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Aglomerular Hemipteran Antennal Lobes—Basic Neuroanatomy of a Small Nose

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Abstract

We have compared the basic organization of the primary olfactory centre, the antennal lobe (AL), in 4 hemipteran species representing the 2 major lineages in this order. The Homoptera were represented by the psyllid *Trioza apicalis* and its aphid relatives the grain aphid *Sitobion avenae* Fabricius and the rose-grain aphid *Metopolophium dirhodum* Walker, whereas the Heteroptera were represented by the pentatomid stink bug *Euschistus heros* Fabricius. The olfactory systems of psyllids and aphids are generally very small, with low numbers of afferents in comparison to other insect groups, and the smallest described so far belongs to *T. apicalis*, comprising less than 50 olfactory receptor neurons (ORNs). Originally, we tried to estimate numbers of olfactory glomeruli in the AL of *T. apicalis*, which in insects generally correspond closely to the number of different types of ORNs. Neither immunocytochemical staining nor anterograde staining of ORNs revealed any glomerular structures in the ALs of *T. apicalis* or the 2 aphids that were included for comparison. In contrast, the ALs of the pentatomid stink bug *E. heros* displayed numerous distinct and well-delineated glomeruli, showing that aglomerular ALs are not typical of all insects within the order Hemiptera. Glomeruli are hallmark features of olfactory lobes in many different phyla, and the absence of glomerular structures in psyllids and aphids appears to be unique in insects that depend on olfactory orientation.

Key words: antennal lobe, aphid, glomeruli, Heteroptera, Homoptera, *Trioza apicalis*

Introduction

The insect's sense of smell is a complex and highly sensitive modality, governing essential decisions such as choice of mates, food, and oviposition sites. The olfactory sensory system is organized in a very similar fashion in most insects. In the antennal lobe (AL), the primary olfactory centre of the insect brain, axons of olfactory receptor neurons (ORNs) from the antenna synapse with AL neurons (local interneurons and projection neurons) in the first steps of olfactory processing. Synaptic interactions in the AL take place within spherical structures called glomeruli (Rospars 1988). Glomerular numbers vary greatly between species, ranging between 10 and 1000 (Rospars 1988). The number and spatial organization of glomeruli is species specific and consistent between individuals (Rospars 1988; Anton and Homberg 1999). The glomeruli are sites of high receptor neuron convergence in most insects, thereby increasing the signal-to-noise ratio (Boeckh J and Boeckh V 1979; Masson and Mustaparta 1990; Anton and Homberg 1999). The size of the glomeruli seems to be roughly correlated to the number of incoming afferents (Anton and Homberg 1999). This is clearly illustrated by the macroglomerular complex (MGC), a male specific, enlarged glomerular aggregation that exclusively handles sex pheromone input, present in species with female produced sex pheromones (Boeckh J and Boeckh V 1979; Matsumoto and Hildebrand 1981). Male sensitivity to pheromones can be extreme, and pheromone-specific ORNs are usually present in great numbers. As a consequence, the units of the MGC are larger than ordinary glomeruli (Boeckh J and Boeckh V 1979; Matsumoto and Hildebrand 1981). The glomeruli form a chemotopic map in the AL as each physiological type of olfactory receptor neuron, expressing one specific olfactory receptor gene, projects into a single glomerulus (Hansson et al. 1992; Vosshall et al. 2000). A given odorant may act as a ligand on more than one receptor type, and thereby

activate more than one glomerulus (Joerges et al. 1997; Carlsson et al. 2002).

