Cell Tissue Res (2013) 352:227–235
DOI 10.1007/s00441-013-1568-z

REGULAR ARTICLE

Species and sexual differences in antennal lobe architecture and glomerular organization in two parasitoids with different degree of host specificity, *Microplitis croceipes* and *Cotesia marginiventris*

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Received: 10 July 2012 / Accepted: 14 January 2013 / Published online: 19 February 2013

Abstract The endoparasitic wasps (Hymenoptera: Braconidae), *Microplitis croceipes* (specialist) and *Cotesia marginiventris* (generalist), are parasitoids of lepidopteran larvae and differ in their degree of host specificity. Recent studies have reported key differences between the two species in the abundance of antennal olfactory sensilla and their response to host-related volatiles. Here, we have compared antennal lobe architecture and glomerular organization in the two parasitoid species by using a combination of axonal tract tracing techniques and confocal microscopy. In *M. croceipes*, the medial half of the antennal lobe is larger with a greater number of glomeruli compared with the lateral half, whereas in *C. marginiventris*, the lateral half is larger than the median half. The volume of the antennal lobe is approximately 2.5 times greater in *M. croceipes* than in *C. marginiventris*. However, the number of glomeruli per antennal lobe is only slightly higher in *M. croceipes* (females: 219–222; males: 220–224) than in *C. marginiventris* (females: 192–194; males: 193–196). A comparison of males and females within each species demonstrated a striking sexual difference in terms of an enlarged glomerulus (macroglomerulus or MG) at the entrance of the antennal nerve and of a complex of 3–4 MG (CMG) in the posterior region of the antennal lobe of males of both species. Being specific to males, both the MG and CMG might be involved in the detection of female-related odor.

This research was funded by a National Science Foundation (NSF) Grant (award number: 0641621) to H.Y.F.

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Keywords Parasitoid · Specialist · Generalist · Antennal lobe · Glomeruli · Confocal microscopy · Macroglomerulus · *Microplitis croceipes*, *Cotesia marginiventris* (Insecta)

Introduction

The antennal lobe is the primary olfactory center in the insect brain. Because the insect antennal lobe is remarkably similar to the vertebrate olfactory bulb (OB), insects are attractive model organisms for investigating neuronal architecture and mechanisms of olfaction in general (Hildebrand and Shepherd 1997; Aungst and Spehr 2005). Both the antennal lobe and OB consist of distinct morphological units called glomeruli, each of which receives input from olfactory receptor neurons (ORNs) expressing the same receptor type (Mombaerts et al. 1996; Vosshall et al. 2000). Briefly, the antennal lobe receives inputs from the ORNs housed within olfactory sensilla located on the antenna, the primary olfactory organ in insects. The ORNs from olfactory sensilla terminate in the glomeruli, in which they make synaptic contact with local interneurons, which interconnect subsets of glomeruli, and with projection neurons, which project to the higher brain centers.

The morphology of the antennal lobe and the glomerular organization has been described in some insect taxa including moths (Lepidoptera; Rospars 1988; Homberg et al. 1989; Boeckh and Tolbert 1993; Hildebrand and Shepherd 1997; Anton and Homberg 1999; Hansson and Anton 2000), ants (Hymenoptera; Zube et al. 2008; Kelber et al. 2009), bees (Hymenoptera; Flanagan and Mercer 1989; Galizia et al. 1999), and flies (Diptera; Vosshall et al. 2000; Wong et al. 2002). Moreover, Smid et al. (2003) have described the architecture of the antennal lobe in two species

of parasitic wasps (Hymenoptera). In many of these studies, axon terminals from ORNs expressing a specific membrane receptor have been shown to innervate a particular glomerulus or glomeruli in the antennal lobe (Gao et al. 2000; Vosshall et al. 2000; Couto et al. 2005; Hallem and Carlson 2006). In moths, plant odor processing occurs mainly in the ordinary glomeruli (Christensen and Hildebrand 2002; Christensen and White 2000), whereas sex pheromone processing in the male occurs in a distinct complex of glomeruli, the macroglomerular complex (Hansson et al. 1991; Berg et al. 1998, 2005; Anton and Homberg 1999). These two systems are, however, not entirely separate because pheromone responses have also been found in neurons within ordinary glomeruli (Kanzaki et al. 1989; Anton and Hansson 1995).

Like moths, parasitic wasps (Hymenoptera) or parasitoids use various types of plant-based volatiles as cues for foraging and locating their herbivore hosts (Dicke and Sabelis 1988; Turlings et al. 1990; McCall et al. 1994; De Moraes et al. 1998; Chen and Fadamiro 2007). However, many aspects of olfactory communication in this important group of insects remain poorly understood, despite the increasing interest in their use as biological pest control agents.

