

Novel antennal lobe substructures revealed in the small hive beetle *Aethina tumida*

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Abstract The small hive beetle, *Aethina tumida*, is an emerging pest of social bee colonies. *A. tumida* shows a specialized life style for which olfaction seems to play a crucial role. To better understand the olfactory system of the beetle, we used immunohistochemistry and 3-D reconstruction to analyze brain structures, especially the paired antennal lobes (AL), which represent the first integration centers for odor information in the insect brain. The basic neuroarchitecture of the *A. tumida* brain compares well to the typical beetle and insect brain. In comparison to other insects, the AL are relatively large in relationship to other brain areas, suggesting that olfaction is of major importance for the beetle. The AL of both sexes contain about 70 olfactory glomeruli with no obvious size differences of the glomeruli between sexes. Similar to all other insects including beetles, immunostaining with an antiserum against serotonin revealed a large cell that projects from one AL to the contralateral AL to densely innervate all glomeruli. Immunostaining with an antiserum against tachykinin-related peptides (TKRP) revealed hitherto unknown structures in the AL. Small TKRP-immunoreactive spherical substructures are in both sexes evenly distributed

within all glomeruli. The source for these immunoreactive islets is very likely a group of about 80 local AL interneurons. We offer two hypotheses on the function of such structures.

Keywords Olfactory system · Neuropeptide · Serotonin · Insect · 3D reconstruction

Introduction

The small hive beetle *Aethina tumida* (Murray 1867, Coleoptera: Nitidulidae) is a parasite and scavenger of colonies of social bees (honeybees: *Apis mellifera*: cf. Neumann and Elzen 2004; bumblebees: *Bombus impatiens*: Spiewok and Neumann 2006; stingless bees: *Trigona carbonaria*: cf. Greco et al. 2010 and *Austroplebeia australis*: cf. Halcroft et al. 2011). Both larvae and adults of the *A. tumida* feed on pollen, honey and bee brood, leading to fermentation of the honey and devastation of the combs, often resulting in the full structural collapse of the entire nest (Lundie 1940; Schmolke 1974; Neumann and Elzen 2004). In its native range in sub-Saharan Africa, *A. tumida* is a rather harmless parasite, mostly affecting weak and stressed colonies (Lundie 1940; Hepburn and Radloff 1998; Neumann and Elzen 2004; Neumann and Ellis 2008). However, *A. tumida* has become an invasive species. It was introduced into the USA (1996), Egypt (2000), Australia (2001) and into Europe twice (2004 and 2014; see Neumann and Ellis 2008; Mutinelli et al. 2014) and now has well-established new populations in North America and Australia (Neumann and Elzen 2004; Neumann and Ellis 2008). In these areas, *A. tumida* can be considered a significant pest of managed honeybees (Neumann and Elzen 2004) and possibly of wild bees (Neumann 2015).

To control this emerging pest, Neumann and Elzen (2004) speculated about the possibility of a *A. tumida* pheromone that

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could be used for trapping systems. Indeed, male-produced aggregation pheromones of other species in the family Nitidulidae are known from *Carpophilus obsoletus* and are used for pest control (Petroski et al. 1994). Today, a great variety of insect pheromones (especially for Lepidoptera and Coleoptera) are known and used for trapping systems (www.pherobase.com). Although pheromone communication has not yet been demonstrated in the small hive beetle, it has been shown that *A. tumida* is highly attracted to volatiles emitted by adult honey bees (*A. mellifera*), bumble bees (*Bombus impatiens*), stored pollen, wax, brood, and honey (Suazo et al. 2003; Graham et al. 2011; de Guzman et al. 2011). Furthermore, *A. tumida* prefer to fly before or after dusk (Schmolke 1974) suggesting that visual cues are less important than olfactory cues when it comes to locating beehives for mating. Altogether, understanding the olfactory system of *A. tumida* might be instrumental in controlling this pest.

In insects, olfactory information is detected by olfactory sensory neurons (OSNs) housed in olfactory sensilla on the antenna and the maxillary palps (reviewed in Hansson and Stensmyr 2011). They pass the information on to the neuronal network in the antennal lobes (AL), the first integration centers for odor information in the insect brain. Typically, AL are substructured in spheroidal compartments, the olfactory glomeruli. Typically, OSNs that express the same specific odorant receptor converge onto the same glomerulus (Vosshall et al. 2000). The glomerulus number ranges between about 40 in Diptera up to several hundred in Hymenoptera (Schachtner et al. 2005; Mysore et al. 2009; Kuebler et al. 2010). In various orders of neopteran insects, including Coleoptera, Dictyoptera, Diptera, Hymenoptera and Lepidoptera, sexual dimorphic glomeruli have been described (Kondoh et al. 2003; Kleineidam et al. 2005; Schachtner et al. 2005; Hu et al. 2011). Such glomerular dimorphism may have been evolved independently where it was needed, e.g., for long-distance pheromone detection or for the detection of specific odors like host plant volatiles or trail pheromone (Hansson and Stensmyr 2011). In the AL, olfactory information is processed by local interneurons (LN) and relayed to projection neurons (PN) that connect to other brain areas including the mushroom bodies (MB) or the lateral horn (LH). Additionally, the AL receives innervation from a few unique centrifugal neurons (CN) that provide efferent input from other brain areas (reviewed in Schachtner et al. 2005).

Antennal lobes across insect species contain a wide range of neuromediators including excitatory and inhibitory transmitters like acetylcholine and GABA (e.g., Bicker 1999; Homberg 2002; Schachtner et al. 2005; Berg et al. 2009; Fusca et al. 2015). In addition, AL neurons contain neuromediators like biogenic amines, gaseous signaling molecules like NO and a large variety of neuropeptides, suggesting important involvement for proper olfactory behavior (e.g.,

Schachtner et al. 2005; Berg et al. 2007; Utz et al. 2008; Carlsson et al. 2010; Binzer et al. 2014; Siju et al. 2014; Fusca et al. 2015). For example, in moths and flies, serotonin (5HT) is able to modulate the sensitivity of odors and sex pheromones (Linn and Roelofs 1986; Gatellier et al. 2004; Hill et al. 2003; Kloppenburg and Hildebrand 1995; Dacks et al. 2009). Another example are the tachykinin-related neuropeptides (TKRP), controlling olfactory sensitivity and locomotor activity in the fruit fly *Drosophila melanogaster* (Ignell et al. 2009; Winther et al. 2006; Winther and Ignell 2010).

Typically, neuromediators are distributed across all glomeruli of the AL (Schachtner et al. 2005; Carlsson et al. 2010; Binzer et al. 2014; Neupert et al. 2012; Siju et al. 2014). However, there are exceptions in which only one or several glomeruli receive innervations by neurons that express specific neuromediators like serotonin in the ant (*Camponotus laevigatus*; Dacks et al. 2006), short neuropeptide F (sNPF) in the mosquito (*Aedes aegypti*; Siju et al. 2014) and in the fly (*Drosophila melanogaster*; Carlsson et al. 2010), or serotonin and several neuropeptides in collembolans (Kollmann et al. 2011a).

The life cycle of *A. tumida* involves long-distance dispersal to new food sources (Neumann et al. 2012), preferentially after dusk (cf. Neumann and Elzen 2004). Therefore, olfaction seems to play a pivotal role for the adult beetles (reviewed in Neumann and Elzen 2004). Given that olfaction is that important for the behavior of the animal, the anatomy of the brain, especially of the central olfactory pathway, might very likely reflect this importance. We hypothesize that brain neuropils involved in processing of olfactory information should be enlarged in relationship to other brain areas. We also hypothesize that a sequential invasion of bee colonies, as was postulated by Elzen et al. (2000), with males first and females following could be reflected by specialized glomeruli, e.g., sexual dimorphic glomeruli as described for several other insect species. In addition, we are looking for any specialization in the central olfactory pathway that could reflect the special life style of *A. tumida*.