Estimates of the number of glomeruli can likely be used as a shortcut for approximate comparisons of the number of physiological types of receptor neurons between most insects because there usually appears to be a near 1:1 relationship between the number of glomeruli and the number of physiological types of receptor neurons (Hansson et al. 1992; Vosshall et al. 2000). Our original aim with the present study was to estimate the number of glomeruli in the AL of the carrot psyllid *Trioza apicalis* Förster (Hemiptera: Homoptera: Psylloidea), which has a very limited olfactory system that may comprise the lowest number of receptor neurons ever described in an adult, odor-guided neopteran insect. Recent studies of the olfactory morphology and physiology of *T. apicalis* have revealed that carrot psyllid antennae bear no more than 20 olfactory sensilla, but possibly even as few as 13 (Kristoffersen et al. 2006). The total number of ORNs is around 50 and the total neuron count, including axons of mechanosensory neurons, at the base of the flagellum is 70–80 (Kristoffersen et al. 2006). Electrophysiological investigations have revealed that *T. apicalis* can detect numerous host odor components, including compounds that occur in its host carrot as well as in the conifer species serving as winter shelter plants (Kristoffersen et al. 2008). Despite the small size of the *Trioza* olfactory system, some redundancy on the neuronal level was observed, showing that the maximal number of olfactory receptor neuron types is considerably lower than 50 (Kristoffersen et al. 2008).

Our investigations of *T. apicalis* ALs revealed that these lacked any recognizable glomerular structures. We then broadened the comparative base of the study to include other hemipteran insects, in order to determine the extent of this unusual feature within the Hemiptera. Aphids (Aphidoidea) are the closest relatives to the Psylloidea and are similar in size, appearance, and general ecology. Both psyllids and aphids belong to the infraorder Sternorrhyncha within the Homoptera. All aphids have olfactory organs called primary rhinaria on the last 2 antennal segments (fifth and sixth), but apterous aphids in addition also have numerous secondary rhinaria, that is, placoid sensilla on segments 3–5 (Bromley et al. 1979). The number of chemoreceptor neurons in adult aphid antennae is up to 10-fold greater than that given for *Trioza* (Chapman 1982). We investigated the grain aphid *Sitobion avenae* Fabricius and the rose-grain aphid *Metopolophium dirhodum* Walker, neither of which showed any evidence of glomerular structures in the ALs. We also included the neotropical stink bug *Euschistus heros* Fabricius (Hemiptera: Heteroptera: Pentatomoidea) as a representative of the Heteroptera (true bugs), which form the other major lineage within the Hemiptera. There are few investigations concerning the antennal equipment of pentatomids and none concerning the genus *Euschistus*. However, both males and females of, for example, the green stink bug *Nezara viridula* L. have been shown to have several different types of olfactory sensilla, occurring in great numbers on the antennae, amassing to well over a thousand (Brézot et al. 1997), and olfactory neuron counts for other Heteroptera are also often given in the thousands (Chapman 1982). Unlike *T. apicalis* and the aphids, *E. heros* displayed numerous glomeruli typical of insect ALs.

Materials and methods

Insects

Trioza apicalis males and females were obtained from a culture originating from insects collected in Finland and southern Sweden and then continuously reared on carrot plants in a 20:4 h light: dark cycle at 20 °C day and 15 °C night temperature. Female apterous *M. dirhodum* were collected from cereal cultures in Svalöv Weibull SEED greenhouses in Svalöv, Sweden. We used alate female *S. avenae* from an Ervibank culture from Koppert Biological Systems, The Netherlands. *Euschistus heros* were obtained from Dr Jeffrey R. Aldrich, USDA-ARS Chemicals Affecting Insect Behavior Laboratory, MD.

Antennal backfills

We made anterograde antennal backfills of ORNs using 2 neuron tracers, neurobiotin (Vector Laboratories, Burlingame, CA) and tetrarhodamine dextran (Molecular Probes, Carlsbad, CA). We used 1% neurobiotin in 0.25 M KCl and 1% rhodamine dextran in distilled water, respectively. *Trioza apicalis* and aphids were inserted into 100-µl disposable plastic micropipette tips, with the head protruding at the tip, whereas *E. heros* were mounted on a microscope glass, ventral side facing up, and fixed with dental wax. In all preparations, parts of both antennae were cut, and glass capillaries (inner diameter 1.17 mm, Harvard Apparatus, Holliston, MA) filled with a solution of one of the given tracers were secured in dental wax and slipped over the antennae. For *T. apicalis*, we also made control preparations with uncut antennae. Preparations were incubated in closed petri dishes with moist paper to prevent desiccation for 36–48 h at +4 °C.