Research by our group (Chen and Fadamiro 2007; Ngumbi et al. 2009, 2010; Das et al. 2011) has focused on olfactory communication in two parasitoids (Hymenoptera: Braconidae) with different degrees of host specificity, *Microplitis croceipes* (Cresson) and *Cotesia marginiventris* (Cresson). *M. croceipes* is a specialist larval parasitoid of *Heliothis* spp., whereas *C. marginiventris* is a generalist larval parasitoid of several caterpillar genera including *Heliothis* spp. and *Spodoptera* spp. In particular, a recent study of the antennal morphology in the two species has revealed important differences in the abundance of antennal chemosensilla: the putative chemosensilla types, namely sensilla placodea and s. basiconica, are more abundant in *M. croceipes* (specialist) than in *C. marginiventris* (generalist; Das et al. 2011).

In the furtherance of our research on mechanisms of olfaction in the two parasitoids species, the present study was conducted to compare the morphology and glomerular organization of the antennal lobe of the parasitoids. Thus, the relative size, position, and number of glomeruli in both sexes of *M. croceipes* and *C. marginiventris* are presented, and key species and sexual differences are discussed.

Materials and methods

Insects

The parent cultures of *M. croceipes* and *C. marginiventris* were provided by the USDA-ARS, Crop Protection and Management Research (Tifton, Georgia) and the Department of Entomology, University of Georgia (Tifton campus, Contact: John Ruberson), respectively.

M. croceipes was reared on the larvae of *Heliothis virescens* Fab., whereas *C. marginiventris* was reared on larvae of *Spodoptera exigua* (Hübner). Colonies of both parasitoid species were maintained in the laboratory as previously reported (Lewis and Burton 1970; Ngumbi et al. 2009). Adult parasitoids (2–3 days old) were first anesthetized by chilling for ~15–20 min at 4°C and then processed for tissue staining for confocal microscopy.

Antennal lobe staining

The neuroanatomical procedures used in this study were modified after Smid et al. (2003) and Zube et al. (2008). Parasitoids were sedated on ice and immobilized by dental wax, leaving the antennae accessible. The antenna was cut at the level of the third or fourth proximal segment of the flagellum under a stereomicroscope (National Microscope, Model direct current 3–420, Meiji, Japan). The axon tracer was prepared as 3.5% biotin dextran amide (Molecular Probes, Ore., USA; MW 10 kDa,) in distilled water. Biotin amide solution was placed into a pointed open-end glass microcapillary to fill the antenna. The cut end of the antenna was inserted to a small extent into the glass capillary and left for 4–5 h at room temperature to allow the tracer to enter through the antennal nerves. The heads of the parasitoids were removed, and the brains were dissected in Ringer's solution by using a stereomicroscope and fixed overnight at room temperature in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0, as performed by Smid et al. (2003). Post-fixation, brains were dehydrated in an ethanol series to 90% and then immersed in n-heptane. The brains were washed four times in phosphate buffer saline for rehydration and then incubated in streptavidin conjugated to Alexa 488 (S-11223, Molecular Probes). The incubation mixture was prepared by diluting the Alexa-488-conjugated streptavidin 1:500 in phosphate buffer containing 1% bovine serum albumin and Triton X-100. The brains were refrigerated (incubation step) for 24 h and washed six times in 0.1 M phosphate buffer for 4 h. Post-washing of brain tissues was followed by dehydration in an ethanol series, clearing in xylene, and mounting in Depex (Fluka). The mounting of delicate brain tissues in Depex was difficult, and great care had to be taken to avoid tissue damage. To avoid any pressure of the cover glass on the brains, spacers were used to mount *M. croceipes* brains, which were larger than the brains of *C. marginiventris*. Of the 64 brains (total for both species and sexes) treated with this protocol, 39 yielded selective labeling of the glomeruli and were digitized by confocal laser scanning microscopy. Three brains per sex of each species were selected for image segmentation and glomerular matching.

