



Morphological and developmental analysis of peripheral antennal chemosensory sensilla and central olfactory glomeruli in worker castes of *Camponotus compressus* (Fabricius, 1787)

Keshava Mysore^{a,b}, Baragur V. Shyamala^b, Veronica Rodrigues^{a,c,*}

^a National Centre for Biological Sciences, Tata Institute of Fundamental Research, GKVK Campus, Bellary Road, Bangalore 560 065, India

^b Department of Zoology, University of Mysore, Manasagangothri, Mysore 570 006, India

^c Department of Biological Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Mumbai 400 005, India

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ABSTRACT

The antennal lobes of different castes of the ant species *Camponotus compressus* show a marked diversity in the organization of their olfactory glomeruli. Notably, there is a significant difference in the number and size of glomeruli between the reproductives and the workers and among the different worker castes. In this report, we investigate the notion that these caste-specific differences in glomerular number might be accounted for, at least in part, by the differences in numbers of olfactory sensilla that target the antennal lobe. For this, we examine the number of sensilla on the antennal flagella of all the individual castes of *C. compressus*. This analysis reveals a striking correlation between sensillar number and the number of antennal glomeruli in a given caste. As a first step in investigating the causal mechanisms that might give rise to this correlation, we carry out an initial characterization of olfactory system development in the minor workers of *C. compressus*. We analyze the temporal pattern of innervations of the developing antennal lobe by olfactory sensory neuron axons. We document the development of the olfactory glomeruli in the antennal lobe during this process, which occurs during early pupal stages. Our findings provide the basis for future manipulative developmental studies on the role of sensory afferent number in glomerular development of different castes within the same species.

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1. Introduction

The olfactory systems of all insects studied to date are characterized by a similar basic organization. Peripheral olfactory sensory neurons (OSNs) are located within sensilla on the surface of the antennal flagella. These OSNs project through the antennal nerve and form a set of identified central antennal tracts to innervate distinct glomeruli in the antennal lobe, where they synapse with local interneurons (LNs) and projection neurons (PNs) (reviewed in Rodrigues and Hummel, 2008; Galizia and Rössler, 2009). The connectivity between the peripheral sensory neurons and their central target neurons in the antennal lobe has comparable structural and functional features in the different insect groups.

Social insects such as ants and bees manifest polymorphisms both in external features and in brain organization which are thought to relate to the specific tasks that they perform within the

colony. Some of these aspects have been reported for the mushroom bodies and other neuropils in the ant brain (Gronenberg et al., 1996; Gronenberg and Hölldobler, 1999; Ehmer and Gronenberg, 2004; Brown et al., 2004; Kühn-Bühlmann and Wehner, 2006). Recently, caste-specific differences in organization of the antennal lobe have been reported for the ant species *Camponotus sericeus* and *Camponotus compressus* (Mysore et al., 2009), and these differences may be involved in the variations in the behavioral responses to various stimuli of which olfaction may be a source of major input, that have been reported for genus *Camponotus* (Hölldobler and Wilson, 1990). In *C. sericeus* and *C. compressus*, the number of central olfactory glomeruli was shown to be different for males, queens and workers. Strikingly, there were also significant differences in glomerular number in the three worker castes, the minor workers, medium workers and major workers (Mysore et al., 2009). Among these castes, the largest number of olfactory glomeruli was observed in the minor workers. In *C. sericeus*, minor workers possess 492 ± 6 antennal glomeruli while in *C. compressus*, minor workers have 501 ± 2 . In both species, the major workers have the smallest number of glomeruli (344 ± 5 for *C. sericeus* and 408 ± 3 for *C. compressus*) and the medium workers of *C. compressus* possess an

* Corresponding author. National Centre for Biological Sciences, (TIFR), GKVK Campus, Bellary Rd., Bangalore 560065, India. Tel.: +91 80 23666230; fax: +91 80 23636662.

E-mail address: veronica@ncbs.res.in (V. Rodrigues).

intermediate number of glomeruli (476 ± 4). Moreover, in minor workers of both the species, it was found that the antennal tracts T4 and T5 innervated a significantly larger number of glomeruli than in the other castes. These marked differences in glomerular number and tract organization in different worker castes are remarkable since all three worker types have a somewhat comparable morphology and importantly share the same genotype. What are the mechanisms that underlie these differences in neuronal architecture of the olfactory system in these worker castes?

In the three cases that have been studied, *Drosophila*, *Apis mellifera* and *Anopheles gambiae*, the overall glomerular number (Laissue et al., 1999; Galizia et al., 1999; Ghaninia et al., 2007) is thought to reflect the number of olfactory receptor (OR) genes that are expressed in a given species (Clyne et al., 1999; Hill et al., 2002; Robertson and Wanner, 2006). Work carried out in *Drosophila* indicates that most OSNs express a single olfactory receptor (OR) and connect to a given glomerulus in the antennal lobe; hence the number of glomeruli corresponds roughly to the diversity of OR gene expression (reviewed by Vosshall and Stocker, 2007). If these findings also apply to the organization of olfactory system in *C. compressus*, minor workers might express a larger number of OR genes than medium workers, which in turn might express a larger number of OR genes than major workers. While this is, in principle, possible and would suggest fascinating gene regulation mechanisms, it is also possible that the number of expressed ORs is the same and other mechanisms for glomerular number control occur. These differences could lie in the differences in the number of OSN axons that project from the antenna to the antennal lobe in the three castes.

A recent study on *Camponotus japonicus*, examined the number of sensory neurons housed in different types of sensilla; *s. basiconica* are innervated by in > 130 OSNs, the *s. trichodea* have only 8–9, while the *s. trichodea curvata* have 50–60 sensory neurons (Nakanishi et al., 2009). We determined the number of chemosensory sensilla on the antennal flagella of the three worker castes using scanning electron microscopy. This revealed a significant correlation between the total number of sensilla and the total number of antennal lobe glomeruli. This correlation was also found for the three different types of sensilla analyzed in the present study, *s. basiconica*, *s. trichodea* and *s. trichodea curvata*, as well as for the number of sensilla on each of the 12 antennal segments in all three worker types. Different number of chemosensory sensilla on the antennal flagella implies different number of total sensory neurons and partially might influence the organization of the antennal lobe in ants.

To investigate the processes that give rise to this correlation between antennal sensillar number and antennal lobe glomeruli number, a study of the development of the olfactory system in the three worker castes is required. As a first step towards this end, we traced the projections of the OSNs from the antennal flagella to the antennal lobe during pupal metamorphosis in minor workers of *C. compressus*. Our findings document the formation of the projections from the OSNs into the antennal lobe and show that the axonal terminals of the OSNs prefigure the formation of the protoglomeruli. Moreover, they reveal a striking increase in the size of the antennal lobe that occurs once the OSN axons invade it. These findings support the notion that major morphological features of the antennal lobe are dependent on the innervating OSN axons and provide the basis for further investigation of mechanisms that determine glomerular numbers in these different worker castes.

2. Materials and methods

2.1. Insects

Mated queens of *Camponotus compressus* were collected from the fields in the vicinity of National Centre for Biological Sciences

(TIFR), Bangalore, India [13° , $43'$ N, 77° , $35'$ E] during the month of March. They were kept in plastic containers and constantly maintained at 12/12 h photoperiod at 25°C . They were fed with 20% sucrose solution and a diet prescribed by Bhatkar and Whitcomb (1970); an additional supplement of 10% honey water improved their survival and egg-laying. These colonies produced adults which were >99% (observation from 10 colonies for a year) minor workers.

When sensillar numbers were to be estimated, the worker castes were collected from the field between April and May, while the reproductives were collected outside the colony during March; but the ages of individuals are unknown.

2.2. Scanning electron microscopy

Individuals were classified into castes as described by Mysore et al. (2009). The size and structure of the antennal flagella was analyzed by scanning electron microscopy (SEM). Antennae ($n = 10$ for workers; $n = 6$ for males; $n = 6$ for queens) were ablated and fixed overnight at 4°C in 4% paraformaldehyde (PFA – Electron Microscopy Sciences, Hatfield, PA.) in phosphate buffered saline (PBS). Samples were taken through an ascending series and stored in 100% methanol: they were air dried, mounted vertically using superglue and coated with a thin layer of gold on all sides for SEM (Leica 440i, Leica Microsystems, Germany). The samples were placed in the chamber, tilted 90° and were imaged across diameter. Each segment was subjected to 4 horizontal scans at an interval of 90° .