Histology and immunocytochemistry

Live animals were decapitated and antennae cut off at the base. Heads were then fixed in 4% paraformaldehyde solution in 0.01 M phosphate buffered saline solution (PBS) overnight at +4 °C. Heads were washed 3 × 10 min in PBS and subsequently dissected. In some cases, paraformaldehyde fixation and washes were done in PBS with added 0.25% Triton-X (PBST).

A mouse antisynapsin antibody (Klagges et al. 1996) was used for visualization of brain structures with high synaptic density. Brains were washed 3 × 30 min in PBST, followed by 30 min in PBST with 2% heat-inactivated normal goat serum (NGS) (Sigma, St. Louis, MO) prior to incubation

with the primary antibody at a dilution of 1:20 in PBST with 2% NGS for 12–48 h at 4 °C. Brains were then washed 3 × 30 min in PBST and 30 min in PBST with 2% NGS before incubation with the secondary antibody at a dilution of 1:100 in PBST with 2% NGS for 12–48 h at +4 °C. Secondary antibodies consisted of goat anti-mouse antibodies with either Alexa Fluor 488 or Alexa Fluor 546 conjugates (Molecular Probes). In some preparations of *T. apicalis* and aphids, TOTO-3 (Molecular Probes) was added together with the secondary antibody at a 1:1000 dilution for visualization of cell body nuclei. After incubation with secondary antibody, brains were washed 3 × 15 min in PBST, 3 × 15 min in PBS, dehydrated in a graded ethanol series to 100% ethanol, and mounted for viewing in methyl salicylate on glass slides with Secure-Seal imaging spacers (Sigma, St. Louis, MO). Several control brains from *T. apicalis* and aphids were processed in parallel with antisynapsin-stained brains, without adding the primary antibody, to confirm that visible structures were a result of antisynapsin staining rather than background fluorescence or unspecific binding of the secondary antibody (data not shown).

Rhodamine dextran–filled brains were either dehydrated and mounted for viewing after fixation and dissection or subjected to antisynapsin staining as described above. Neurobiotin fills were visualized by incubation with avidin conjugated to Oregon Green 488 (Molecular Probes) at a 1:1000 dilution. Avidin incubation was performed either as a 1-step process identical to that of the primary antibody described above or neurobiotin-filled brains were also subjected to synapsin staining, in which case the avidin was added together with the secondary antibody.

Confocal microscopy

A Zeiss LSM 510 confocal microscope equipped with a 40 × oil immersion objective lens was used for scanning whole-mount brains. Whole-mount brains were scanned in multitrack mode with up to 3 different excitation wavelengths simultaneously (488 nm for Alexa 488 or Oregon Green, 543 nm for rhodamine, and 633 nm for TOTO-3) for visualization of antennal nerve fills, antisynapsin staining, and cell body staining. Light from the preparation was split at 635 nm for the blue channel and at 545 nm for the red and green channels. The red channel was filtered with a long-pass filter at 560 nm and the green with a band-pass filter at 505–530 nm. Image data were captured as serial stacks through the tissue at 1-μm intervals.

Results

Background data and results from the different species included in the study are provided in Table 1. Immunocytochemical staining with antisynapsin antibodies was overall rather strong in all species, with antibodies penetrating through the whole brain of the smaller *T. apicalis* and aphids and at least through the whole ALs of the much larger *E. heros*. In *T. apicalis* and the aphids, large parts of the brain appeared relatively undifferentiated, with no unambiguously visible mushroom bodies with calyces. Nonetheless, other morphological structures with high synaptic density, like the central complex and optic lobes, were evident (Figure 1a,b).