Materials and Methods

Experimental animals

Adult *Aethina tumida* were collected from naturally infested colonies of the African honeybee subspecies *Apis mellifera scutellata* at the experimental farm of the Department of Zoology and Entomology, University of Pretoria, South Africa. After collection, the beetles were immediately sexed following a routine procedure (Neumann et al. 2013). According to European legislation, import of live *A. tumida* to Germany is illegal (Commission Decisions EC No 2003/881 and Commission Regulation (EC) N° 1398/2003). Therefore, the

beetles were decapitated in Pretoria and the heads were fixed for 12 h at 4 °C in 4 % FA (formaldehyde FA; Roth, Karlsruhe, Germany) in PBS (phosphate-buffered saline, 0.01 M, pH 7.4). They were rinsed for 15 min in PBS and afterwards stored in PBS in glass vials in a customized cooling device and then sent by express delivery to the Philipps University of Marburg (Marburg, Germany).

Immunohistochemistry

Primary antibodies In the current study, we used antibodies against the synaptic vesicle protein synapsin, the biogenic amine serotonin and an antiserum recognizing tachykinin-related peptides (summarized in Table 1).

The monoclonal antibody from mouse against a fusion protein consisting of a glutathione-S-transferase and the first amino acids of the presynaptic vesicle protein synapsin I coded by its 5'-end (SYNORF1; 3C11, #151101) was used to selectively label neuropilar areas. It was used in combination with one additional primary antibody raised in rabbit. The synapsin antibody was kindly provided by Dr. Erich Buchner (University of Würzburg, Germany). This antibody was first described by Klagges et al. (1996) and has been used in many insect studies to label neuropilar areas (e.g., Utz et al. 2008; Heuer et al. 2012; Binzer et al. 2014). The antibody was used at a dilution of 1:100.

The polyclonal antiserum against serotonin (5HT) was raised in rabbit against paraformaldehyde-coupled conjugates of BSA (bovine serum albumin) and 5HT (DiaSorin, Dietzenbach, Germany). Its specificity for the insect nervous system has been shown in several studies (e.g., Dacks et al. 2006). It was used at a dilution of 1:5000.

The polyclonal TKRP antiserum was kindly provided by Dr. H. Agricola (University of Jena, Germany). It was raised in rabbits against locustatachykinin-2 (Lom-TK II, APLSGFYGVRamide) glutaraldehyde-conjugated to bovine thyroglobulin (Veenstra et al. 1995). The antiserum is also known to detect tachykinin-related peptides (TKRPs; consensus sequence FXGXRamide) in other insects (e.g., Vitzthum and Homberg 1998; Heuer et al. 2012; Binzer et al. 2014). In beetles, specificity for the anti-TKRP antiserum has so far been confirmed in *Tribolium castaneum* by preabsorption of the antiserum with synthetic Lom-TK II (Binzer et al. 2014). In the current study, we used the anti-TKRP antiserum to

reveal morphological structures of the brain of *A. tumida*. It was used at a dilution of 1:2000.

Secondary antibodies Goat anti-mouse antibodies conjugated to Cy5 (GAM-Cy5) and goat anti-rabbit antibodies conjugated to Cy3 (GAR-Cy3) were used as secondary antibodies (each 1:300; Jackson ImmunoResearch, Westgrove, PA, USA).

Whole mount double immunostainings Brains of *A. tumida* were dissected out of the head capsule, fixed overnight at 4 °C in 4 % FA in PBS, followed by rinsing (4×10 min) with PBS at RT (room temperature). Afterwards, brains were preincubated for 2 days in PBT (PBS containing 0.3 % Triton-X 100; Sigma Aldrich, Steinheim, Germany) with 5 % NGS (normal goat serum; Jackson ImmunoResearch). The primary antibody anti-synapsin (1:100) was used in combination with the anti Lom-TK II (1:5000) or anti 5HT antiserum (1:2000) diluted in PBT with 1 % NGS. Brains were incubated for 2 days at 4 °C. After rinsing (4×10 min) with PBT at RT, brains were incubated in secondary antibodies (GAM-Cy5 and GAR-Cy3; 1:300) in PBT with 1 % NGS at 4 °C for 2 days in the dark. After rinsing (6×10 min) with PBT at RT and washing in distilled H₂O for 10 min, brains were dehydrated in an ascending alcohol series (30, 50, 70, 90, 95 %, 2×100 % ethanol, 5 min each). Followed by clearing the tissue in methyl salicylate (10 min; Merck, Darmstadt, Germany) the brains were finally mounted in resin (Permount; Fisher Scientific, Pittsburgh, PA, USA). During the immunostaining procedure, all washing and incubation steps were performed on a laboratory orbital shaker (MS 3 digital; IKA, Staufen, Germany).

Data processing

Fluorescence was analyzed with a confocal laser scanning microscope (Leica TCS SP5 Microsystems; Leica, Wetzlar, Germany), with the object lenses ×20 oil objective (HCX PL APO lambda blue ×20/NA=0.70 Imm UV, working distance: 260 μm; Leica), ×40 oil objective (HCX PL APO lambda blue ×40/NA=1.25 Oil UV, working distance: 100 μm; Leica), and ×63 glycerol objective (HCX PL APO ×63/NA=1.30 Glyc 21 °C CS working distance: 260 μm; Leica). We scanned with a resolution of 1024×1024 or 512×512 pixels, a line average of 2, speed of 200 Hz, a digital zoom of 1–2,

Table 1 List of antibodies used, including dilution, source, donor and reference for each antibody

Name, anti-	Shortcut	Dilution	Source	Donor	Reference
<i>D. melanogaster</i> Synapsin I	α-Synapsin	1:100	Mouse	Dr. Buchner	Klagges et al. 1996
Serotonin	α-5HT	1:2000	Rabbit	DiaSorin	–
<i>Locusta migratoria</i> Tachykinin II	α-Lom-TK II	1:5000	Rabbit	Dr. Agricola	Veenstra et al. 1995

and z-steps varying from 0.5 to 1.0 μm for detailed scans and from 3.0 to 5.0 μm for overview scans.

Image segmentation, reconstruction and visualization

Brain structures were 3-D reconstructed using AMIRA 5.2 (Visage Imaging, Berlin, Germany). Segmentation and reconstruction were performed according to Kurylas et al. (2008) and El Jundi et al. (2009). In short, data of the CLSM image stacks were opened in the segmentation editor of AMIRA. From all three spatial directions (lateral to lateral, anterior to posterior and dorsal to ventral) of the respective structure, 3–12 layers (depending on the size of the structure) were labeled and finally wrapped to obtain a voxel-based 3-D model. By using the tool “SurfaceGen”, we transferred the voxel-based 3-D model into a polygonal surface model. Standard color codes were used for the reconstructed neuropils (Brandt et al. 2005). For further global processing (i.e., contrast and brightness optimization) and final figure arrangements, snapshots were taken in AMIRA and subsequently processed in Corel Draw 13 (Corel, Ottawa, ON, Canada). Diagrams generated with Excel XP (Microsoft, Redmond, WA, USA) were imported and revised in Corel Draw 13 without any further modification. For statistical analyses, we used a two-tailed *t* test in Origin 6.0 (OriginLab, Northampton, MA, USA) and Excel XP.