The scans were overlapped using clear landmarks to minimize errors in counting of the sensilla. Only *sensilla basiconica*, *sensilla trichodea* and *sensilla trichodea curvata* were analyzed from the worker antenna. These sensilla were described as chemosensory earlier by Fresneau (1979), Hashimoto (1990) and Renthal et al., (2003).

Differences between two groups of samples were computed using Student's unpaired *t*-test with 0.001 as the significance level. Data comprising more than two groups were compared by one-way ANOVA and Scheffe's post-hoc tests using Origin 6.0 software. At least 10 samples were used in each case.

2.3. Analyses of the developing olfactory sense organs

Pupal stages were determined as described by Ishii et al. (2005; Table S1). Briefly, cocoon- spinning larvae (L4s), pre-pupae and pupae were removed from the nest and incubated individually at 25°C and 60% relative humidity. Pupae were aged to 40 h (~7% of pupal life), 75 h (~12% of pupal life) and 125 h (20% of pupal life) and removed from the pupal case. An incision was made along the axis of the antenna using a fine microsurgical knife and a few crystals of micro-ruby (Cat. No. D7162, Molecular Probes, Invitrogen Corporation, CA) were loaded on a fine capillary and applied on the incisions. Preparations were left in a dark and moist chamber for 8 h at room temperature. The antennae were fixed in 4% paraformaldehyde (PFA) overnight, washed extensively in PBS and mounted in 70% glycerol. Imaging of micro-ruby stained profiles was carried out using an Olympus FV 1000 confocal microscope (Olympus Corporation, Japan).

2.4. Developmental analysis of antennal lobes by anterograde tracing and immunohistochemistry

Timed pupae were removed from their case, the antenna ablated and a few crystals of micro-ruby loaded on a fine capillary applied on the cut end of the antenna; preparations were left in a humid

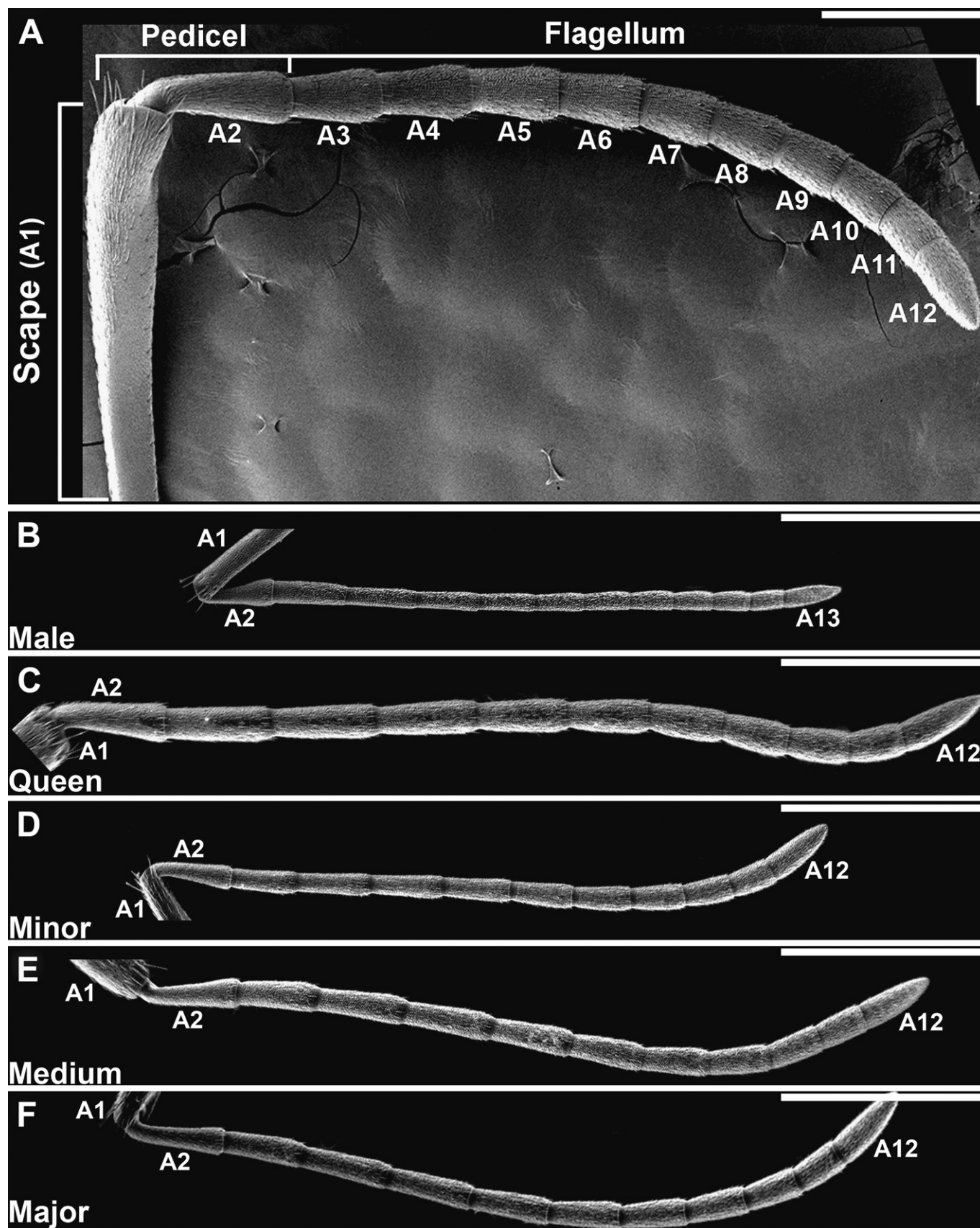


Fig. 1. Antennal organization in different castes of *Camponotus compressus*. (A) Antenna from a worker of *C. compressus*; the scape and components of the funiculus, pedicel and flagellum, are labeled. (B–F) Funiculi of males, queen and worker castes of *C. compressus*. Scale bar in A = 500 μ m, B–F = 1 mm.

