconic sensilla (*bs*) are located in a pit (*asterisk*) surrounded by microtrichia (*m*). *Scale bar*=2 μm. **c** A coeloconic sensillum (*cl*) lies in a deep pit (*star*) with microtrichia (*m*) impending over it. *Scale bar*=1 μm

**Table 1** Morphometric data on funicular sensilla of female *O. ovis* flies

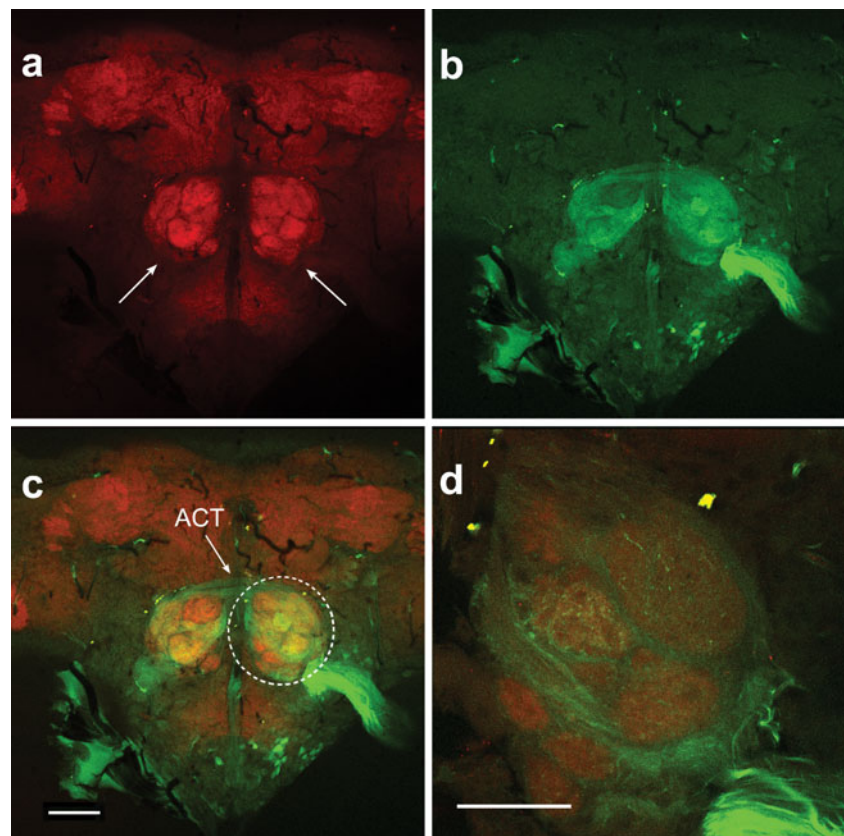
Surface	Sensilla			
	Type	Density ( $n/5,600\mu\text{m}^2$ )	Length ( $\mu\text{m}$ )	Pore density ( $n/\text{nm}^2$ )
Anterior	Trichoideum	40.0±2.7	17.3±0.3	14.5±1.8
	Basiconicum	15.1±0.7	6.4±0.2	25.0±1.3
	Coeloconicum	4–5	2.3±0.2	Undetermined
Posterior	Trichoideum	–	–	–
	Basiconicum	12.5±0.5	6.5±0.1	25.7±1.2
	Coeloconicum	4–5	2.3±0.1	Undetermined

Mean values±SE ( $n=8$ ). A dash indicates absence

A continuous airflow of a 1 L/min, adjusted by a Syntech air and stimulus delivery controller (CS-55), purified through a charcoal filter and humidified in a wash bottle, was blown over the preparation through a glass tube (inner diameter of 8 mm) which ended 2 cm from the insect's antennae. The tip of a Pasteur pipette containing an odour-loaded filter paper (5 mm × 25 mm) was inserted into a small hole (diameter of 3 mm) in the glass tube, 12 cm from the outlet. Odour stimulation was administered by injecting a puff of purified air (0.5 s at 10 mL/s airflow) through the Pasteur pipette using the Syntech stimulus delivery controller. Three microlitres of a stimulating solution was loaded on the filter paper.