Results

General organization of the brain

A 3-D reconstruction of the brain of *Aethina tumida* was created based on confocal sections of an adult female stained with anti-synapsin antibody (Fig. 1) (movie of a rotating 3-D reconstruction and a camera path through the synapsin staining of a brain can be found in the digital supplements, ESM 1 and 2; 3-D reconstructions of single male and female AL can be found in ESM 3). The brain contains all typical neuropils known from most insects including neuropils of the optic lobes, the antennal lobes, mushroom body and neuropils of the central complex (e.g., *Drosophila melanogaster*, Rein et al. 2002; honeybee *Apis mellifera*, Brandt et al. 2005; desert locust *Schistocerca gregaria*, Kurylas et al. 2008; sphinx moth *Manduca sexta*, el Jundi et al. 2009; red flour beetle *Tribolium castaneum*, Dreyer et al. 2010). We reconstructed all neuropils that were clearly identifiable and separable (8 paired and 3 unpaired neuropils).

In the optical lobes, we reconstructed the paired medulla (Me), lobula (Lo), lobula plate (LoP) and accessory medulla (aMe). The central complex is located in the center of the protocerebrum. Its reconstruction includes the unpaired upper and lower unit of the central body (CBU, CBL), the paired

Noduli (No) and the dorso-posteriorly located unpaired protocerebral bridge (PB).

The paired mushrooms bodies are placed lateral to both sides of the central complex. We reconstructed calyx (Ca) and pedunculus (Pe) separately. The Pe contains the vertical lobe (vL) and medial lobe (mL). The Pe and the lobes are separated into an inner core region, which is densely stained with synapsin antibody (Fig. 1; non-transparent-shaped part of the MB) and a less densely anti-synapsin stained exterior region (Fig. 1; transparent-shaped part of the MB).

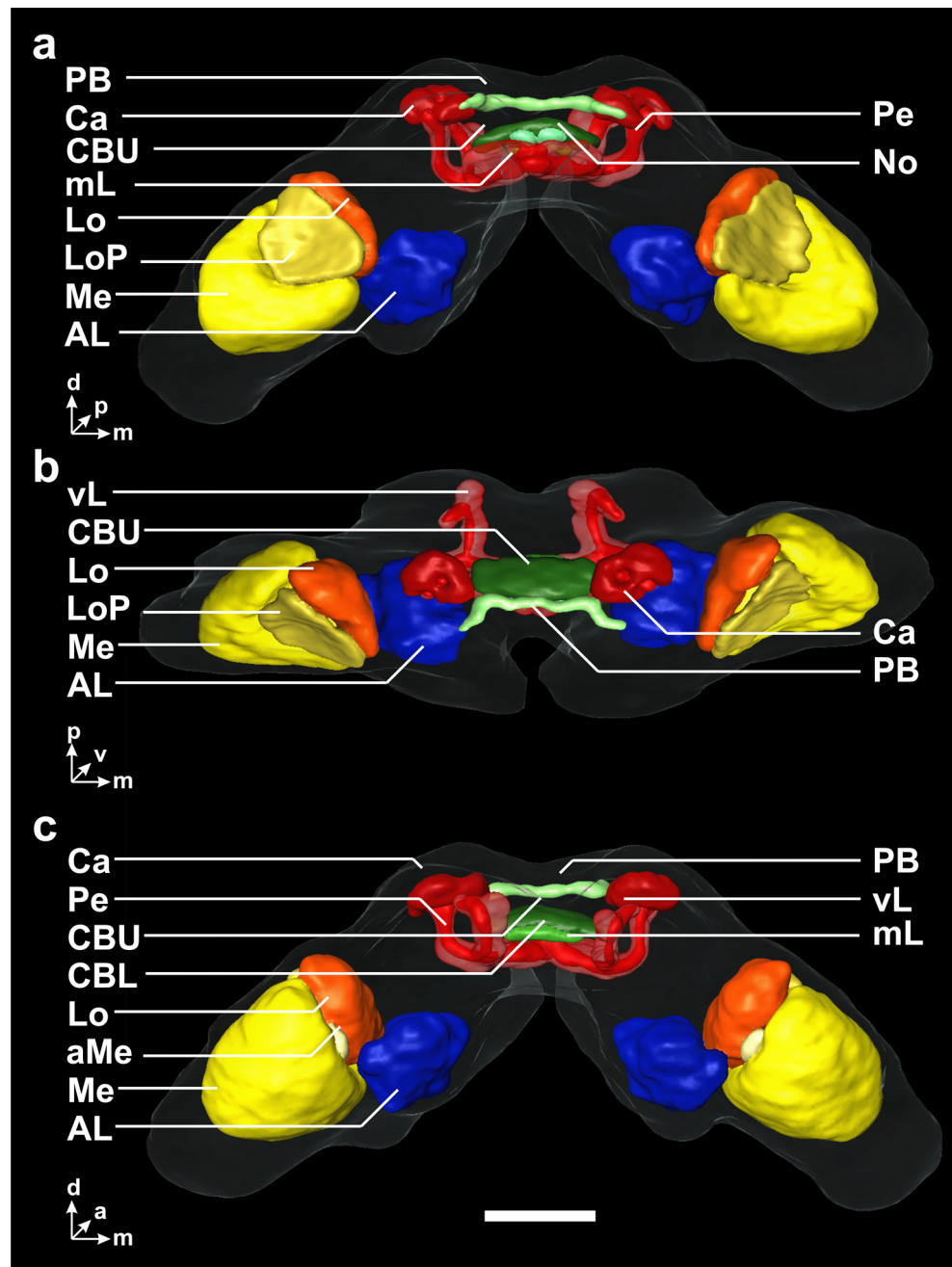
Organization of the antennal lobe

In total, we analyzed 12 ALs of 7 males and 9 ALs of 5 females. Characteristically for insects (Schachtner et al. 2005), in *A. tumida* the AL are organized in small, spherical substructures, the olfactory glomeruli, which are arranged around a central coarse neuropil. The small hive beetle possesses 72.0 ± 3.9 glomeruli per AL in males ($n=12$ ALs) and 71.1 ± 3.4 glomeruli per AL in females ($n=9$ ALs) ($p=0.588$).

The average size of one glomerulus is $10.7 \pm 1.8 \mu\text{m}^3$ in males ($n=12$ ALs / $n=864$ glomeruli) and $10.1 \pm 1.8 \mu\text{m}^3$ in females ($n=9$ ALs / $n=640$ glomeruli). For approximation of the AL size, the volumes of all glomeruli within the AL were summed; extraglomerular space and central coarse neuropil were not included. Mean volume of male AL is $730.0 \pm 120.5 \mu\text{m}^3$ ($n=12$ ALs), compared to $719.2 \pm 114.9 \mu\text{m}^3$ in females ($n=9$ ALs). Taken together, male and female ALs of *A. tumida* were statistically indifferent in regard of glomeruli number ($p=0.588$), overall glomeruli size ($p=0.875$), or on the level of the AL volume ($p=0.997$).

It is known from several insect species that conspicuously larger glomeruli appear in one of the sexes, typically at the entrance site of the antennal nerve (Schachtner et al. 2005; Hu et al. 2011). To verify whether this might also be true for the small hive beetle, we grouped the different-sized glomeruli of males ($n=12$ ALs; $n=864$ glomeruli) and females ($n=9$ ALs; $n=640$ glomeruli) according to their volume ($0\text{--}999 \mu\text{m}^3 = \text{group 0}$; $1000\text{--}1999 \mu\text{m}^3 = \text{group 1}$; ... $35,000\text{--}35,999 \mu\text{m}^3 = \text{group 35}$), followed by analyzing the relative abundance of these different size groups (Fig. 2) (data of glomeruli from all ALs of one sex were pooled). No conspicuous sexual dimorphism could be seen. Middle-sized glomeruli with a volume between $4000 \mu\text{m}^3$ and $11,999 \mu\text{m}^3$ seemed to be the most common ones, while small glomeruli (between 1000 and $2999 \mu\text{m}^3$) and large glomeruli (between $17,000$ and $20,999 \mu\text{m}^3$) were less abundant. In addition, a varying number of a few larger glomeruli (between $21,000$ and $35,999 \mu\text{m}^3$) occur in both sexes. Analysis of the position of the three largest glomeruli per AL revealed that they are not clustered and that they distribute randomly within the anterior half of the AL, with no particular correlation to the site where the antennal nerve enters the AL.