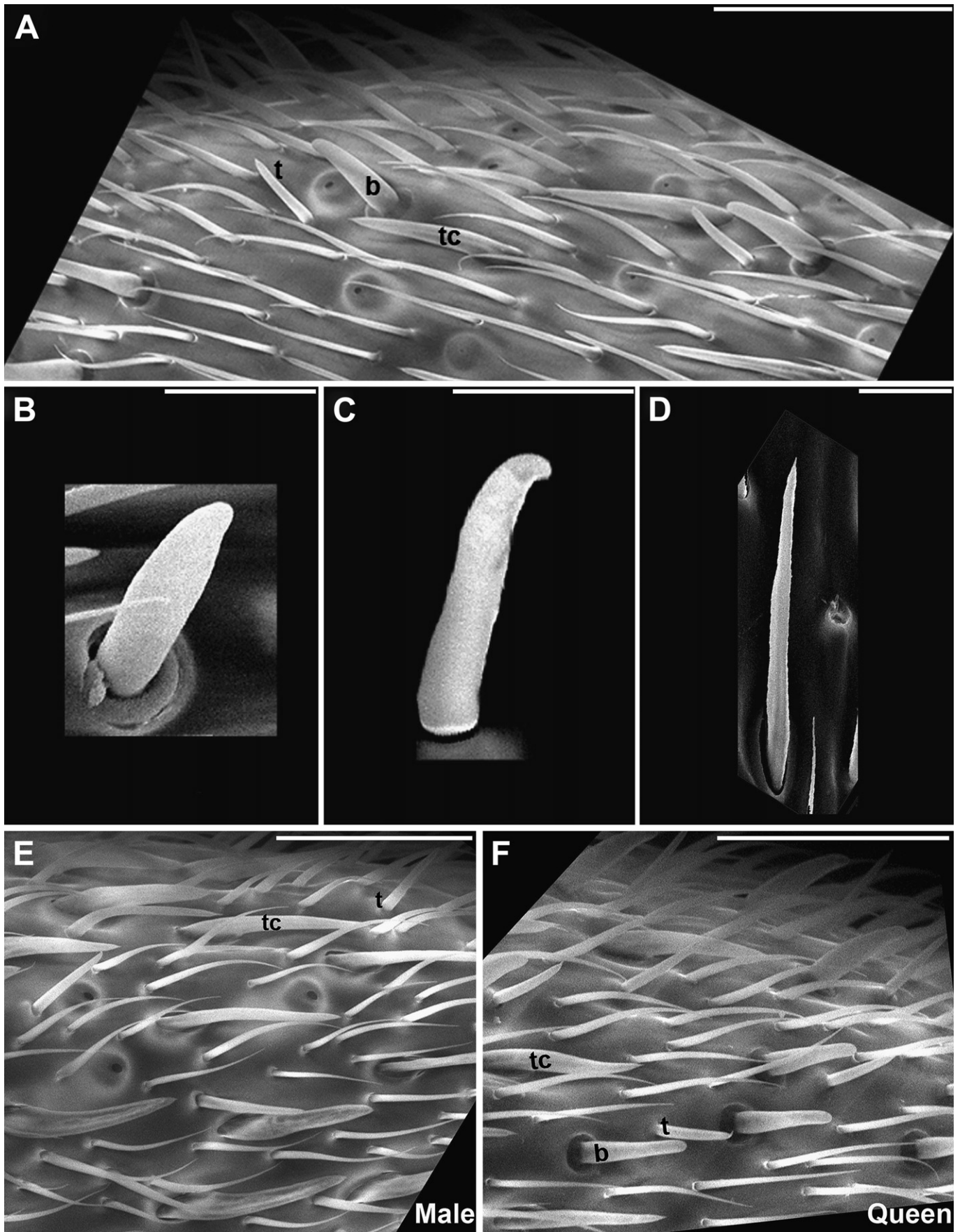


Fig. 2. Scanning Electron Microscopic images of sensilla on the antennal flagella of the ant. (A) Region of antennal flagella of *C. compressus* indicating the sensillar types- *s. basiconica* (b), *s. trichodea* (t) and *s. trichodea curvata* (tc). (B–D) These sensilla are enlarged in high resolution micrographs. (E–F) Distal region of the antenna from a male and queen. Males lack *s. basiconica*. Scale bar in A, E & F = 30 μ m and in B = 6 μ m, C & D = 10 μ m.

Table 1

Distribution of sensilla on the antennal flagella of individuals of *C. compressus* colony. Values are mean \pm standard deviation of counts from SEM micrographs from 6 antennae in case of males and queens and 10 antennae in each of workers. Significance was assessed using one-way ANOVA.

Caste	<i>s. basiconica</i>	<i>s. trichodea</i>	<i>s. trichodea curvata</i>	Total sensilla	Glomerular number
Male	0	244 \pm 6	802 \pm 11	1047 \pm 14	198 \pm 8
Queen	553 \pm 8	303 \pm 7	1002 \pm 14	1555 \pm 19	373 \pm 6
Minor worker	708 \pm 69 ^b	333 \pm 12	1102 \pm 31 ^c	2143 \pm 89 ^a	501 \pm 2 ^d
Medium worker	661 \pm 59	312 \pm 15	1017 \pm 30	1990 \pm 76	476 \pm 4
Major worker	550 \pm 49	260 \pm 24	847 \pm 24	1658 \pm 62	408 \pm 3

^a Significance at $p = 0.0001$, minor workers compared with other worker castes for total number of sensilla.

^b Significance at $p = 0.01$, total *s. basiconica* in minor worker compared with other worker castes.

^c Significance at $p = 0.001$, total *s. trichodea curvata* in minor worker compared with other worker castes.

^d Significance at $p = 0.001$, number of glomeruli in minor workers compared with other worker castes (Mysore et al., 2009).

and dark chamber for at least 10 h. A minimum of 5 animals were sampled at each time point.

Brains were dissected in cold PBS, fixed in 4%PFA and blocked with 10% normal goat serum for 1 h. They were incubated in primary antibody mAb7B1 (1:10; BioKlone, Chennai) for 48 h. Following washing, samples were incubated in secondary antibodies: anti-mouse Alexa 488 (1:400, Molecular Probes, Invitrogen Corporation, CA) and anti-rat Alexa 568 (1:400, Molecular Probes, Invitrogen Corporation, CA) secondary antibodies for 4 h at 4 °C. Following washing in PBS containing 0.3%Triton-X100 (PTx) for 1 h, brains were mounted in 70% glycerol and imaged as described in Section 2.5.

To assess cell-division dynamics, animals of each stage were injected with 3 μ l of 20 mM BrdU (Cat. No. 586064, Boehringer Mannheim GmbH, Mannheim, Germany). They were kept in a moist chamber for 8 h at room temperature; brains were dissected in chilled PBS and transferred immediately into 4% PFA solution in PTx overnight. Brains were washed in 0.3% PTx and incubated for 2 h in a solution of 50% formamide/2X SSC buffer (17.53 g NaCl, 8.82 g sodium citrate/litre pH 7.0) at 65 °C, followed by several washes in PBS. They were incubated for 30 min in 2 N HCl at 37 °C and neutralized in 0.1 M boric acid

for 10 min. Samples were incubated with anti-BrdU (1:100; Cat. No. ab6326, Abcam, Cambridge, MA) antibody for 48 h followed by treatment with secondary antibodies as described above.

2.5. Antibodies used in this study

2.5.1. Monoclonal antibody 7B1

Mouse mAb7B1 was generated by BioKlone, (Chennai, India). Briefly, mice were immunized with \sim 5 mg brain homogenate of *C. sericeus* and those showing a positive reaction in ELISA tests against the antigen were used to generate hybridoma cell lines. Supernatants were tested for immunoreactivity against brain tissues in wholemount stainings. mAb7B1 recognizes brain neuropile of *C. sericeus* and *C. compressus* at developmental stages and that of the adult. It does not cross-react with *Drosophila* brains.

2.5.2. Anti-BrdU

Rat monoclonal antibody (abcam, Catalog#ab6326, 1:100) generated against 5-bromo-2'-deoxyuridine-BSA (Lessner et al., 2002). The antibody was used to visualize 5-bromo-2'-deoxyuridine (BrdU) that is incorporated into dividing cells.

2.6. Confocal laser scanning microscopy and image processing

Stacks of \sim 60–100 images were collected with step sizes 0.4 μ m (20 \times objectives with 1.3–1.5 \times digital zoom for antennal lobe) at a resolution of 512 \times 512 pixels. Preparations labeled with Alexa 488 were excited with an argon laser at 488 nm and fluorescence detected through a 505 nm long-pass filter. Alexa 568 and micro-ruby labeled preparations were excited with HeNe laser at 543 nm and detected using a 560 nm long-pass filter. Images obtained from a confocal microscope were processed using ImageJ (<http://rsb.info.nih.gov/ij/>; Wayne Rasband, NIH, USA) and Adobe Photoshop CS3.

The volume of the antennal lobe was determined as per Mysore et al. (2009) using Amira 4.1 software (TGS, France). Briefly, the “label field” option was used and the antennal lobe region or antennal lobe anlagen was outlined in each section. Further the volume was determined using “tissue statistics” of Amira 4.1.

Table 2

Distribution of sense organs on different segments of the antenna on the individuals of *C. compressus* colony. Values are mean \pm standard deviation of counts from SEM micrographs from 6 antennae in case of males and queens and 10 antennae in case of workers. Significance between minor and the other workers was assessed using one-way ANOVA.