Confocal microscopy and three-dimensional reconstruction

The brain samples were examined with a BioRad MRC-1024 confocal laser-scanning microscope, equipped with an argon/krypton laser. The Alexa fluorophore (conjugated to streptavidin) was excited at a wavelength of 488 nm in order to obtain digital image stacks from laser scanning. Images from brain tissues were obtained by using Zeiss ×40 NA 1.3 oil-immersion objectives, at a resolution of 512×512 pixels. The optical sections from each antennal lobe were obtained in a stack of 100–200 images (80–120 μm). Complete stacks of images were imported into the three-dimensional (3D) analysis software AMIRA 5.3.3 (Visage Imaging, San Diego, Calif., USA). To maintain similarity in glomerular identification, we used the nomenclatures of Smid et al. (2003) to describe the various landmark glomeruli in *C. marginiventris*. However, glomeruli nomenclatures were different in *M. croceipes*, since its glomerular organization and positions were not like those of *C. marginiventris*. A contour enclosing different glomeruli was given a specific color to incorporate the glomerulus in the same glomerular layer. A randomly chosen number was provided to each landmark glomerulus. The volume of the antennal lobe mass was calculated, and the 3D surface model was constructed manually from optical section stacks by using AMIRA for analysis. Images of slices from confocal scans and Amira reconstructions were further processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, Calif., USA) in order to adjust for brightness and contrast. For 3D reconstructions, three antennal lobe image stacks, each from an individual wasp/sex, were analyzed for comparison.

To identify glomerular variance in each species, antennal lobe specimens were compared to match different glomeruli in each layer. Optical image stacks were matched to confirm species and sexual differences in antennal lobe organization. The size of each glomerulus, the morphology of each confocal section, and the position of glomeruli in 3D reconstructions of the antennal lobe were utilized to provide identification.

Results

Architecture of the antennal lobe

An image of the full brain of *M. croceipes* was superimposed onto a 3D model of the antennal lobe (Fig. 1) to indicate the position of the antennal lobe and of the antennal nerves innervating the glomerular mass. The antennal lobes of the two wasp species have different external morphology and glomerular organization. Visually distinct sections/layers of the antennal lobes for the two species are depicted in different colors in Figs. 2, 3. The average width of *M. croceipes* brains is 1,320±45 μm (n=3), compared with 852±25 μm (n=3) in *C. marginiventris*. The antennal lobe is oval, and the height is smaller compared with its width (Fig. 4). The antennal lobe volume ranges from $867\times10^3\,\mu m^3$ to $897\times10^3\,\mu m^3$ in *M. croceipes* compared with $347\times10^3\,\mu m^3$ to $384\times10^3\,\mu m^3$ in *C. marginiventris*. This species difference in antennal lobe

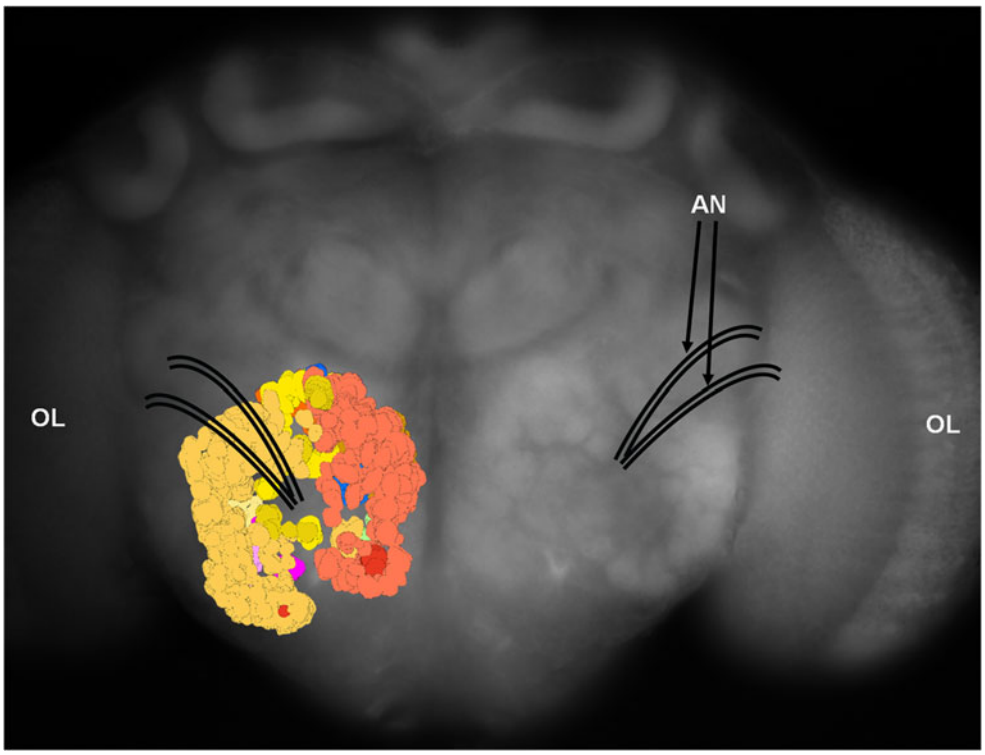
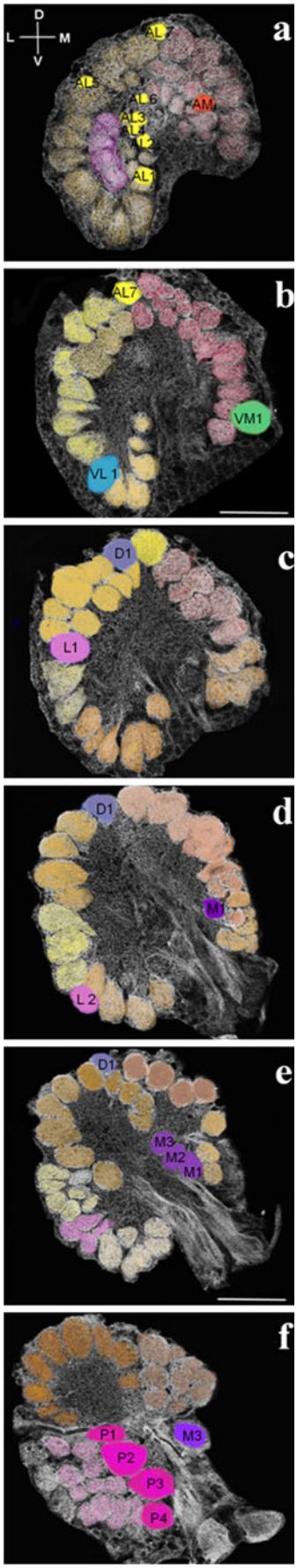