Fig. 1 3-D reconstruction of the female brain of *Aethina tumida* in **a** anterior, **b** dorsal, and **c** posterior view. The neuropils were reconstructed with the AMIRA tools SurfaceGen and SurfaceView. The color code of the labeled neuropils is consistent with Brandt et al. (2005). *AL* antennal lobe, *Ca* Calyx, *CBL* lower unit of the central body, *CBU* upper unit of the central body, *aMe* accessory medulla, *Lo* lobula, *LoP* lobula plate, *Me* medulla, *No* noduli, *PB* protocerebral bridge and *Pe* pedunculus with lobes. Orientation bars: *a* = anterior, *d* = dorsal, *m* = median, *p* = posterior, *v* = ventral. Scale bar 100 μ m



In summary, we found no evidence for any sexual dimorphism in the AL on the level of AL size, glomerulus number and individual or overall glomerulus size.

Tachykinin-related peptides in the antennal lobes

Immunostaining with the anti TKRP antiserum in *A. tumida* revealed small, spherical substructures within all glomeruli (Fig. 3; see also ESM 4). In the anti-synapsin staining, these internal substructures are only slightly more strongly labeled in comparison to the surrounding neuropil of the glomerulus (Fig. 5b, c). Each AL contains about 230 of these

substructures (males: 245.7 ± 14.3 , $n=3$ ALs; females: 224.6 ± 19.1 , $n=5$ ALs). We did not find a sexual dimorphism ($p=0.154$). All observed glomeruli contain between one and ten substructures, which are never attached to the outer rim of a glomerulus but usually distributed evenly across the glomerular volume (Fig. 3; ESM 4). In both sexes, the number of substructures in a glomerulus correlates with the volume of the glomerulus (males: $n=3$ ALs, $n=224$ glomeruli, $R^2=0.99$; females: $n=5$ ALs, $n=350$ glomeruli, $R^2=0.95$ (Fig. 4).

The staining of TKRP-ir glomerular substructures most likely originates from a set of about 80 ± 18 ($n=6$ ALs) neurons

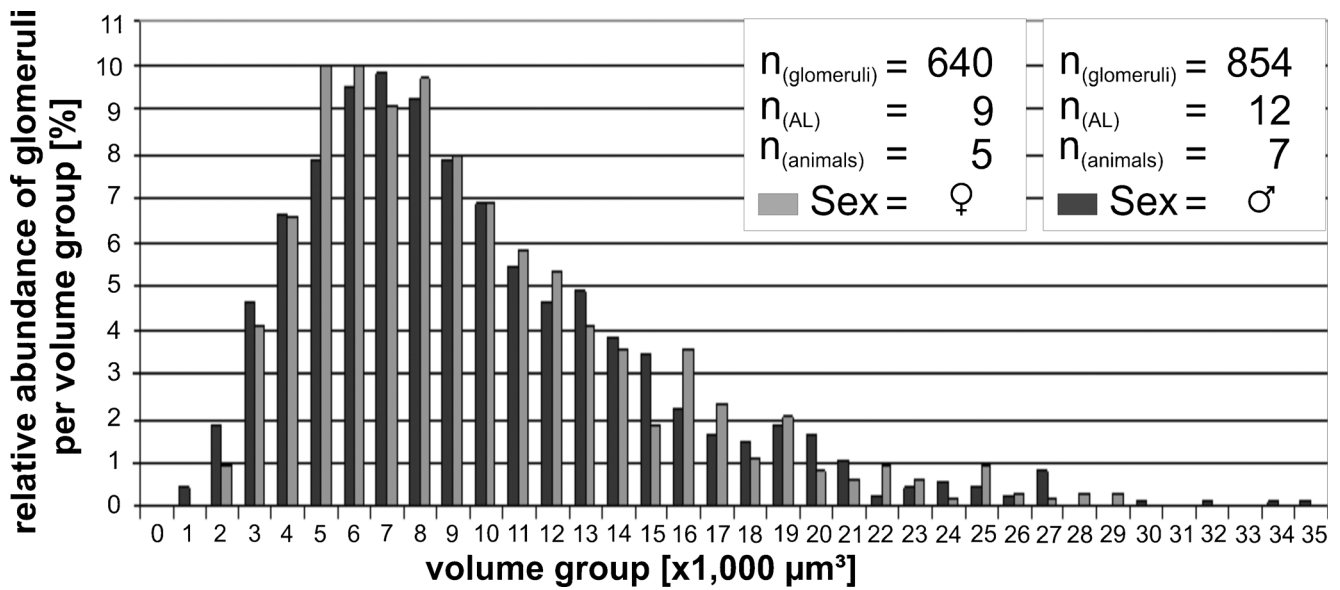


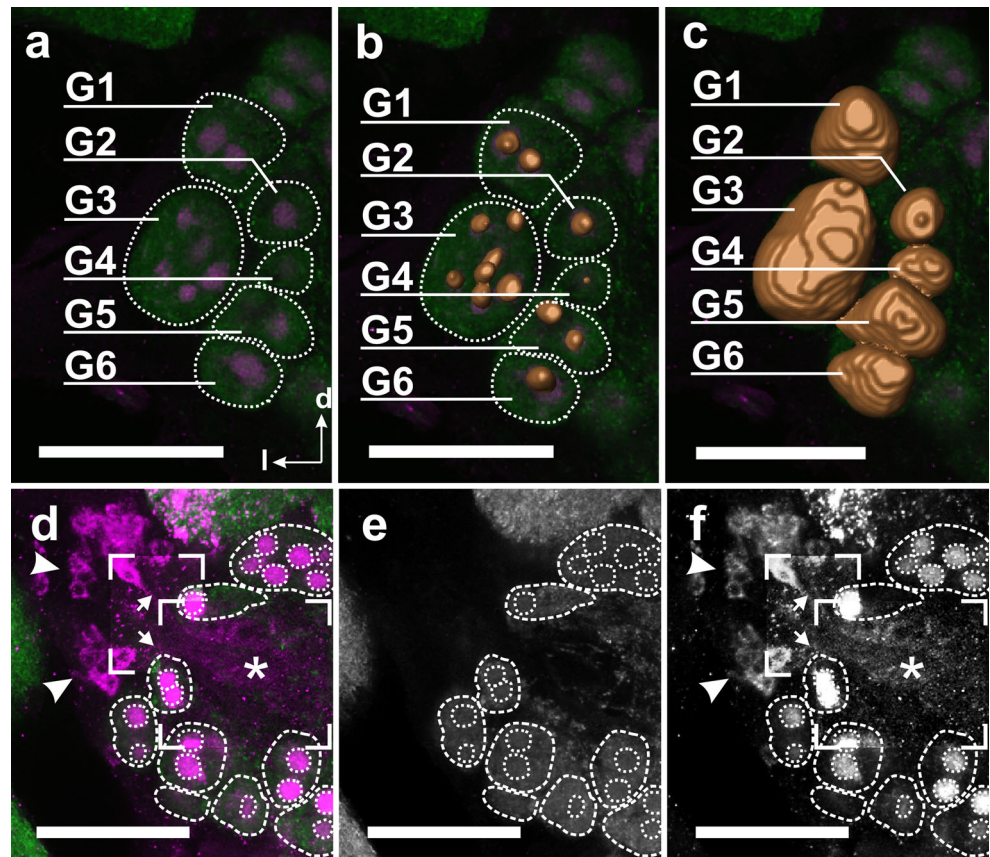
Fig. 2 Relative abundance of different sized glomeruli of both sexes of *Aethina tumida*. The x-axis represents the different glomerular volumes, shown as volume groups (0–999 μm^3 =group 0; 1000–1999 μm^3 =group

1; ...35,000–35,999 μm^3 =group 35). The y-axis represents the relative abundance in percent of the different size groups

located laterally in the AL (Fig. 3d–f, arrowheads), presumably exclusively local interneurons, which project their processes into the AL (Fig. 3d–f, arrows; see also electronic supplementary material, ESM 5). As the antennal nerve shows no immunoreactivity to the TKRP antibody, we exclude that

the staining of TKRP-ir glomerular substructures originates from OSNs. We also did not find any TKRP-ir fibers leaving the AL or entering the AL from other brain regions, excluding that the TKRP-ir glomerular substructures originate from projection neurons (PN) or centrifugal neurons (CN).