Caste	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	Type of Sensilla
Male	0	0	0	0	0	0	0	0	0	0	0	0	0	<i>s. basiconica</i>
Queen	0	0	7 \pm 3	15 \pm 3	19 \pm 2	24 \pm 4	34 \pm 2	58 \pm 5	63 \pm 6	75 \pm 5	93 \pm 2	165 \pm 4	NP	
Minor worker	0	0	10 \pm 5	23 \pm 5	28 \pm 6	38 \pm 10	47 \pm 8**	70 \pm 10	79 \pm 10**	96 \pm 10**	114 \pm 6**	204 \pm 10**	NP	
Medium worker	0	0	9 \pm 4	21 \pm 5	26 \pm 5	35 \pm 9	44 \pm 7	70 \pm 10**	73 \pm 9**	89 \pm 10**	105 \pm 6**	188 \pm 10**	NP	
Major worker	0	0	8 \pm 4	18 \pm 4	22 \pm 4	29 \pm 7	36 \pm 6	51 \pm 9	61 \pm 7	74 \pm 8	87 \pm 5	157 \pm 8	NP	
Male	0	0	6 \pm 1	8 \pm 1	9 \pm 1	11 \pm 2	21 \pm 1	25 \pm 5	26 \pm 1	30 \pm 1	33 \pm 1	35 \pm 1	39 \pm 2	<i>s. trichodea</i>
Queen	0	7 \pm 2	10 \pm 2	11 \pm 1	14 \pm 2	26 \pm 1	30 \pm 7	32 \pm 1	37 \pm 1	41 \pm 1	44 \pm 1	49 \pm 2	NP	
Minor worker	0	0	11 \pm 2	14 \pm 3	18 \pm 4	22 \pm 4	31 \pm 3	35 \pm 3	37 \pm 5	48 \pm 4	55 \pm 2	61 \pm 6	NP	
Medium worker	0	0	13 \pm 4	14 \pm 4	17 \pm 4	20 \pm 4	28 \pm 3	33 \pm 3	35 \pm 4**	45 \pm 3**	51 \pm 2**	57 \pm 5**	NP	
Major worker	0	0	11 \pm 3	12 \pm 3	14 \pm 3	17 \pm 3	24 \pm 2	25 \pm 9	26 \pm 10	37 \pm 3	42 \pm 2	43 \pm 16	NP	
Male	0	0	10 \pm 1	37 \pm 3	55 \pm 3	61 \pm 2	71 \pm 1	76 \pm 3	84 \pm 2	85 \pm 3	92 \pm 2	107 \pm 5	123 \pm 4	<i>s. trichodea curvata</i>
Queen	0	13 \pm 2	47 \pm 4	68 \pm 4	77 \pm 3	89 \pm 1	95 \pm 4	105 \pm 3	106 \pm 3	114 \pm 3	134 \pm 6	154 \pm 6	NP	
Minor worker	0	0	58 \pm 11	75 \pm 6**	80 \pm 6**	91 \pm 8**	100 \pm 4**	117 \pm 7**	123 \pm 10**	133 \pm 11**	143 \pm 9**	181 \pm 15**	NP	
Medium worker	0	0	53 \pm 10	69 \pm 5	74 \pm 6	84 \pm 7	93 \pm 4***	108 \pm 6***	113 \pm 10**	123 \pm 10***	132 \pm 8***	167 \pm 14***	NP	
Major worker	0	0	44 \pm 8	58 \pm 4	61 \pm 5	70 \pm 6	77 \pm 3	90 \pm 5	94 \pm 9	102 \pm 9	110 \pm 7	139 \pm 12	NP	

*** $p < 0.001$, ** $p < 0.01$. Comparison between the medium and major workers was carried out by the unpaired student *t*-test. *** $p < 0.001$, ** $p < 0.01$. NP-not present.

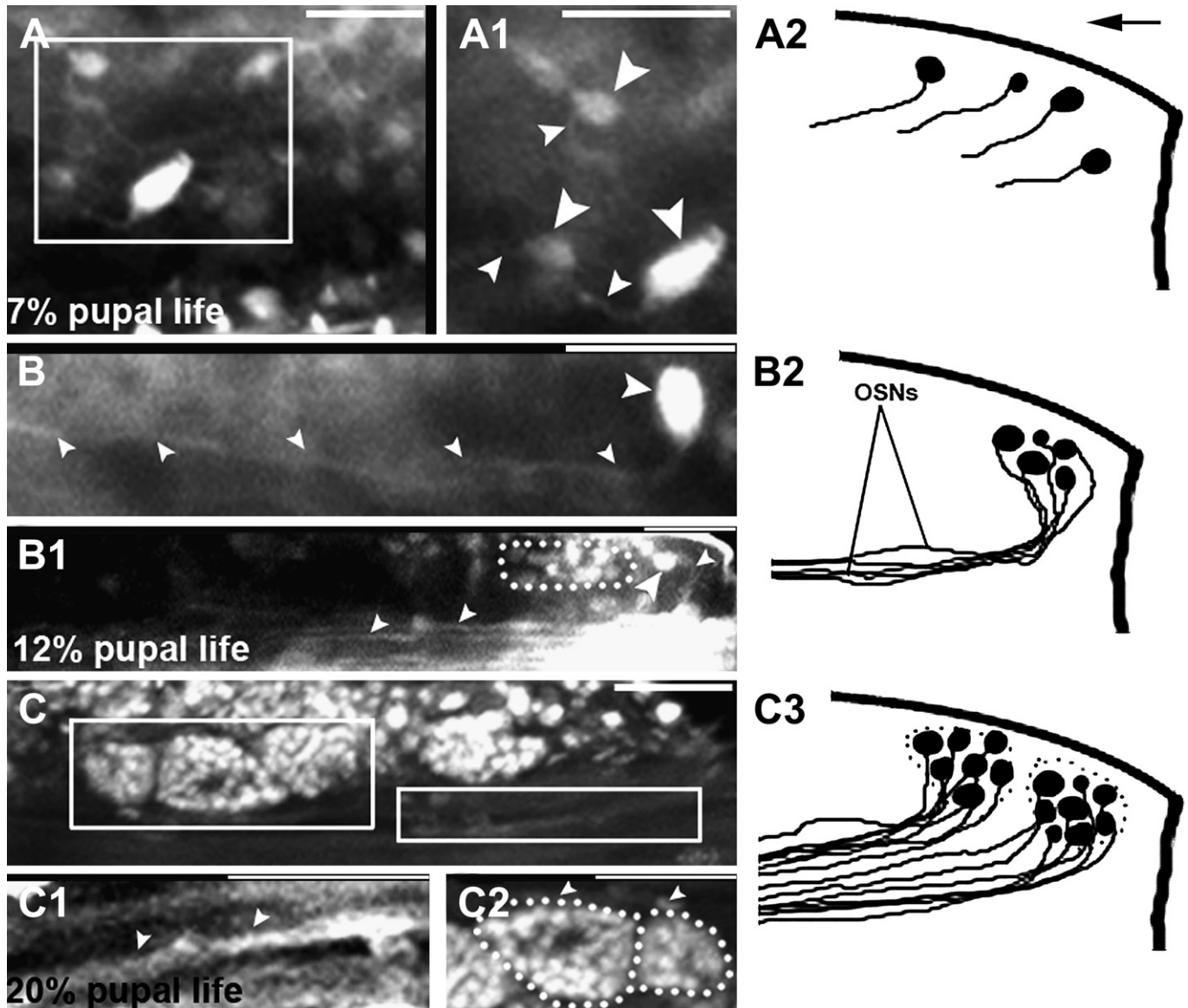


Fig. 3. Development of olfactory sense organs in antennae of developing workers of *C. compressus*. (A) At 40 h APF a few differentiated neurons are labeled by micro-ruby. A region demarcated in the white box is magnified in A1. Large arrowheads indicate the cell soma; projections are labeled with a small arrowhead. (B) A region of the antennal flagellum at 70 h APF where cell bodies (large arrowheads) can be seen with longer projections (small arrowheads) that project towards the brain. A single labeled cell is shown in B1. (C) 125 h APF antenna. The neurons have differentiated in most regions of the antenna (enlarged in C1) and processes traverse in the developing antennal nerve (enlarged in C2). (A2, B2 and C3) Schematics showing the development of OSNs in the antennal flagellum. Scale bar = 50 μ m.