Odour stimuli were applied according to a random sequence, allowing a 3-min interval between successive stimulations to avoid receptor adaptation. Each series started and ended with a control stimulation (pure air) followed by a blank one (solvent). Because of their stimulating and attractant effects on several dipteran species (Dethier and Yost 1952; Hribar et al. 1992; Tømmerås et al. 1996; Jeanbourquin and Guerin 2007), the following synthetic compounds were used: 1-hexanol; dimethyl disulphide (DMDS); dimethyl trisulphide (DMTS); butanoic, pentanoic and hexanoic acid; and  $\text{NH}_3$ . All chemicals were diluted in hexane at a 1% concentration, with the exception of 1-hexanol (10% in hexane) and  $\text{NH}_3$  (10% in

**Fig. 4** The ALs of a female *O. ovis* fly. **a** Immunocytochemical staining with anti-synapsin reveals a couple of defined morphological structures in the brain with the typical glomerular organisation (arrows). **b** Anterograde fills from the antennal flagella with neurobiotin reveal the projection of the antennal nerve axons into the brain. **c** Anterograde fills with neurobiotin combined with anti-synapsin staining (merged) show antennal nerve axons projecting to the ALs interconnected by an antenno-commissural tract (ACT). **d** The left AL enlarged (dashed circle in **c**) clearly showing innervations of the antennal glomeruli by ORN afferents. Scale bars=50  $\mu\text{m}$



water). Because of airflow water content, only hexane was used for blank stimulations. Recordings were made on 13 specimens.

## Results

Adult *O. ovis* flies are equipped with a pair of relatively small antennae located in a pair of shallow depressions of the head capsule area between the eyes (Fig. 1a). A fly antenna consists of a scapus, a pedicellus and a flagellum (Fig. 1a, b). The latter comprises a proximal subsegment, the subspherical and small-sized funiculus and a distal subsegment, the arista (Fig. 1b).

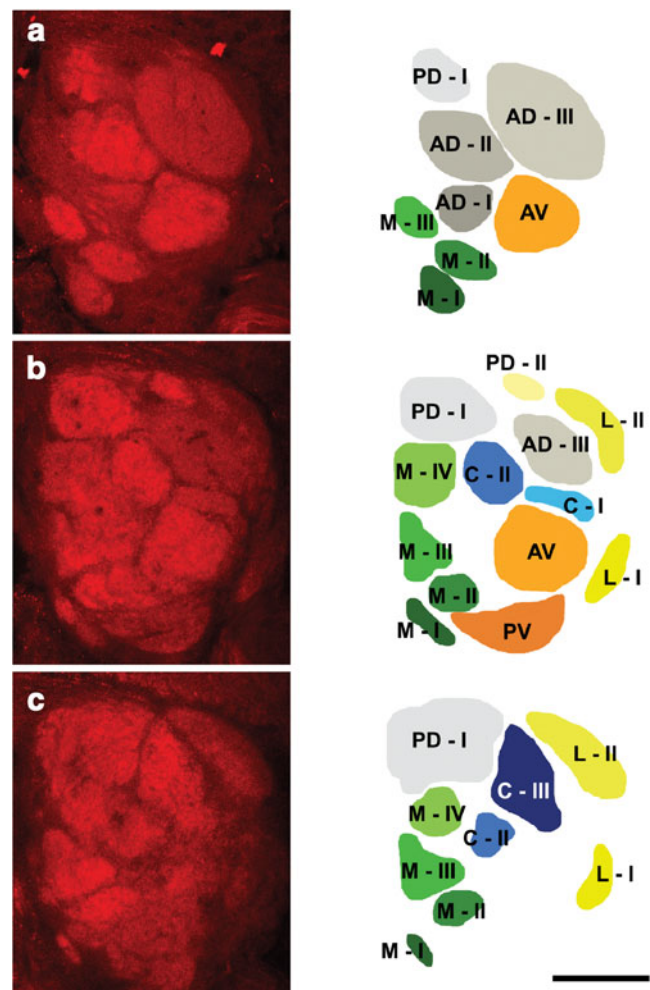
The funiculus bears several morphological types of sensilla, whose distribution is visible in Fig. 2. The anterior funicular surface contains trichoideum and basiconicum sensillum types (Fig. 2a, b). The trichoidea ones are elongated hair-shaped structures, ending with a rather pointed tip and presenting a multiporous wall (Fig. 3a). They extend well above microtrichia and average 17  $\mu\text{m}$  in length (Table 1). Sensilla basiconica are small structures, lying one to six in a single pit and displaying a blunt tip with a ball-shaped formation and a wall pierced by numerous pores (Fig. 3b). They are rather short, averaging 6  $\mu\text{m}$  in length (Table 1). Of these sensillum types, only the basiconicum one was found on the posterior funicular surface (Fig. 2a, c). Sensilla coeloconica, showing a wall with a typical cone-shaped palisade of cuticular fingers meeting at the tip, were also identified on both funicular surfaces (Fig. 3c, Table 1). They are the least abundant and the smallest funicular sensilla, averaging 2  $\mu\text{m}$  in length. Coeloconica sensilla are, however, not visible from previous figures because they are located in a deep pit and not extending above the level of microtrichia.