In the homopteran species, immunocytochemical staining failed to reveal any apparent glomerular structures characteristic of ALs in other insects (Figure 1a,c). In these species, the location of the ALs was revealed through anterograde staining with rhodamine dextran from the antennae, whereas neurobiotin proved unsuccessful (Figures 2a and 3). The ALs of the homopteran species were approximately 20 μm in diameter, slightly smaller in *Trioza* than in the aphids, and located ventrally near the esophageal connectives. The ALs were surrounded by small clusters of cell bodies visible in some preparations and which appeared to number less than 100 in total. Presumably, many of these cell bodies represent projection neurons and local interneurons of the ALs, although there was no way to determine the identity of the TOTO-stained cell bodies (Figure 1c). Rhodamine-stained axons in *T. apicalis* primarily entered and filled the ALs, with a few axons bypassing the ALs and terminating in what appears to be the antennal mechanosensory and motor complex (AMMC) located more ventrally–posteriorly adjacent to the AL (Figure 2a). In the aphids, we did not precisely determine the termination site of neurons bypassing the ALs.

In contrast to the homopteran species, the heteropteran *E. heros* displayed clearly defined ALs, approximately 140 μm

Table 1 Species included in this investigation and approximate data about their body size, number of ORNs, brain size, AL size, and number of glomeruli

Species	Suborder	Superfamily	Body	ORNs	Brain[a]	AL	Glomeruli
Trioza apicalis	Homoptera	Psylloidea	2 mm	~50[b]	100 μm	20 μm	Absent
Sitobion avenae	Homoptera	Aphidoidea	3 mm	>200[c]	120 μm	20 μm	Absent
Metopolophium dirhodum	Homoptera	Aphidoidea	3 mm	>200[c]	120 μm	20 μm	Absent
Euschistus heros	Heteroptera	Pentatomoidea	12 mm	≥1000[c]	450 μm	200 μm	60–80

[a]Excluding optic lobes.
[b]Kristoffersen et al. (2006).
[c]Generic number based on Chapman (1982).