3. Results

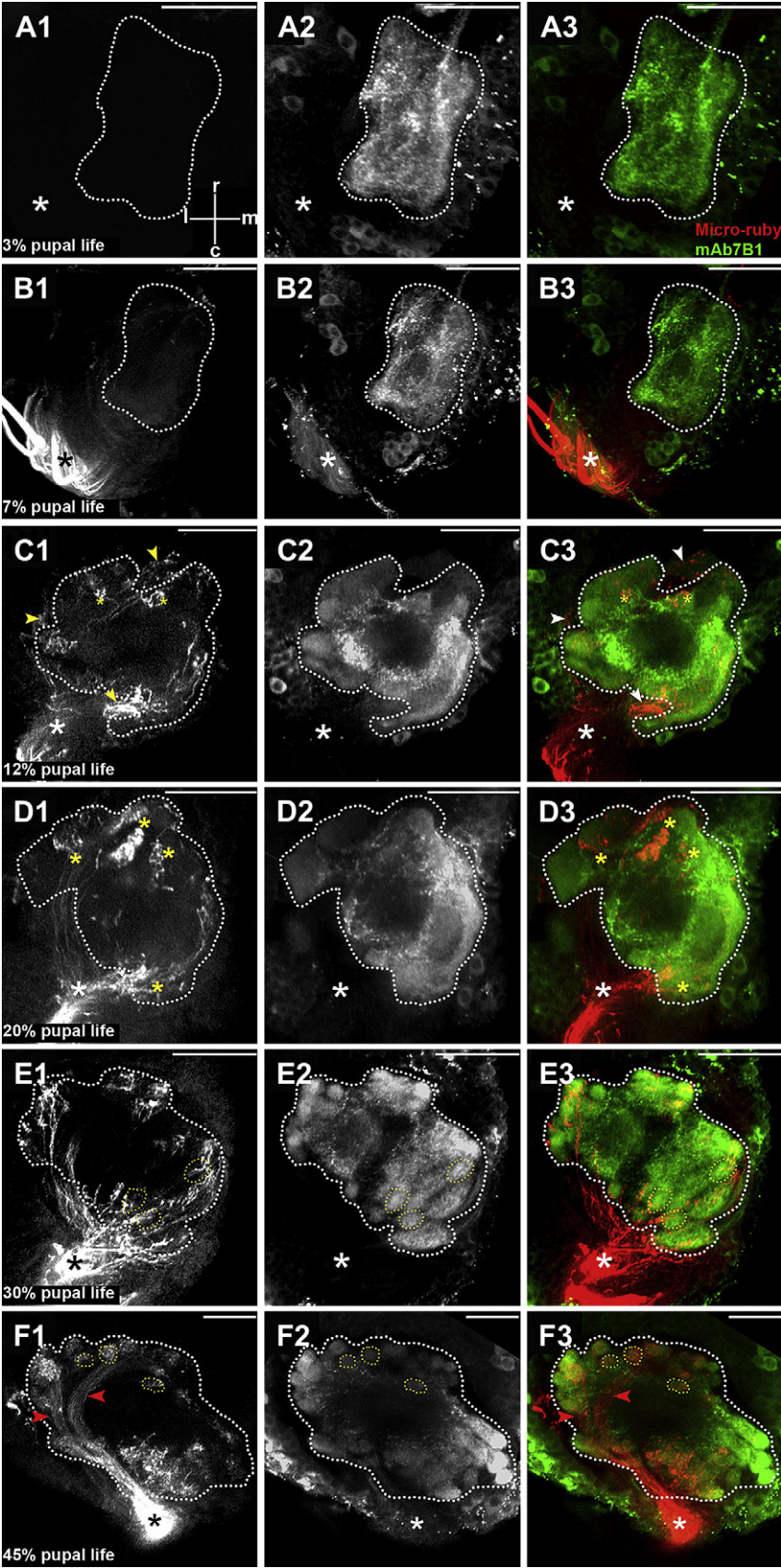
3.1. Analysis of antennal chemosensory sensillar number in different castes of *C. compressus*

In all insects, the glomeruli of the antennal lobe are first order centers of olfactory information processing. Previous studies have shown that the number of antennal glomeruli differs in the castes of *C. compressus* (Mysore et al., 2009). To determine if there are comparable differences in the number of peripheral antennal sense organs in the castes, we used scanning electron microscopy (SEM) to visualize the antennal sensilla in each antennal segment and based on this, performed sensillar counts for males, reproductive females, and the three different worker types.

The antennal flagellum in *C. compressus* has 13 segments in the male and 12 in the female reproductives and workers (Fig. 1A–F). The length of the antennal flagella varies among the different castes

of *C. compressus* (Fig. S1). Males have the shortest antennae (3.3 ± 0.017 mm, $n = 6$) while queens have longest (4.9 ± 0.01 mm, $n = 6$; $p < 0.001$). Among the three worker types, the length of the antenna in majors (4.5 ± 0.03 mm, $n = 10$) and in medium workers (4.5 ± 0.06 mm, $n = 10$) is similar, while the minor workers have a significantly shorter (3.8 ± 0.04 mm, $n = 10$; $p < 0.0001$) antenna (Fig. S1). However, the antennal flagellum was still found to be disproportionately large relative to the small size of other external structures in the minors (Mysore et al., 2009). The minors can be referred to as ‘walking antennae’.

Three types of chemosensory sensilla are readily distinguished on the antennal flagella of all *C. compressus* castes. These are *sensilla basiconica*, *sensilla trichodea* and *sensilla trichodea curvata* (Fig. 2A–D). Based on ultrastructural and electrophysiological studies performed on other insects, all three of these sensillar types are likely to have an olfactory function (Fresneau, 1979; Hashimoto, 1990; Renthal et al., 2003; Ozaki et al., 2005). The male antenna is