Female *O. ovis* flies have clearly defined ALs protruding from the anterior part of the brain (Fig. 4). The staining with the anti-synapsin antibody, through its restricted labelling of synapses, reveals the set of characteristic subunits, the so-called glomeruli (Fig. 4a). Anterograde fills from the antennal flagella with neurobiotin and subsequent labelling with fluorescein–avidin reveal the projection of antennal nerve axons into the brain (Fig. 4b). The anterograde neurobiotin and anti-synapsin combined stainings (Fig. 4c, d, merged) show antennal nerve axons projecting to and interconnecting the ALs (antenna-commissural tract; Fig. 4c) and targeting AL glomeruli (Fig. 4d).

A careful analysis of anti-synapsin stainings allowed the demarcation and identification of 16 AL glomeruli, located in the three planes shown from anterior to posterior in Fig. 5. The glomerular nomenclature used is based on the map developed for *Drosophila melanogaster* (Stocker et al. 1990), where the general position of a single glomerulus in

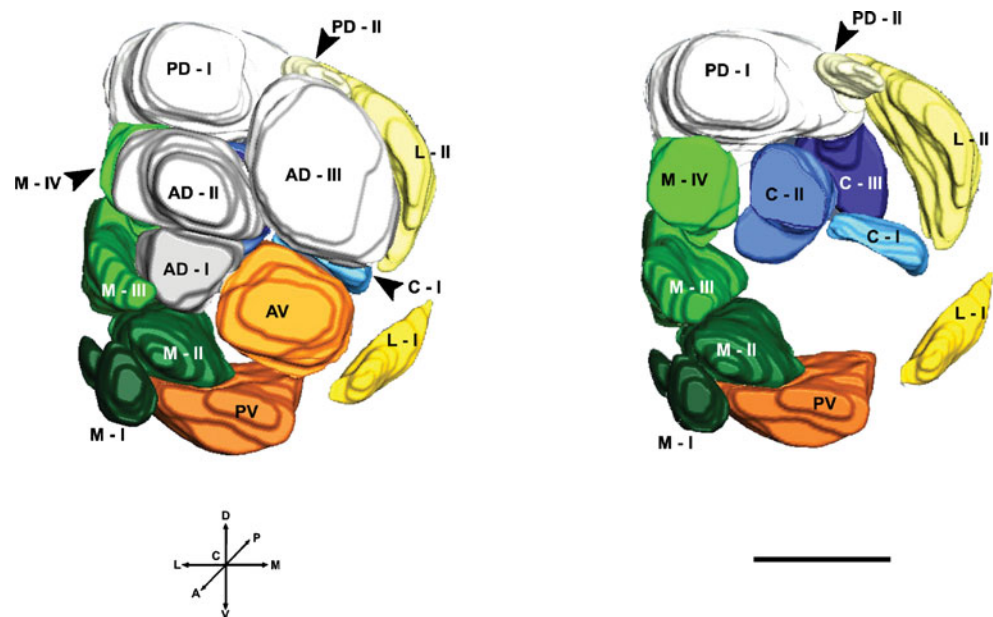
the AL is marked by one or two capital letters denoting the position, with numbers used as additional classifiers (A, anterior; D, dorsal; L, lateral; M, medial; P, posterior; V, ventral). Central glomeruli (C) are defined as having no contact with the AL periphery.

In the first plane (Fig. 5a), eight glomeruli were identified: three anterior-dorsal, one posterior-dorsal, three medial and one anterior-ventral. In the second plane (Fig. 5b), the identity of 13 glomeruli was recognisable: one anterior-dorsal, two posterior-dorsal, four medial, one anterior-ventral, three central, two lateral and one postero-ventral. In the third plane (Fig. 5c), nine glomeruli were identified: one posterior-dorsal, four medial, two central and two lateral. However, a precise delineation of some glomerular-like structures in the postero-ventral AL area was not always possible due to unclear borders of individual structures. A



**Fig. 5** Representative confocal stack of the left AL of a female of *O. ovis* as shown by anti-synapsin staining. Three frontal planes from anterior to posterior (a–c) were selected from a total of 77 images at 0.18- $\mu\text{m}$  intervals. Data are arranged pairwise, where left images display unaltered confocal micrographs and right images display the identified demarcated glomeruli. Scale bar=50  $\mu\text{m}$