Fig. 3 The antennal lobe (AL) of *Aethina tumida* stained with an antibody against synapsin (green) and Lom TKII (magenta). **a** Single optical section of an AL. Several individual glomeruli (G1–G6, dotted lines), containing TKRP-ir glomerular substructures. **b, c** 3-D reconstructions of glomerular substructures (**b**) and of the glomeruli (**c**) of the set of glomeruli outlined in (**a**). **d–f** Staining with antibodies against synapsin (**e**) and TKRP (**f**) and overlay of both (**d**; synapsin in green, TKRP in magenta) showing TKRP-ir local AL interneurons (arrowheads) and their axons (arrows) projecting into the core area of the AL (asterisk), from where they give rise to TKRP-ir substructures. Boxed areas in (**d**) and (**f**) are enhanced in brightness and contrast to better visualize fibers of the local AL interneurons. Orientation bars in (**a**) valid for all subfigures: l=lateral, d=dorsal. Scale bars 50 μm



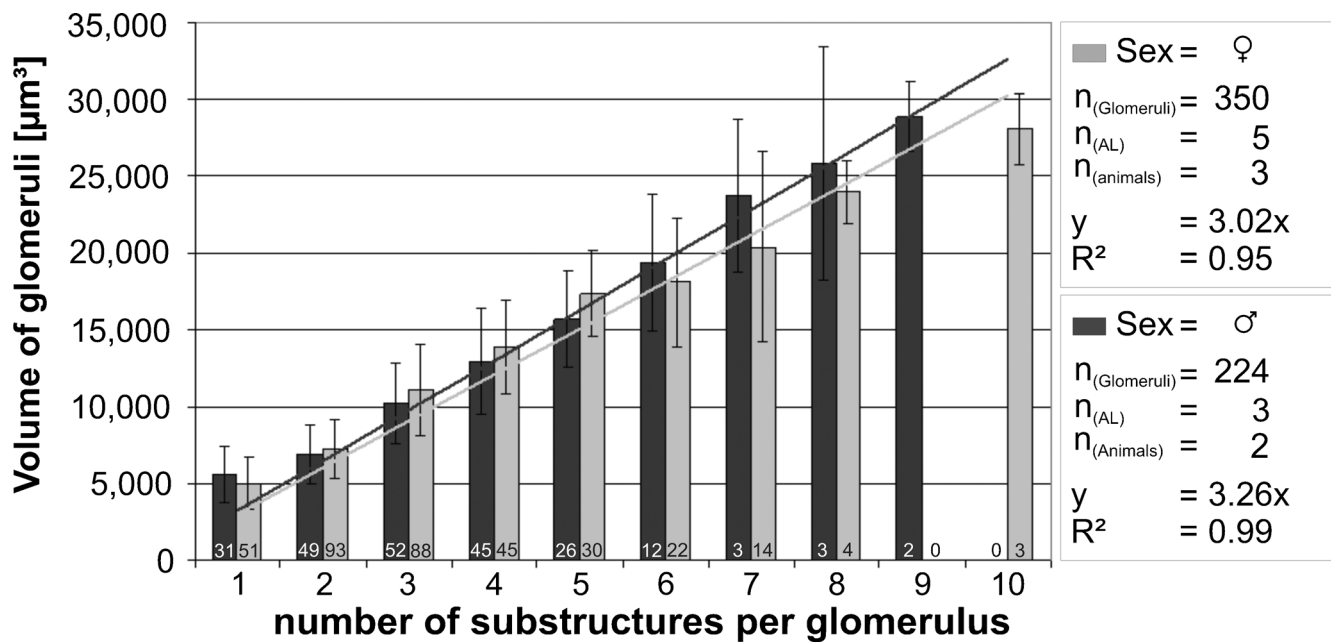


Fig. 4 Abundance of glomerular substructures (x-axis) in relation to glomerulus size (y-axis). Numbers at the base of the bars represent sample number (number of glomeruli). The diagram shows a linear

relationship between glomerulus size and number of glomerular substructures, with a coefficient of determination of 0.95 for male and 0.99 for female animals

Serotonin in the antennal lobes

All glomeruli of one AL are innervated by axons branching from one, brightly stained main fiber, entering the AL at its medio-ventral side (Fig. 5a, unfilled arrowheads). The origin of this 5HT-ir main fiber is most likely a single cell body, dorso-lateral to the AL at the contralateral side (as demonstrated in Fig. 5a; arrow). The primary neurite runs through the AL without obvious branching and exits the AL at its dorsal side (Fig. 5a, filled arrowheads). From here, the fiber runs dorsally and crosses to the contralateral hemisphere of the superior protocerebrum where a divergent branch forms putative dendritic arborizations. The main fiber continues ventrally, to enter the contralateral AL (Fig. 5a, unfilled arrowheads). The TKRP-ir glomerular substructures are not innervated by the 5HT-ir branches although some are touched just at their surface (Fig. 5b–d).

Discussion

General organization of the *A. tumida* brain

The overall anatomy of the brain of *A. tumida* (Fig. 1) compares well to other beetle brains regarding major neuropils including the optical lobes (OL), antennal lobes (AL), mushroom bodies (MB) and central body complex (CBX) (e.g., Van Haeften 1993; Breidbach and Wegerhoff 1994; Larsson et al. 2004; Dreyer et al. 2010; Hu et al. 2011).