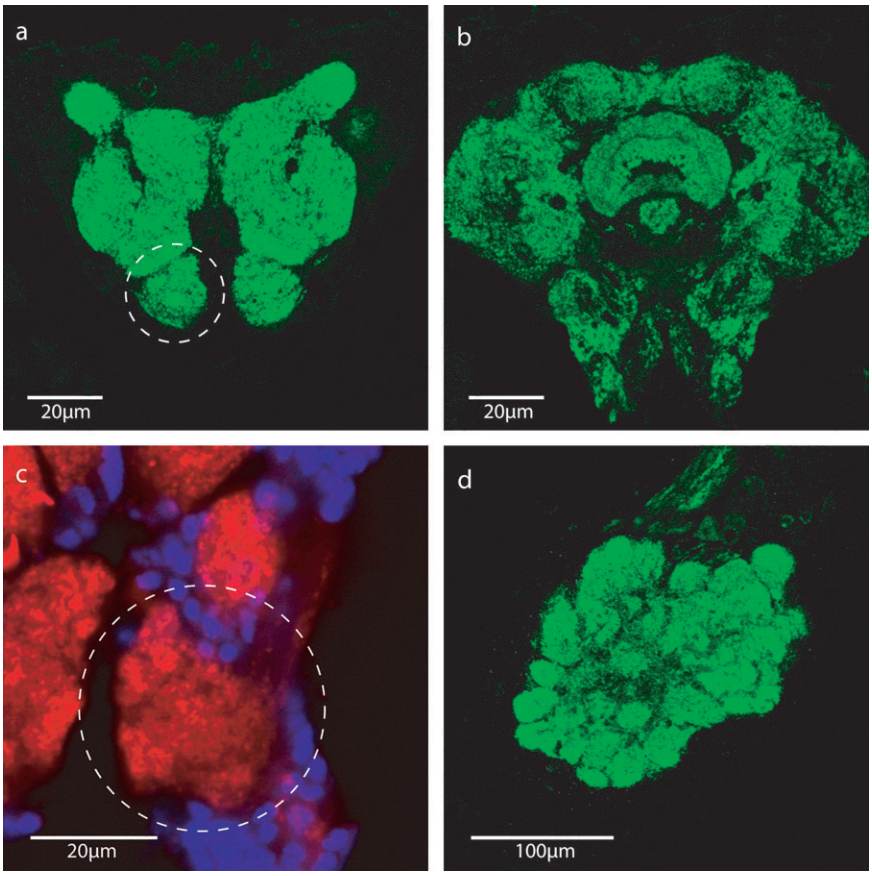

Figure 1 Immunocytochemical staining with antisynapsin revealed morphological structures in hemipteran brains. **(a)** Frontal section of the brain of a male *Trioza apicalis*. The left AL is indicated with a white dashed circle in the posteroventral part of the brain. No glomerular structures are apparent in the ALs, whose location became evident only through subsequent anterograde fills from the antennae. **(b)** A deeper section from the same brain showing a clearly delineated central complex, indicating that structures with high synaptic density were indeed visualized by the antisynapsin antibody. **(c)** Combined antisynapsin and TOTO-3 staining in a frontal section of an apterous female *Metopolophium dirhodum* aphid shows the left AL (white dashed circle) surrounded by cell bodies, most of which are likely to be AL interneurons. Neither the aphid AL has any apparent glomeruli, although its somewhat heterogeneous texture suggests the presence of synaptic aggregations. **(d)** In contrast to the homopteran species, antisynapsin staining of the AL of the heteropteran bug *Euschistus heros* reveals many clearly delineated glomeruli.

in diameter, situated ventrally below the esophagus, near the esophageal connectives, and with characteristically distinct glomeruli individually delineated by antisynapsin staining (Figure 1d). We did not determine the precise number of glomeruli but estimated the total to be around 60–80. In *E. heros* rhodamine staining was unsuccessful, whereas neurobiotin-stained axons partially filled glomeruli near the entrance of the AL. Neurobiotin staining was rather selective, with most neurobiotin-stained axons bypassing the AL and either targeting the AMMC or continuing further along the esophageal connectives (Figure 2b,c).

In the 3 homopteran species, all successful antennal neuron fills included several neurons, also in individuals whose antennae were left intact during the filling process. Nevertheless, at most only a fraction of all neurons appeared to be filled in any individual. All 3 species exhibited similar neuronal architectures (Figure 3). Terminal branching of individual neurons was not distinguishable with certainty in any preparation, but the bulk of filled neurons exhibited extensive arborizations, apparently covering great portions of the AL. Furthermore, neurons were sparsely equipped with rather large boutons, representing synaptic terminals (Figures 2a and 3). Synapsin staining in the ALs of the homopteran species appeared comparatively weaker than in the rest of the brain (Figure 3), and while closer examination of antisynapsin-stained homopteran brains at high magnification showed slightly heterogeneous structures in the ALs, nothing suggested the presence of delineated glomeruli (Figure 1c). Neither anterograde filling nor antisynapsin staining allowed delineation of defined glomerular units in the ALs of the homopteran species.

Discussion

Here, we have studied a group of insects whose tiny olfactory systems are devoid of clearly defined glomerular structures, which otherwise form the hallmark of olfactory systems in a great number of animal phyla (Hildebrand and Shepherd