Fig. 1 Representation of brain structure of *Microplitis croceipes* with superimposed three-dimensional (3D) model of antennal lobe. Anterior view (*AN* antennal nerves, *OL* optic lobe)

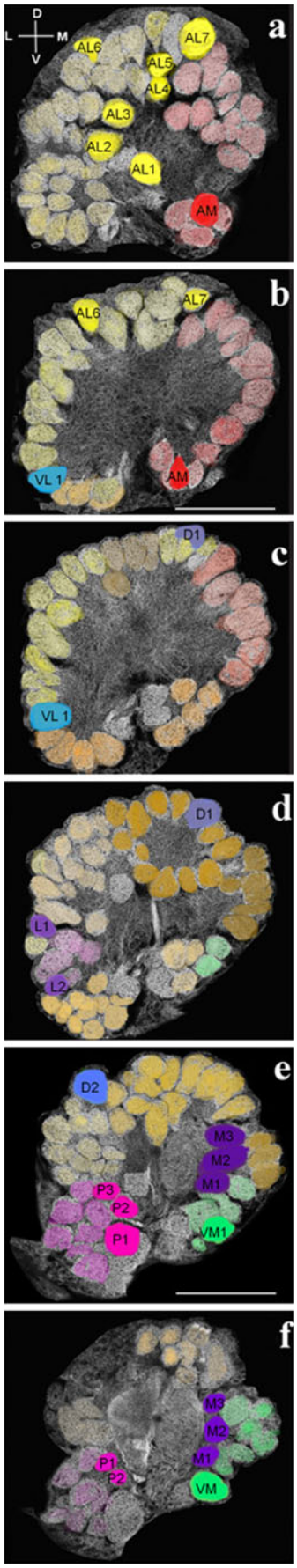


◀ **Fig. 2 a–f** Optical sections showing the different glomerular layers by *color* from anterior to posterior through the antennal lobe of female *M. croceipes*. Dimensions: stack size 109 μm, 204 optical sections, 15-section interval. Landmark glomeruli are named in subgroups with respect to their positions: antero-lateral (*AL1–7*), antero-median (*AM1*), dorsal (*D1*), median (*M1–3*), ventro-lateral (*VL1*), ventro-median (*VM1*), lateral (*L1–2*), and posterior (*P1–3*). *Colors* indicate glomeruli in subgroups. Orientation of antennal lobe sections: dorsal (*D*), ventral (*V*), median (*M*), lateral (*L*). *Bars* 50 μm

volume is probably attributable to *M. croceipes* having a larger head, and hence greater antennal lobe volume, than *C. marginiventris*. The glomerular number per antennal lobe is 219–222 (females) and 220–224 (males) in *M. croceipes* compared with 192–194 (female) and 193–196 (male) in *C. marginiventris*.