**Fig. 6** Three-dimensional reconstruction of the left AL of a female of *O. ovis* as seen from an anterior (left) and a posterior perspective (right). Anterior glomeruli are removed from the complete model (left) in order to uncover the posterior glomeruli (right). A anterior, P posterior, L lateral, M medial, V ventral, D dorsal, C central. Scale bar=100  $\mu$ m



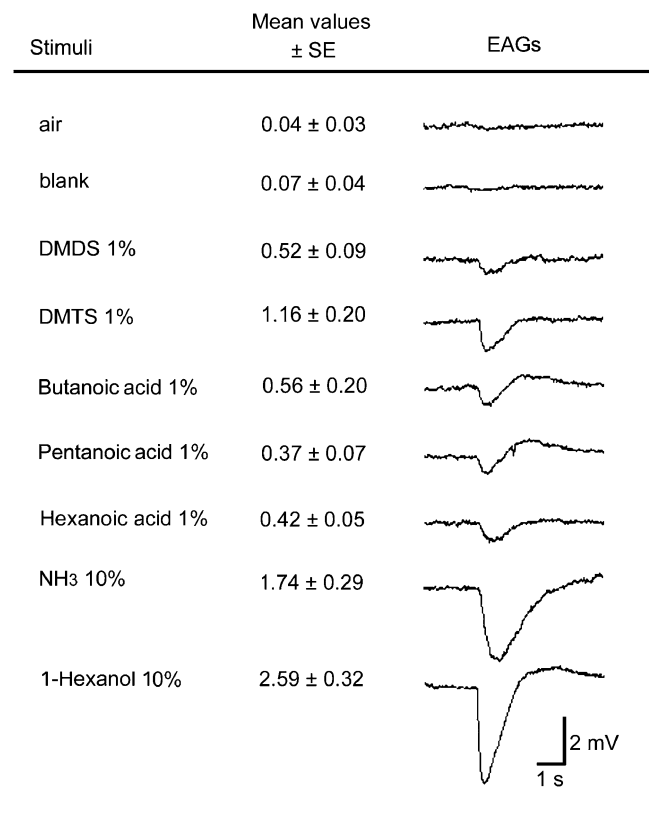
representative preparation was chosen for the development of the 3D model of the left AL shown in Fig. 6.

EAG responses of female *O. ovis* to stimulation with several odour compounds were recorded (Fig. 7). Results indicate that DMDS evoked the strongest response, compared with the other four stimuli applied at the same concentration (1%), i.e. DMDS, butanoic acid, pentanoic acid and hexanoic acid. A 10% concentration of 1-hexanol triggered a higher response compared to that of an equivalent concentration of ammonia.

## Discussion

Our neuroanatomical results demonstrate that the female nose of *O. ovis* possesses a well-developed olfactory system. Moreover, the EAG data show that female *O. ovis* flies are sensitive to several volatile compounds. Female insects may likely identify odour compounds of importance in behavioural performances. The results of a field study showed that the larviposition behaviour of *O. ovis* was mainly influenced by climatic factors and possibly affected by the sight of potential host movements, whereas it seemed independent of olfactory cues (Cepeda-Palacios and Scholl 2000). The experimental conditions and the kind of chemicals used for analysing the behaviour of wild specimens in the field may account for the above-mentioned lack of responsiveness to odour stimulation in *O. ovis*.

The external morphology of the three sensilla types identified on the antennae of female *O. ovis* flies conforms in many aspects to characters of antennal sensilla described in the female oestrid fly *Hypoderma bovis* (Hunter and Adserballe 1996) as well as in other dipteran species (Dethier et al. 1963; Keil 1999; Shanbhag et al. 1999). As in the case



**Fig. 7** EAG responses of female *O. ovis* flies. Sample of EAG responses (traces) to olfactory stimulation in a single specimen, and correspondent mean values  $\pm$  SE obtained from 13 flies are shown. Three microlitres of a stimulating solution (% = odour concentration) loaded on a filter paper was used. All chemicals were diluted in hexane, with the exception of ammonia (dissolved in water). DMDS dimethyl disulphide, DMTS dimethyl trisulphide

of *H. bovis* (Hunter and Adserballe 1996), *O. ovis* fly's funicular surface presents a high number of microtrichia and trichoidea sensilla and bears basiconica sensilla lying in pits. Less abundant coeloconica sensilla are singly located in deep pits on the funicular surface. The multiporous wall that the trichoidea and basiconica sensilla display implies that these sensilla accommodate olfactory receptor neurons (ORNs). On the other hand, it remains to be verified whether or not coeloconica sensilla have wall pores, a character typical of olfactory sensilla (Steinbrecht 1997; Keil 1999). As already described for very similar sensillar structures, a possible thermoreceptor or hygroreceptor function cannot be ruled out for coeloconica sensilla in *O. ovis* female flies (Keil 1999). Sensory information from funicular sensilla may guide female flies towards the proper host and larviposition site.