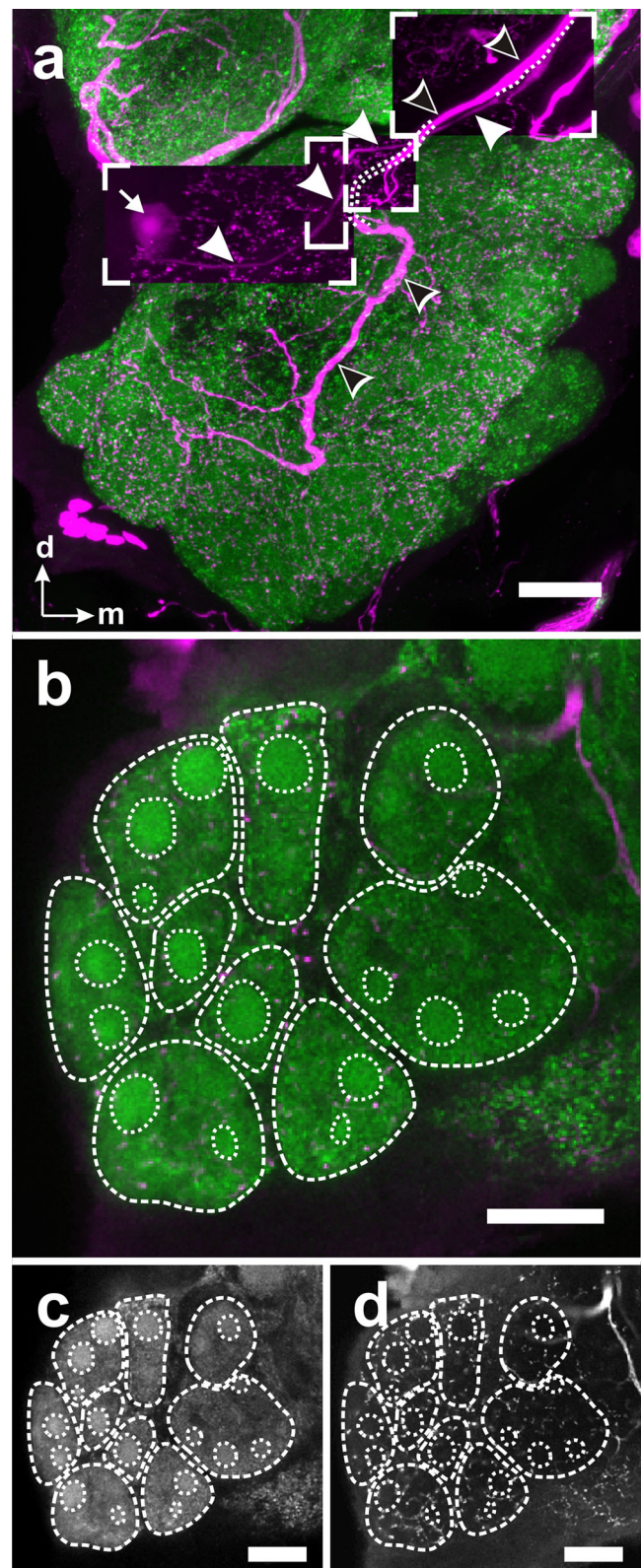
The OL of *A. tumida* contain the paired medulla (Me), lobula (Lo), lobula plate (LoP) and accessory medulla (aMe). The LoP has so far only been found in Ephemeroptera, Trichoptera, Coleoptera, Lepidoptera, Diptera (Strausfeld 2005) and Heteroptera (Settembrini and Villar 2005). The AL consists of about 70 glomeruli, which seems to be a typical number for beetles; there are about 70 glomeruli in the red flour beetle *Tribolium castaneum* (Dreyer et al. 2010), about 60 glomeruli in the scarab beetle *Holotrichia diomphalia* (Hu et al. 2011), and about 70 glomeruli in the cockchafer *Melolontha hippocastani* (third instar; Weissteiner et al. 2012). The AL will be discussed in more detail below. The paired MB contains the calyx (Ca) and the pedunculus (Pe), which is divided in the vertical and medial lobe (vL and mL). The Pe, vL and mL can be separated in a densely synapsin-stained core region and a less densely stained exterior region. This separation is in accordance with observations in the red flour beetle (Zhao et al. 2008; Binzer et al. 2014) and the African scarabid beetle *Pachnoda marginata* (Larsson et al. 2004). The medial part of the right mL is overlapping the medial part of the left mL, as has been observed in other beetles like *T. castaneum* (Dreyer et al. 2010) or the blind cave beetle *Neaphaenops tellkampfi* (Ghaffar et al. 1984). The unpaired central complex (CBX) can be separated into the protocerebral bridge (PB) and the central body (CB), which consists in the upper and lower unit (CBU and CBL), as well as the paired noduli (NO). This organization of the CBX is paralleled in other beetles like *T. castaneum* (Dreyer et al. 2010) or the mealworm beetle *Tenebrio molitor* (Breidbach and Wegerhoff 1994), as well as in many other insects (Homberg 2008).

Fig. 5 Antennal lobe (AL) of *Aethina tumida* stained with anti-synapsin (green) and anti-serotonin (magenta) antibodies. **a** The maximum projection shows the branching of a single serotonin immunoreactive (5HT-ir) fiber, entering the AL at the dorsal site (unfilled arrowheads). Dorsolateral to the AL, a single 5HT-ir cell body can be observed (arrow) projecting dorsal (filled arrowheads) without any branching or varicosities out of the AL. **b–d** Single optical section of an AL. The glomerular substructures in the AL glomeruli are distinctly brighter stained with the synapsin antibody than the surrounding area of the glomeruli (**b, c** dotted lines). 5HT-ir fibers clearly stay outside the glomerular substructures (**d**). Orientation bars: *m*=median, *d*= dorsal. Scale bars 20 μ m

Comparison with relative brain neuropil volumes of other insects reveals that the AL of *A. tumida* are comparably large, only surpassed by *T. castaneum* and the Madeira cockroach *Rhyparobia maderae* (Table 2). The AL of *A. tumida* take up about a fifth of the compared relative neuropil volumes, resembling the ratio found in *T. castaneum*, while the relative AL volume of *R. maderae* is even larger. *T. castaneum* is considered as an insect relying dominantly on olfactory cues (Dreyer et al. 2010), as are cockroaches (*Periplaneta americana*, Sakura and Mizunami 2001). In summary, this result supports the hypothesis that, for *A. tumida*, similar as stated for *T. castaneum*, olfactory cues are of major importance for their specialized behavior.

The relative volumes of the MB are remarkably smaller in *A. tumida* (11.5 %) compared to *T. castaneum* but still larger than in the majority of compared insects (Table 2). MBs are higher integrative centers of the insect brain that are best known for their involvement in olfactory learning (e.g., McGuire et al. 2001; Menzel 2001; Heisenberg 2003; Davis 2004). However, insect MB are not solely higher centers of the olfactory pathway but are involved in the integration and processing of a broad range of sensory modalities including processing of visual, gustatory and mechanosensory information as well as contributions to sleep regulation, place memory and temperature preference (reviewed in Heuer et al. 2012). Farris and Roberts (2005) demonstrated that generalist plant-feeding scarab beetles (Scarabaeidae) have larger MB, while specialist dung-feeding scarab beetles have smaller MB. Interestingly, this difference in MB volume is independent of size and glomerulus number of the AL, the primary input olfactory neuropil of the MB. This observation may offer a possible explanation for the difference in MB volume between feeding specialist *A. tumida* and feeding generalist *T. castaneum*, which evolved as saprophytic insects and naturally occur under the bark of trees, in rotten wood and infrequently in the nests of some Hymenoptera (Sokoloff 1977; Grimm 2001; Arnaud et al. 2005).

In *A. tumida*, the OLs are with about two-thirds of the relative neuropil volume, larger than the OLs of *Tribolium*. In insects, larger OLs typically correlate with larger complex eyes (e.g., Ghaffar et al. 1984; Gronenberg and Liebig 1999; Ehmer and Gronenberg 2004; Beutel et al. 2005; Kuebler et al.



2010). *Tribolium* has relative small compound eyes (80–83 ommatidia per eye; Friedrich et al. 1996) compared to other insect species including *A. tumida*.

Table 2 Comparison of relative neuropil volume of *Aethina tumida* with eight different insect species including sex and sample number (*Drosophila melanogaster*, Rein et al. 2002; *Apis mellifera*, Brandt et al. 2005; *Schistocerca gregaria*, Kurylas et al. 2008; *Rhyssalus maderae*, Wei et al. 2010; *Manduca sexta*, el Jundi et al. 2009; *Godyris zavaleta*, Montgomery and Ott 2014; *Heliothis virescens*, Kvello et al.