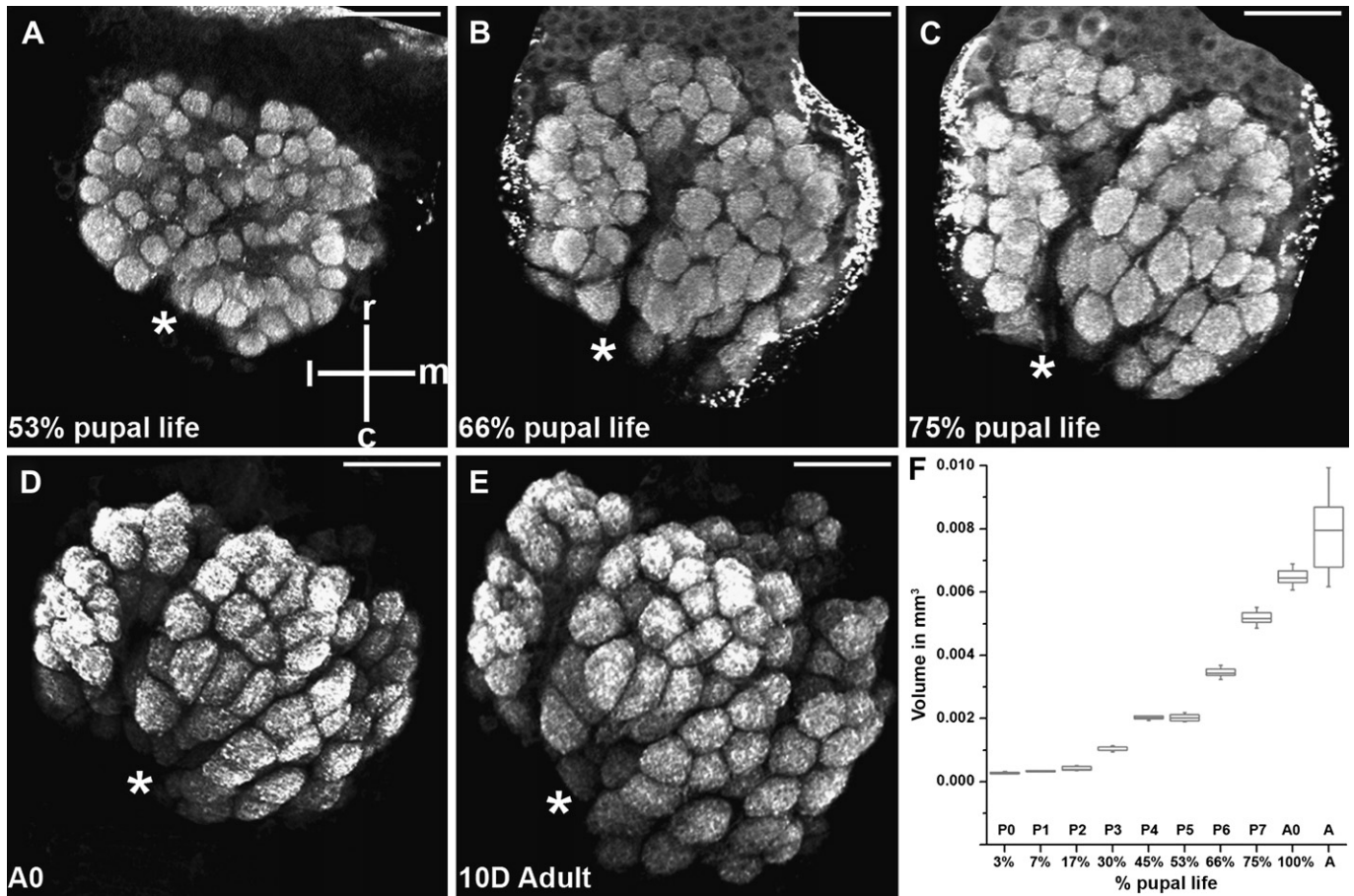


Fig. 5. Late postembryonic development of antennal lobes in minor workers of *C. compressus*. (A–E) Labeling with mAb7B1 reveals the glomerular development and maturation at late stages of AL development in *C. compressus* minor workers. The orientation of images is indicated in A (m-medial; r-rostral; l-lateral; c-caudal). Scale bar = 50 μm. White asterisk indicates the entry point of the antennal nerve. (F) The volume of the antennal lobe at different stages of development determined by Amira 5.2 (TGS, France) indicated in mm³. Data from at least 4 antennal lobes are used for each stage.

distinct in that it lacks *s. basiconica*, while females -queens and all the worker classes- possess all three types of chemosensilla (Fig. 2E, F).

The three different worker classes show significant differences in the total numbers of chemosensilla present on the flagella (Table 1). Remarkably, despite the small size of the antenna, the minor workers have the largest number of sensilla (2143 ± 89 , $n = 10$; $p < 0.0001$; Table 1; one-way ANOVA) as compared with other workers (medium workers: 1990 ± 76 , $n = 10$; major workers: 1658 ± 62 , $n = 10$; Table 1) and reproductives (males: 1047 ± 14 , $n = 6$; queen: 1555 ± 19 , $n = 6$; Table 1). There is also a significant difference in total sensilla number between medium and major workers ($p < 0.001$; student *t*-test). Hence, the differences in total sensillar number in the three worker castes correlate with the previously reported differences in the number of antennal lobe glomeruli in the three worker castes; minor workers (501 ± 2) have more antennal lobe glomeruli than medium workers (476 ± 4) and major workers (406 ± 3) (Mysore et al., 2009). The number of sensilla on the antenna of the queen (1555 ± 19 , $n = 6$) are less than

on the major workers correlating with a smaller number of glomeruli (373 ± 6); males have the smallest number of sensilla (1047 ± 14) and glomeruli (198 ± 8). The correlation between sensillar and glomerular numbers is strong, particularly in the female castes with ~ 4.2 sensilla per glomerulus. However, this correlation must be interpreted with caution since we have not examined the numbers of OSNs innervating each sensillum.

The difference in sensillar number between the minor workers and other castes is most significant for the *s. trichodea curvata* ($p < 0.001$; one-way ANOVA) and *s. basiconica* ($p < 0.01$; one-way ANOVA; Table 1). The number of *s. trichodea* are also significantly different between minor workers and major workers ($p < 0.001$) but not between minor and medium workers ($p = 0.0139$; one-way ANOVA followed by Scheffe's post-hoc test). The numbers of sensilla differ across the segments of the antennal flagellum (Table 2). As reported previously, the scape is devoid of any chemosensilla (Letzkus et al., 2006). The A2 segment also lacks all three sensillar types. For the remaining segments, the number of sensilla increases from proximal to distal antennomers. The difference in sensillar

Fig. 4. Early postembryonic development of antennal lobe in minor workers of *C. compressus*. (A1–F1) Dye backfilling using micro-ruby label OSNs from the antennal flagella; (A2–F2) lobe anlagen is immunostained with the neuropile marker mAb7B1. (A3–F3) Merged images from first two columns. All images are shown in the orientation indicated in A (m-medial; r-rostral; l-lateral; c-caudal). The entry of the antennal nerve is marked with *. The antennal lobe is demarcated with white dots. Scale bar = 50 μm. (B) Micro-ruby labeled neurons are first detected within the brain at 40 h APF (7% of pupal life), although terminals have still not entered the antennal lobe (B1–3). mAb7B1 stains the lobe neuropile prior to this (A2) suggesting that interneurons are present within the lobe well before OSN entry. By 75 h APF (12% of pupal life) the first OSN axons invade the lobe (yellow arrowheads in C) and these form loose structures termed protoglomeruli (yellow asterisks in D) by 125 h APF (20% pupal life). By 190 h APF (30% pupal life) (E1–3) some glomeruli have matured from the protoglomeruli and formation continues in a staggered manner (highlighted with yellow dots in E and F). Tracts carrying OSNs to defined glomerular clusters can be identified by 270 h APF (45% of pupal life) (red arrowheads in F1–3).

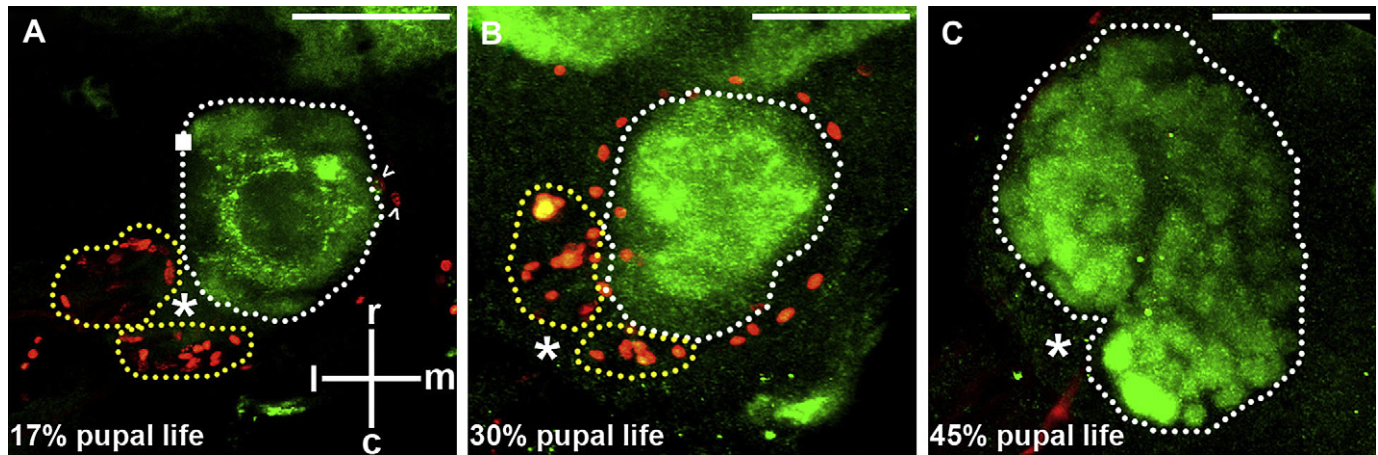


Fig. 6. Cell division profiles in the developing antennal lobe in minor workers of *C. compressus*. (A–C) Dividing cells were labeled by injection of BrdU; incorporation was revealed by labeling with anti-BrdU 8 h after injection (red). (A) BrdU-positive cells first appear at ~17% of pupal life where two clusters of cells (yellow dotted lines) are located at the entry of the antennal nerve (*) and a few cells (arrowheads) are seen at the periphery of the lobe (demarcated with white dots). (B) By 30% of pupal life, BrdU labeled cells are located around the lobe. (C) No further labeled cells are observed in injections after 45% pupal life. The orientation of images is given in A (m-medial; r-rostral; l-lateral; c-caudal). Scale bar = 50 μ m.

number between the three worker castes is most pronounced in the distal antennomers (A10–A12; $p < 0.01$, Table 2; one-way ANOVA).