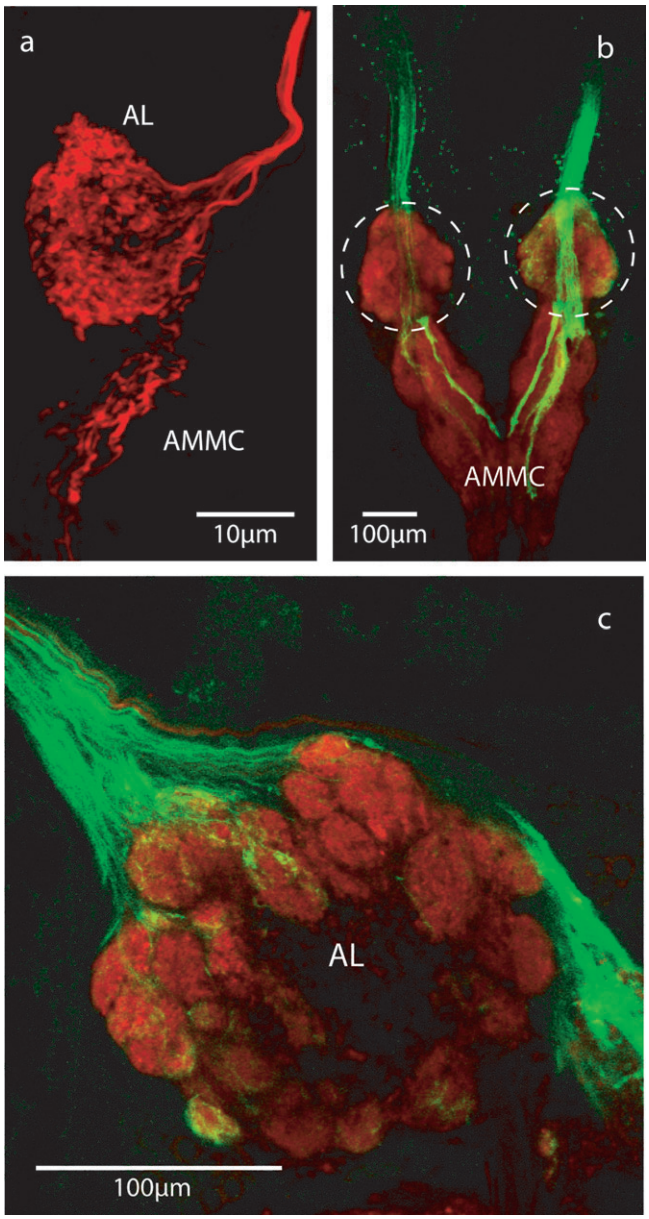

Figure 2 Anterograde fills from the antennae with rhodamine dextran or neurobiotin reveal the projection of antennal nerve axons into the brain. **(a)** Anterograde rhodamine dextran fill in a male *Trioza apicalis* shows olfactory neurons targeting the left AL and presumably mechanosensory neurons targeting the AMMC near the esophageal connective (frontal view). **(b** and **c)** Anterograde neurobiotin staining combined with antisynapsin staining in *Euschistus heros* shows antennal nerve axons targeting the AL (white dashed circles in b, ventral view) and AMMC. In *E. heros*, a relatively small fraction of stained neurons entered the AL.

1997). The general appearance of the brains and ALs of *T. apicalis* and the aphids are very similar. Most insect groups have between 40 and 200 glomeruli (Rospars 1988; Masson and Mustaparta 1990; Anton and Homberg 1999), which are thought to represent landmarks in a chemotopic map where afferent ORNs of the same functional type, that is, expressing the same olfactory receptor gene, cluster together in the same glomerulus (Vosshall et al. 2000). In a few groups of insects, no glomeruli have been found and the ALs are reduced, including Ephemeroptera, Odonata, Plecoptera, and Cicadoidea (Panov 1961; Strausfeld et al. 1998). Most of these insects are wholly or nearly anosmic, in contrast to aphids and psyllids, which are very dependent on olfactory cues, for example during host finding and migration to and from the winter shelter plants.