Results on brain morphology reveal that the arrangement of nervous pathways from flagella to the brain of *O. ovis* flies conforms in many aspects to that described ever since in other dipteran species (Strausfeld 1976; Stocker et al. 1990, 2001). Our findings demonstrate that one or more of the three funicular sensilla types accommodate first-order neurons of the fly's nervous pathway, which carry olfactory information to ALs. Compared to other dipteran species (Stocker et al. 1990, 2001; Steinbrecht 1997), a reduced number of funicular sensilla as well as of AL glomeruli were identified in the female nose botfly. However, even a sparsely equipped 'nose' could be of importance in guiding the behaviour of a female fly, which does not feed as adult. In this regard, odour perception has been demonstrated in *Trioza apicalis*, an insect species with an olfactory system comprising few antennal ORNs and aglomerular ALs (Kristoffersen et al. 2008a, b).

The EAG recordings demonstrate that several volatile compounds evoke antennal olfactory responses in female *O. ovis* flies. Among chemicals tested at a lower concentration (1%), DMTS was the most powerful olfactory stimulus for the nose botfly. Dimethyl disulphide was also highly effective in evoking an olfactory response. It has been proven that two reindeer parasite fly species belonging to the family of Oestridae, *H. tarandi* and *C. trompe*, are specifically able to sense DMTS emanating from the interdigital pheromone gland of the host (Tømmerås et al. 1996). Oligosulphides have been identified in volatile blends of several organic odour sources, such as livestock wastes (Mackie et al. 1998), emanations from the human skin (Bernier et al. 2000), dead-horse arum florets (Stensmyr et al. 2002) and cheese flavour (van Kranenburg et al. 2002). Dimethyl sulphide compounds play an important role in host location by *S. calcitrans* (Jeanbourquin and Guerin 2007) and strongly attract muscid and calliphorid fly species in the field (Nilssen et al. 1996; Stensmyr et al. 2002). Three compounds, butanoic acid, pentanoic acid and hexanoic acid, evoked EAG responses. These acids are components of the

steer–rumen volatile blend and evoked antennal responses in the stable fly *S. calcitrans*, a cattle parasite (Jeanbourquin and Guerin 2007). Based on the assumption that oligosulphide and acid compounds can also be released by *O. ovis* host, we hypothesised their detection over a short-distance range and tested them at a low chemical concentration (1%).

Responses to a 10% concentration of ammonia as well as of 1-hexanol show that both chemicals are effective olfactory stimuli for female *O. ovis* flies. In nature, high ammonia concentrations are mainly released from animal manure and fertilisers (Lee and Park 2002). Therefore, this chemical may serve as a host-related cue for *O. ovis* flies tracking herd of sheep or goats, even when moving over wide areas. Ammonia strongly attracts several insect pest species (Kendra et al. 2005), and its role in host location has been recognised in several dipteran species (Hribar et al. 1992; Geier et al. 1999; Smallegange et al. 2005). When tested at a high concentration, 1-hexanol vapours evoked high-amplitude EAG responses in *C. trompe* (Tømmerås et al. 1993) and *H. tarandi* (Tømmerås et al. 1996). Behavioural responses to olfactory stimulation with 1-hexanol (Dethier and Yost 1952), as well as EAG (Angioy et al. 1987) and single sensillum (Kaib 1974) responses, have been obtained in blowflies. In nature, volatile blends containing 1-hexanol are emitted by green plant sources (Kaib 1974; Budavari 1989; Bowen 1992). A release of this chemical volatile from grass trodden by a grazing herd could serve as a host-related cue for *O. ovis* flies. By assuming that *O. ovis* can detect ammonia and 1-hexanol released from herd tracks over long distances, the two chemicals have been tested at a 10% concentration.

The present study shows that female *O. ovis* flies are equipped with olfactory sensilla, which allow for the detection of various host-related odour volatiles. Axons of olfactory receptor neurons project to ALs, conveying olfactory information to target glomeruli. By demonstrating the ability of female flies to perceive odours, we provide a basis for future investigation on control strategies of this insect pest.

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