3.2. Development of the sensory neurons within the antennal flagellum

The olfactory sensilla on the surface of the antenna are innervated by OSNs that connect to the antennal glomeruli in the antennal lobe (Mysore et al., 2009). In holometabolous insects, the development of the sensory neurons has been shown to arise from progenitor cells in the antennal disc during pupal life. Such an analysis is not yet feasible in the ant, due the lack of antibody and other cellular markers to label developing cells. We were however, able to mark mature OSNs by backfilling from a distal lesion by placing crystals of micro-ruby on a damaged region. We focused on the pupal development of minor workers of *C. compressus* since this caste had the highest relative number of antennal sensilla and olfactory glomeruli; also the young colonies of the species makes only minor workers during the initial time of starting a colony making it possible for us to study the development of the OSNs in this caste only. In an initial staging study, we were able to recognize 7 pupal stages, P1–P7, that corresponded to those described by Ishii et al. (2005) for *Camponotus japonicus* (Table S1). The entire pupal duration lasts 23 ± 4 days/504–648 h at 25 °C and we classified each stage as percentage of total pupal life in order to facilitate a comparison between our findings and those from other holometabolous insects.

The developing antennal flagellum can first be recognized in the pupa 14–17 h after pupal formation (APF). At this time (~3% of pupal life), we could not observe any micro-ruby labeled cells within any region of the antennal flagellum (data not shown). We first observed labeled cells that showed features of sensory neurons at ~40 h APF (~7% pupal life) (Fig. 3A). The cell bodies (large arrowheads in Fig. 3A1) possessed short projections (small arrowheads in Fig. 3A1). By ~70 h APF (~12% pupal life), many more cell somata could be detected near the site of the backfill (large arrowheads and region within dotted lines in Fig. 3B). Several cells showed long axonal projections that traversed the length of the antennal flagellum (small arrowheads in Fig. 3B1). It appears that additional OSNs are formed during development and fills at ~120 h APF (~20% pupal life) labeled large clusters of cell somata

(Fig. 3C1) and their projections fasciculate into distinct nerve bundles (Fig. 3C2, diagram in 3C3).

3.3. Projection of the OSN axons to the brain and antennal lobe during pupal life

We next traced the projections of the developing axons to the antennal lobe glomeruli using backfilling with micro-ruby as described above. In these experiments we dissected the pupal brain and in addition to visualizing micro-ruby filled axons also immunostained with the neuropile marker mAb7B1. No labeling could be seen at ~15 h APF (Fig. 4A1–3% of pupal life), supporting our findings described above that OSNs were not yet mature. In contrast, immunostaining with mAb7B1 revealed a compact structure within the developing antennal lobe suggesting that some interneurons had already projected into the antennal lobe at this time (Fig. 4A2, A3). The earliest time point when OSNs could be seen within the antenna is ~40 h APF (~7% pupal life) (Fig. 3A); some axons appear to have projected into the brain. However these did not enter the developing antennal lobe neuropile (Fig. 4B1,B3; axons indicated by asterisk; antennal lobe anlage demarcated with white dotted lines). Immunostaining of the developing antennal lobe neuropile with mAb7B1, at this stage was relatively homogeneous and localized to a compact structure similar to that observed in the earlier stage (Fig. 4B2). By early P2 (~75 h APF, ~12% pupal life), OSN axon projections began to encircle the developing antennal lobe (Fig. 4C1; yellow arrowheads). Moreover, at this stage the first labeled axons invaded the antennal lobe neuropile (Fig. 4C1, 3; yellow asterisks). The number of OSN axons that projected into the developing antennal lobe increased with time and by late stage P2 (~125 h APF, ~20% pupal life), labeled terminal arbor-like structures first became apparent within the antennal lobe (Fig. 4D1, D3; yellow asterisk). Structures which resembled protoglomeruli described in other insects (Malun et al., 1994; Oland et al., 1998) were recognizable at late stage P2 (Fig. 4D1–D3; yellow asterisk) as well as at stage P3 (Fig. 4E1–E3; highlighted by yellow dots; 190 h APF, ~30% pupal life). Distinct glomerular structures innervated by labeled axons were clearly observed at stage P4 (Fig. 4F1–F3; ~270 h APF, ~45% pupal life).

Distinct glomeruli were present in the developing antennal lobe from stage P4 onward. To determine the number of glomeruli that were present, careful analyses of confocal sections through 4

immunostained antennal lobes were carried out at this stage as well as at stage P5. At stage P4, a total of 421 ± 21 glomeruli were identified. Moreover, at this stage organization of OSN axons into distinct tracts within the antennal lobe also became apparent (Fig. 4F1, F3; red arrowheads). At stage P5, the total adult complement of ~ 500 glomeruli in minor workers was already present (Fig. 5A; Movie 1; ~ 320 h APF, $\sim 53\%$ pupal life). The number of glomeruli in the antennal lobe and their general structural organization remain unchanged until emergence of adults (Fig. 5A–D). An analysis of the total volume of the developing antennal lobe revealed a marked, approximately linear increase in the volume from stage P2 onward (Fig. 5F). Thus, the onset of this volume-increase correlates with the onset of antennal lobe innervations by OSN axons.

3.4. Analysis of mitotic activity in cells associated with the developing antennal lobe

In addition to our developmental analysis of ORN projections and glomerular formation, we observed evidence for mitotic activity in cells that were closely associated with the antennal lobe during the first half of pupal development. Since this suggests neuronal and/or glial progenitors might be undergoing proliferative division during the time when the antennal lobe begins to increase in size, we studied this phenomenon in more detail. We injected the nucleoside thymidine analogue BrdU and examined incorporation of the label by immunoreactivity; injections were carried out at several pupal stages and labeled cells were visualized 8 h later. During early pupal life up to about stage P3 (~ 190 h APF, $\sim 30\%$ pupal life), cells that incorporated BrdU, and hence were probably engaged in mitotic division were observed in the vicinity of the antennal lobe (red in Fig. 6A, B). In contrast, evidence for dividing cells near the antennal lobe could no longer be detected when BrdU was administered after stage P4 (Fig. 6C; 270 h APF, $\sim 45\%$ pupal life); BrdU-incorporation into cells was still observed at this stage near the developing mushroom bodies (data not shown). These findings define a window for cell division in the vicinity of the antennal lobe between the start of pupation and ~ 300 h APF. Our experiments showed a lack of cell division throughout the central nervous system beyond stage P5, consistent with earlier reports in other ant species (Ishii et al., 2005).

Interestingly, specific clusters of dividing cells in the early pupal stages (P1 to P2) were localized at the position where the antennal nerve enters the antennal lobe (Fig. 6A, B). Moreover, when a pulse of BrdU was given between 100 and 200 h ($\sim 30\%$ of pupal life) labeled cells were observed at the periphery of the antennal lobe (Fig. 6B). Mitotically active glial cells have been reported at a comparable position around the antennal lobe, in *Drosophila* (Jhaveri et al., 2000). The identity of these cells in our preparations remains unknown; we are unable to test whether they are glia because of the lack of specific markers.