The comparatively weak antisynapsin staining of the ALs in *T. apicalis* and the aphids indicates an unusually low density of synapses, which is likely a consequence of low numbers of neurons at all synaptic levels in the ALs. Judging by the low number of cell nuclei clustering around the ALs, the afferent neurons entering through the antennal nerve may synapse onto very few AL interneurons. This is in stark contrast to ALs in most other insects, including the heteropteran *E. heros*, where the glomeruli represent some of the densest synaptic aggregations in the brain. In our antennal backfills, axons from ORNs project into the AL and axons from mechanosensory antennal organs project past the AL to the AMMC near the esophageal connective (Figure 2) (Rospars 1988; Anton and Homberg 1999). The relationship between neurons projecting to the *T. apicalis* AL and the AMMC observed in this study agrees with the information provided by a recent morphological study of antennal sensilla (Kristoffersen et al. 2006). In *T. apicalis*, neurons from the antenna appear to terminate either in the AL or the AMMC, which agrees with the observation that no taste sensilla were found on the antenna. In contrast, antennal neurons projecting past the AL and AMMC in *E. heros* may correspond to projections from taste sensilla, which are usually present on heteropteran antennae (Brézot et al. 1997). In aphids, taste sensilla appear to be found on the antennae (Bromley et al. 1979, 1980), but in aphids, we did not determine whether neurons projecting past the AL always terminated in the AMMC or whether some neurons continued along the esophageal connective.

The indistinct organization of the ALs in *T. apicalis* and the aphids stand in sharp contrast to the many well-defined glomeruli found in *E. heros*. In this species, the ALs are very similar to those found in most other insects, both with regards to number and appearance of the olfactory glomeruli. This striking difference is not primarily due to the small size of the ALs of *T. apicalis* and the aphids, whose diameters approximate that of an average glomerulus in *E. heros*. Comparisons with ALs of *Drosophila* (Vosshall et al. 2000), parasitoid wasps (Smid et al. 2003), and mosquitoes (Ignell et al. 2005), which are not considerably larger than those of the homopterans in this study, reveal that these sport typical glomeruli, equally distinct as those found in much larger insects. For comparison, the adult fruit fly has 600 sensilla, approximately 1300 ORNs and its AL contains approximately 45 glomeruli (Stocker et al. 1990; Stocker 1994; Laissue et al. 1999). The AL of the predatory mite *Phytoseiulus persimilis* is similar to the AL of *T. apicalis* in size as well as in the