2009; *Tribolium castaneum*, Dreyer et al. 2010; and *Aethina tumida*, this work). With exception of the lobula plate, only neuropils with complements in all examined animals were compared (medulla, lobula complex, and lobula plate; antennal lobes, mushroom calyces and pedunculi and the upper and lower unit of the central body)

Order	Diptera				Hymenoptera			Orthoptera			Blattodea			Lepidoptera			Coleoptera			
	Species	<i>D.melanogaster</i>	<i>A. mellifera</i>	<i>S. gregaria</i>	<i>R.maderae</i>	<i>M. sexta</i>	<i>G.zavaleta</i>	<i>H.Virescens</i>	<i>T. castaneum</i>	<i>A.tumida</i>	Sex	♀	♂	♀	♂	♀	♂	♀	♂	♀
N	28	20	10	20	12	12	8/8	10	20	20	1									
AL (%)	9.5	8.5	9.7	35.6	12.8	15.0	8.7	15.7	22.4	24.5	21.6									
MB (%)	7.5	32.7	16.0	37.2	6.9	6.8	6.1	13.6	21.9	23.8	11.5									
OL (%)	79.6	57.9	72.6	23.0	79.4	77.3	84.4	64.5	50.1	45.1	62.1									
CB (%)	3.4	0.9	1.7	4.2	0.9	0.9	0.8	6.1	5.6	6.6	4.8									

The role of the central body is probably best described as a central coordinator in sensory and motor integration (for reviews, see Strauss 2002; Wessnitzer and Webb 2006; Homberg 2008). With 4.8 %, the relative volume of the central body of *A. tumida* is smaller than the same structure in *Tribolium* and *Heliothis virescens* but still larger than in the fly, honey bee, locust, cockroach or two Lepidoptera species. This suggests for the two beetles a more prominent function of the central complex than in most other insects. In this context, it would be interesting to have more comparable central complex volumes of other Coleoptera with different lifestyles, e.g., water beetles or non-flying beetles.

Olfactory driven behavior and sexual dimorphism

Males of the related beetle *Carpophilus obsoletus* release an aggregation pheromone that attracts both sexes (Petroski et al. 1994), leading to the hypothesis that a similar pheromone could guide *A. tumida* into host beehives that have already been parasitized (Elzen et al. 2000; Neumann and Elzen 2004). However, a sequential arrival of male and female *A. tumida* could not be observed (Spiewok and Neumann 2012). This does not rule out sex-specific differences in olfaction; females seem to be more responsive to beehive volatiles than males (Suazo et al. 2003) and this might be reflected in a sexual dimorphism of the olfactory system of *A. tumida*.

Sexual dimorphism has been described in various insect species on different levels of the olfactory pathway ranging from the periphery to the central nervous system including the antenna (e.g., beetles: Kaissling 1971; Ågren 1985; Allsopp 1990; Renou et al. 1998; Diptera: Clements 1999; Ruther et al. 2000; Stocker 2001; Hymenoptera: Streinzer et al. 2013; moths: Schneider 1992; Rospars and Hildebrand 2000; Huetteroth and Schachtner 2005), the specificity, number and/or distribution of olfactory receptors (e.g., moths: Miura

et al. 2009; Nakagawa et al. 2005; or Diptera: Bohbot et al. 2007), the morphology and number of glomeruli in the AL (Kondoh et al. 2003; Schachtner et al. 2005; Hu et al. 2011; Kelber et al. 2010; Streinzer et al. 2013) and higher order brain structures (e.g., *Drosophila*: Cachero et al. 2010). Detailed analyses of the antennae of *A. tumida* are missing. So far, there is no evidence that demonstrates a sexual dimorphism at the level of the antenna of the small hive beetle and information on the distribution of olfactory receptors for beetles is rare. For *T. castaneum*, transcription analyses of female and male antenna show no sexual dimorphism in the expression of odorant receptors (Dippel et al., in preparation); data for the small hive beetle are so far lacking.

Differently sized sexual dimorphic glomeruli have been observed in a wide range of insects, including beetles, cockroaches, bees, ants, moths, flies, and mosquitoes (Schachtner et al. 2005; Vosshall and Stocker 2007; Hu et al. 2011). Typically, these are one up to five glomeruli of a so-called “macroglomerular complex” in males to detect sex pheromones or in ants to detect trail pheromones, or “female sex-specific glomeruli” to detect host plants for oviposition. Such glomeruli are normally positioned at the entrance area of the antennal nerve into the AL (Hansson 1997; Anton and Homberg 1999; Rospars and Hildebrand 2000; Schachtner et al. 2005; Kleineidam et al. 2005). Hu et al. (2011) demonstrated such a sexual dimorphism for the first time in a beetle, identifying a single macroglomerulus in the AL of the Korean black chafer (*Holotrichia diomphalia*). The current study addresses the AL morphology of *A. tumida* in detail but neither male macroglomeruli nor female sex-specific glomeruli could be found.

A difference in number of glomeruli between sexes is common among insects. Yellow fever mosquito females have one additional glomerulus (Ignell et al. 2005) and *Pieris brassicae* female butterflies have three glomeruli more than their male counterparts (Rospars 1983). In hymenopterans, female

honeybee workers possess about 160 glomeruli compared to about 106 glomeruli in males (Arnold et al. 1985; Flanagan and Mercer 1989; Brockmann and Brückner 2001). In beetles, varying glomerulus numbers between sexes have so far not been described. In *A. tumida*, we found with about 70 glomeruli in both sexes no sexual dimorphism. In summary, the absence of a sexual dimorphism in the AL of *A. tumida* does not favor the work of Spiewok and Neumann (2012) or of Neumann and Elzen (2004) and can on this level of analysis not add to a better understanding of why female *A. tumida* respond more strongly to beehive volatiles than males (Suazo et al. 2003). A detailed analysis of the *A. tumida* antenna including distribution of olfactory sensillae and the identification and distribution of olfactory receptors would be necessary. Such insights could be helpful to create/optimize olfactory beetle traps. In addition, analysis of higher brain centers in the olfactory pathway including mushroom body and lateral horn might provide additional insights.

Serotonin-ir neuron in the AL

The observed innervation of all glomeruli of one AL by only one 5HT-ir cell body is common among insects (Dacks et al. 2006), as well as for ancestral hexapods, as observed in collembolans (Kollmann et al. 2011a). The described anatomy of the 5HT-ir neuron has been described for Coleoptera before and can also be found in Lepidoptera, Trichoptera, non-Schizophoran Diptera and Neuroptera (Dacks et al. 2006), and is very likely also true for aphids (Kollmann et al. 2011b). A side branch of the 5HT-ir neuron into the ipsilateral lateral protocerebrum as observed in many insects (including Coleoptera; Dacks et al. 2006) could not be found in *A. tumida*.

Glomerular substructures

Immunostaining with an antiserum recognizing tachykinin-related peptides (TKRPs) resulted in the discovery of small, spherical substructures, which are evenly distributed among all glomeruli, with no obvious difference between both sexes. These substructures seem to originate from a cluster of cell bodies, presumably local interneurons, lateral in the AL. Immunostaining with the same antiserum in two other beetles, *Tenebrio molitor* (Wegerhoff et al. 1996) and *T. castaneum* (Binzer et al. 2014), resulted in a cluster of local interneurons located in a similar position lateral in the AL. They provide the glomeruli with a dense meshwork of projections but did not give rise to spheroidal structures as described here. Similar to *A. tumida*, the antiserum did not reveal fibers in the antennal nerve or fibers belonging to either projection or centrifugal neurons (Wegerhoff et al. 1996; Binzer et al. 2014). Similarly, in all other insects where TKRP stainings were performed, the antisera labeled only local interneurons in the AL and no other neuron types (Schachtner et al. 2005; Carlsson et al. 2010;

Neupert et al. 2012; Binzer et al. 2014; Siju et al. 2014). In summary and in contrast to all other insects examined before, the TKRP positive local neurons provide each glomerulus with particular islet-like projections.