4. Discussion

In previous studies we have shown that different castes of the ants *C. compressus* and *C. sericeus* possess different numbers of glomeruli within the antennal lobe (Mysore et al., 2009). What might be the basis for these caste-specific differences in developmental patterning? We discuss two possible non-exclusive mechanisms which could be tested: i) In most species studied the number of OR genes correlate strikingly with the numbers of glomeruli. Since each OSN expresses only one OR gene, the glomeruli appear to serve as 'collection centers' for OSN expressing the same OR (reviewed in Vosshall and Stocker, 2007). In this case, we would expect each caste to express a different number and/or

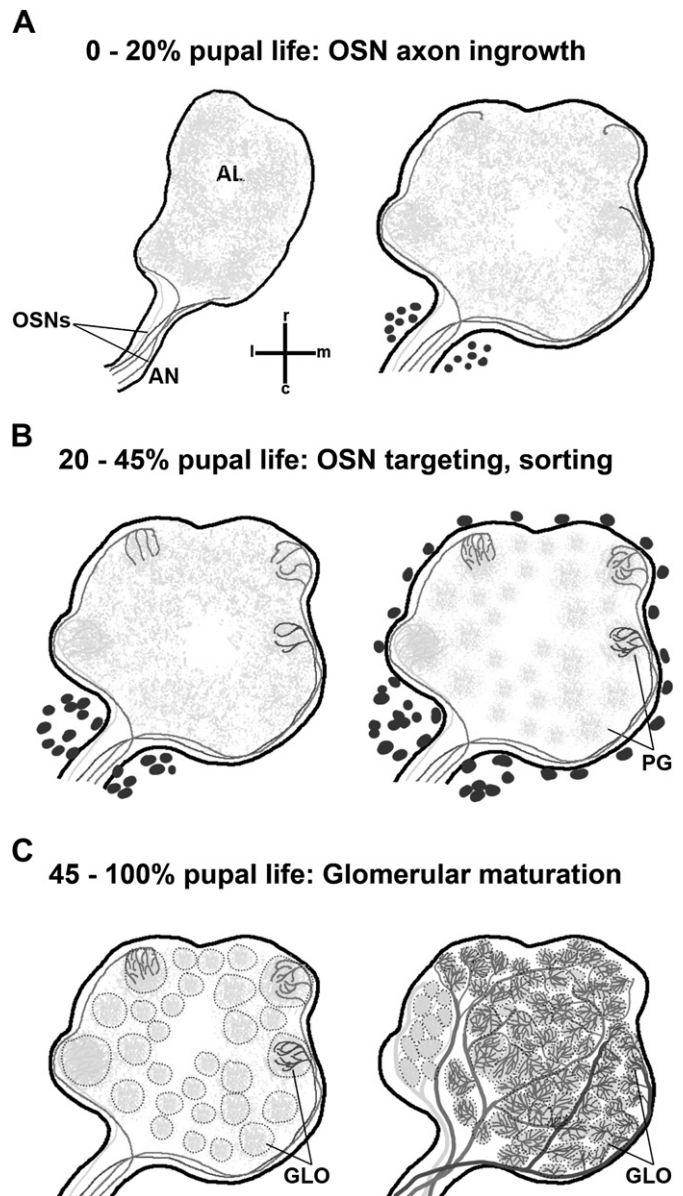


Fig. 7. Summary of development of antennal lobe in *C. compressus* minor workers. (m-medial; r-rostral; l-lateral; c-caudal). (A) 0 to 120 h APF (0–20% pupal life). Antennal OSNs, first reach the antennal lobe by 70 h APF ($\sim 12\%$ pupal life) and stall at the periphery of the lobe. Synaptic markers (shaded regions) are present homogeneously within the lobe anlagen suggesting that, while the secondary interneurons are already present, they lack a distinct organization. Cells (large dots) that had recently undergone division are located at the entry of the antennal nerve. (B) 120 to 250 h APF (20–45% pupal life). The first OSNs target the antennal lobe by 120 h APF and terminate in specific regions which prefigure formation of protoglomeruli. This is followed by glomerular formation in a staggered manner across the antennal lobe. As glomerular formation proceeds, nascent cells labeled with BrdU (large dots) appear around the antennal lobe. (C) 250 to ~ 600 h APF (45% to 100% pupal life). The numbers of glomeruli are comparable to that of the adult. However, the lobe continues to grow in size up till the point of adulthood. During this period, individual glomeruli possibly undergo maturation to form the adult structure.

repertoire of OR genes. ii) Glomerular number and pattern is known in several studies to dependent on afferent innervation (reviewed by Oland and Tolbert, 1996; Tolbert et al., 2004). This notion is supported by data from *Manduca sexta* (Hildebrand et al., 1979). Trans-sexual grafting of a male antennal disc resulted in formation of a male-specific macroglomerulus in the female antennal lobe (Rössler et al., 1999b). In view of these observations, we reasoned

that glomerular number in different worker castes of the same species might correlate with the numbers of OSNs terminating within the antennal lobe.

The analysis of antennal sensillar number demonstrates that castes with the largest number of glomeruli, i.e. the minor workers, also have the largest number of sensilla (~ 2200 and 501 ± 2 glomeruli), while the medium workers with ~ 2000 sensilla have 476 ± 4 glomeruli and the major workers have ~ 1700 sensilla and 408 ± 3 glomeruli. These findings indicate a correlation between sensillar number, and by inference OSN number, and the number of glomeruli in the antennal lobe of the corresponding worker caste. To determine if this correlation has a causal basis, an analysis of the development of antennal lobe innervation and glomerular formation is to be performed.

The sequence of events that underlie developmental patterning of the antennal lobe has been examined in several insects including *Drosophila melanogaster* (Jhaveri et al., 2000; Jefferis et al., 2004), the hawk moth *M. sexta* (Malun et al., 1994; Oland and Tolbert, 1996; Oland et al., 1998) and the honeybee *A. mellifera* (Groh and Rössler, 2008). In all of these cases, there is a broad conservation of the developmental events leading to formation of the antennal lobe glomeruli. Studies in *Drosophila* and *M. sexta* have shown that incoming OSNs target defined regions within the antennal lobe to induce formation of protoglomeruli (Malun et al., 1994; Oland et al., 1998). In Lepidopterans, there is evidence that the OSNs are organized by axon–glial interaction in the sorting zone at the entrance of the antennal lobe to form distinct tracts (Rössler et al., 1999a). Studies performed in *Drosophila* indicate that antennal lobe interneurons are already present within the antennal lobe prior to OSN entry (Jefferis et al., 2004) and synaptic connectivity within the protoglomeruli occurs upon arrival of the OSNs, allowing these to mature into glomeruli. Taken together these studies provide evidence that the OSNs are key elements in specifying glomerular morphology (Hildebrand et al., 1979; Oland and Tolbert, 1987; Tolbert, 1989; Rössler et al., 2000).

Since nothing was known about the development of the olfactory system in worker castes of *C. sericeus* and *C. compressus*, we carried out an initial study of OSN development and axonal in-growth and the formation of the olfactory glomeruli in *C. compressus* minor workers. These investigations identify the main phases of olfactory system development in this ant caste. Moreover they provide the basis for future manipulative developmental studies which are needed to test the idea that caste-specific differences in glomerular number can be accounted for, at least in part, by the differences in numbers of OSNs targeting the antennal lobe in the different worker castes.

Our characterization of antennal lobe development of the minor worker of *C. compressus* indicates that the overall developmental process can be divided into three stages (summarized in Fig. 7). During the first stage, between 0 and 150 h APF (0–20% of pupal life) OSN development in the antenna and axon in-growth occurs. Dye-fills from the developing antennal flagellum, first labeled axons in the CNS by ~ 75 h APF suggesting that this is the time point when the first sensory axons arrive in the brain. Subsequent axonal projections reach the antennal lobe until about 150 h APF. During this period, OSN terminals do not invade the antennal lobe but remain at its periphery. During the following second stage, which lasts until about 250 h APF (20–45% pupal life), OSN targeting, sorting and glomerular formation occurs in the antennal lobe. During this phase, OSN axons segregate within the antennal nerve into tracts and project to different regions of the antennal lobe. Their terminals arborize within regions of the antennal lobe to form protoglomeruli-like structures in the first half of this phase (within 30% of pupal life). Protoglomeruli are loose collections of OSN terminals which form a template for

induction of glomeruli (Malun et al., 1994; Oland et al., 1998; Lipscomb and Tolbert, 2003). Formation of definite glomeruli occurs in a spatially staggered manner across the antennal lobe and based on investigations in other insects, most likely involves the formation of synapses between OSNs, PN and LN. The antennal lobe volume increases during this period and there is evidence of cell division around the antennal lobe between 20% and 45% of pupal life. During the final third phase, antennal lobe volume continues to increase until adulthood and this is probably due to an increase in the size of individual glomeruli. We term this phase as glomerular maturation, although we have not used any markers to characterize this change.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.asd.2010.04.003.

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