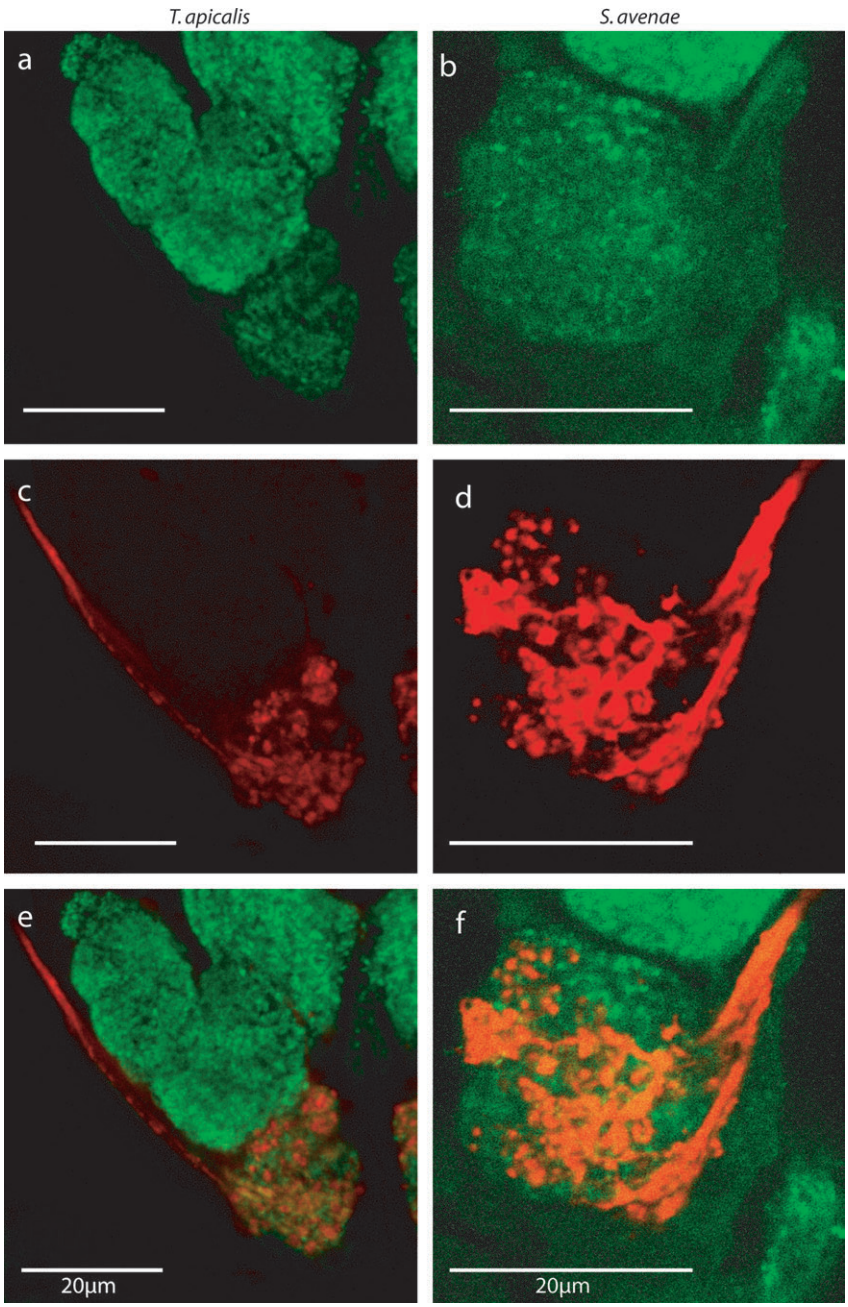

Figure 3 Antisynapsin staining **(a** and **b)** and anterograde rhodamine dextran backfills **(c** and **d)** in combination (merged in **e** and **f**) in frontal views of a female *Trioza apicalis* (left AL) and an alate female *Sitobion avenae* aphid (right AL) show very light antisynapsin staining in the ALs, indicating overall low synaptic densities. Aggregations of axon terminals are suggested only by the rhodamine fills.

number of afferents entering the AL (up to 25 ORNs) (van Wijk et al. 2006). Anterograde dye fills reveal that the AL of *P. persimilis* also contains distinct glomeruli, with approximately a single ORN per glomerulus, showing that the absence of glomeruli in the homopteran ALs is not a consequence of their low numbers of afferent ORNs.

Among other insects, the only olfactory system with a similarly low number of components as in *T. apicalis* that has been studied before is that of the *Drosophila melanogaster* larva, which has been characterized by means of genetically expressed labeling systems driven by *Drosophila*-specific GAL4 drivers. The olfactory system in each hemisphere of the *Drosophila* larva is made up of 21 ORNs emanating from a single olfactory organ and projecting to 21 individual glomeruli (Python and Stocker 2002; Kreher et al. 2005; Ramaekers et al. 2005). Its primary olfactory centre lacks neuronal convergence, displaying a 1:1:1 relationship between afferent neurons, glomeruli, and projection neurons.

Trioza apicalis is a specialist migratory herbivore whose summer host (carrot) and coniferous winter shelter plants share many of their odorants (Valterová et al. 1997; Kristoffersen et al. 2008). The olfactory system of *T. apicalis* is intriguing because of its exceptional level of reduction, coupled with rather complex biology and daunting olfactory tasks, involving host alternation and long-distance migration events. *Trioza apicalis* passes the winter in the adult stage on conifer trees, surviving temperatures down to –18 °C or lower (Rygg 1977; Kristoffersen and Anderbrant 2007). The small number of ORNs found in *Trioza* may be an adaptive reduction, preventing desiccation during winter. Moreover, both carrot and conifers (*Daucus carota* and various Pinophyta, respectively) are rather strong smelling and typically occur in large stands, which may lessen the need for exceptionally sensitive host detection abilities.

This study provides a first perspective of the hemipteran brain and its primary olfactory centre. Our results show the absence of morphologically distinct glomeruli in homoptera. Without methods to distinguish projections of individual receptor types, however, we can not further characterize the spatial organization of the antennal lobe of these insects.

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