In ALs of insects, all or only a subpopulation of glomeruli can be the target of individual neurons or of populations of neurons and a variety of innervation patterns of olfactory glomeruli by AL neurons (LNs, PNs) or CNs has so far been described either by filling of single neurons or by immunostaining (summarized in Fig. 6; reviewed in Schachtner et al. 2005; Seki and Kanzaki 2008; Husch et al. 2009; Carlsson et al. 2010; Chou et al. 2010; Seki et al. 2010; Neupert et al. 2012; Binzer et al. 2014; Siju et al. 2014). Comparing the different patterns of glomerulus supply via central neurons (CNs, LNs, PNs), innervation can occur (a) only at the surface, (b) scattered throughout the whole glomerulus, (c) or only through parts of the glomerulus, (d) up to massive dense innervation of the whole, or (e) a distinct area of a glomerulus, or (f) as described in this study, through islet-like projections, which are evenly distributed throughout the glomerulus (Fig. 6). From our analysis, we cannot distinguish, whether an individual islet structure is supplied via a single axon or by more axons and whether islets of a single glomerulus are innervated by several or only by one neuron as suggested in Fig. 6f.

The role of LNs in the AL network is to shape the olfactory representation within and between the olfactory glomeruli to eventually form the output profile of the PNs via complex inhibitory and excitatory interactions (Stopfer et al. 1997; Sachse and Galizia 2002; Wilson and Laurent 2005; Olsen et al. 2007, 2010; Root et al. 2007; Shang et al. 2007; Silbering and Galizia 2007; Olsen and Wilson 2008; Okada et al. 2009; Tanaka et al. 2009; Chou et al. 2010; Seki et al. 2010; Wilson 2013; Nagel et al. 2015). It is interesting that the small hive beetle developed, at least for a subpopulation of LNs, a morphologically different pattern of glomerulus innervation compared to other insects. With the available data, we can only speculate on the function of the islet-like innervation.

We offer two hypotheses. Our first hypothesis argues that the islet-like innervation is another effective way to provide the glomerular network with information carried by neuromediators, e.g., neuropeptides. Such neuromediators can act as paracrine neurohormones, affecting a broad area surrounding the release site, also known as “volume transmission”, in contrast to “wiring” (Agnati et al. 1995; Nässel 2002). Considering the facts that the islet number is linearly correlated to glomerulus volume (Fig. 4) and that they are evenly distributed in each glomerulus, the islet contents could affect the glomerulus network over prolonged periods of time or in an otherwise temporally unique fashion. Our second hypothesis interprets the islets as a specific adaptation of the beetles to their lifestyle to cope with the complex chemical communication in a beehive with an olfactory system of a beetle. The TKRP immunostaining unmasks a group of LNs,

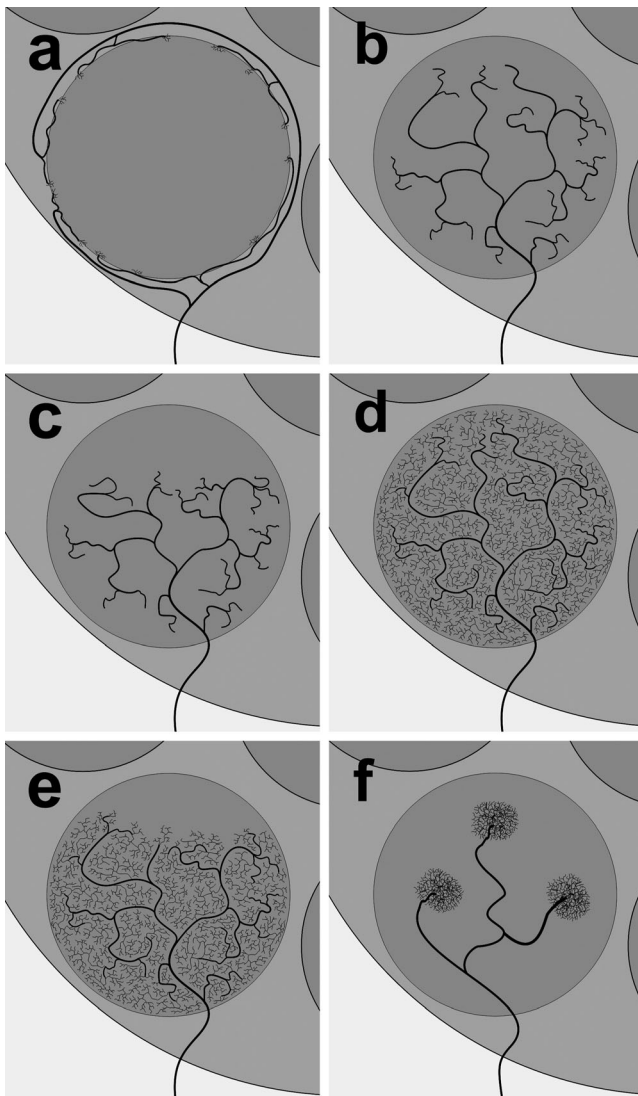


Fig. 6 Schematic drawing of the principal innervation pattern of glomeruli. **a** Glomeruli are just sparsely innervated at the surface. **b** The innervation is scattered through the whole glomerulus. **c** The innervation is just scattered through a distinct area of the glomerulus. **d** The whole glomerulus is densely innervated by fine branches, which appears in immunohistological stainings as a bright, uniform staining. **e** A distinct area of the glomerulus is densely innervated by fine branches. **f** Branching appears just in several, small, distinct areas of the glomerulus (glomerular substructures), which are evenly distributed throughout the glomerulus

which are part of such a particular network. It is known that chemical communication mainly based on pheromones is very important for bees (Slessor et al. 2005; Trhlin and Rajchard 2011). To manage this olfactory task, worker bees have about 64,000 OSNs (Esslen and Kaissling 1976), a large number of glomeruli (152–166 per AL in workers; Arnold et al. 1985) and about 170 ORs (Robertson and Wanner 2006). For a parasitic insect living in a beehive survival and breeding success may highly depend on the ability to understand at least parts of the chemical communication of its host. As the repertoire of the beetle is restricted to about 70 glomeruli per AL, the islets

could be part of a system that allows the beetle to compensate for this disadvantage by expanding the glomerular coding space. The genome sequence of another beetle, *T. castaneum*, revealed a much higher number of functional ORs than olfactory glomeruli and it is still enigmatic what role these surplus ORs could play. The islets could be innervated by OSNs carrying different ORs than the OSNs that principally innervate the glomerulus. Following this line, the islets would functionally stand as specific “glomeruli” within the ordinary glomeruli and thus exaggerate the potential of the olfactory system of the beetle to cope with a more complex odor environment. The synapsin immunostaining is slightly stronger than in the surrounding parts of the glomeruli, suggesting a higher synaptic density within the islets. This argues for specialized zones with high synaptic communication between the involved neurons. If these islet-like zones are targeted by specialized OSNs and/or LNs, PNs remain to be shown in the future by backfills from the antenna and a thorough analysis of intrinsic end extrinsic antennal lobe neurons.

Summary

Analyzing the brain of *A. tumida* by means of immunohistochemistry and 3-D reconstruction revealed a basic brain neuroarchitecture comparable to other beetle and insect brains. In relationship to other brain areas and in comparison to other insects, the AL are relatively large, suggesting that olfaction is of major importance for the beetle. The AL of both sexes house about 70 glomeruli with no obvious size differences of the glomeruli between males and females. In accordance to what is typically found in other insects, staining with a 5HT antiserum revealed a large cell that projects from one AL to the contralateral AL to densely innervate all glomeruli. Immunostaining with an antiserum recognizing TKRPs revealed small spherical substructures, which are in both sexes evenly distributed among all olfactory glomeruli. The source for the TKRP-ir structures is very likely a group of about 80 local AL interneurons. The number of substructures ranges between one and ten and correlates linearly with the volume of the glomeruli. In total, one AL contains about 230 of these islets. For this unusual finding, we offer two hypotheses. First, these evenly distributed substructures could act as massive releasing sites to deliver the neuromediator throughout the particular glomerulus. Second, the islets act as specialized subcompartments that expand the functional coding space of the beetle’s olfactory system.

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