The antennal lobe in both species is separated into two halves: medial and lateral. Adjacent glomeruli in these two halves are either attached or overlap each other (Figs. 2, 3). The medial and lateral halves are mostly continuous in all specimens; however, they are separated at the ventral side and partly separated at the posterior side. The receptor neuron axons form two antennal nerves before entering each antennal lobe. The two antennal nerves innervate the glomerular mass anteriorly and project posteriorly through the antennal lobe as two main tracts, one median and one lateral. Each tract then branches into the subgroups of glomeruli within its respective region (half) of the antennal lobe (medial or lateral). A confocal image obtained dorsally from the antennal lobe of *C. marginiventris* (Fig. 5) shows the major division of the tracts.

Key morphological differences were observed between the antennal lobes of the two species. In *M. croceipes*, the medial half is larger compared with the lateral half (Figs. 2, 4a, c). In contrast, the lateral half in *C. marginiventris* is larger than the medial half (Figs. 3, 4b, d). In *C. marginiventris*, a small set of glomeruli is attached to the lateral half in the posterior region; however, this structure is absent in *M. croceipes*. In *M. croceipes*, the median tract enters into the medial half, travels in the ventromedian direction, and then innervates the ventro-median, median, and dorsal glomeruli. In *C. marginiventris*, the lateral tract branches and penetrates into the ventro-lateral and lateral glomeruli. The presence of a greater number of glomeruli in the medial half suggests that this region processes information about more odors than the lateral half in *M. croceipes*. The innervating pattern is reversed in *C. marginiventris*. The lateral tract enters the lateral, ventro-lateral, and dorsal glomeruli and reaches the posterior glomeruli. The lateral tract seems to have ORN axons projecting to a relatively large number of glomeruli, as it innervates the lateral glomerular mass, which has a greater number of glomeruli than the medial half. The median tract in *C. marginiventris* innervates the



◀ **Fig. 3 a–f** Confocal sections of *color*-marked glomeruli from anterior to posterior through the antennal lobe of female *Cotesia marginiventris* showing the different glomerular layers. Abbreviations for landmark glomeruli are same as Fig. 2. Dimensions: stack size 94 μm, 130 optical sections, 15-section interval. *Bars* 50 μm

ventro-median and median glomeruli. In contrast to *M. croceipes*, more glomeruli are present in the lateral half of the antennal lobe of *C. marginiventris* that in the medial half. This suggests that the lateral half processes information about more odors than the medial half in *C. marginiventris*.

Glomerular organization in the antennal lobe

The glomeruli that were consistently seen in the different image stacks were considered as landmark glomeruli in both species. The anterior glomerular mass was divided into median (antero-median or AM) and lateral (antero-lateral or AL) anterior glomerular clusters. In both species, the ventro-median (VM) and median (M) glomeruli were separated by landmark glomerulus VM1 (Fig. 4c, d). The ventro-lateral (VL) and lateral (L) glomeruli were separated by landmark glomerulus VL1. Each glomerular cluster was further divided into different subgroups and landmark glomeruli within the subgroups depending on their position (Fig. 4) in the different regions. In the two species, the antero-lateral mass had a set of landmark glomeruli grouped according to their position (AL1–7), and the antero-median mass had one landmark glomerulus AM1 (Figs. 2a, 3a). The ventro-lateral region had a single landmark glomerulus, VL1. The ventro-median also had one landmark glomerulus, VM1 (Figs. 2b, 3b). The lateral glomerular mass had two landmark glomeruli, L1 and L2 (Figs. 2c, d, 3d), whereas the median region had three landmark glomeruli, M1, M2 and M3 (Figs. 2e, 3e). In addition, one landmark glomerulus (D1) lay in the dorsal layer (Figs. 2e, 3e), and a group of 3–4 landmark glomeruli (P1, P2, P3, P4) were recognized in the posterior region (Figs. 2f, 3e, f).

Key differences were recorded between the two species in the size, shape, and position of glomeruli at several locations (Figs. 2, 3). In general, *M. croceipes* has larger (size) glomeruli than *C. marginiventris*. Remarkable sexual differences were also recorded. In males of the two species, a prominently enlarged macroglomerulus (MG) was found close to the entrance of antennal nerves (Fig. 6). Analyses of three complete confocal image stacks confirmed that the MG was larger than the ordinary glomeruli in all specimens. In addition to the MG, a complex of 3–4 macroglomeruli (complex of macroglomeruli or CMG) was observed in the posterior region of the antennal lobe of males of the two species (Fig. 7). One glomerulus that was consistently found (in all observed antennal lobe specimens) adjacent to the MG in the anterior glomerular region of males in both

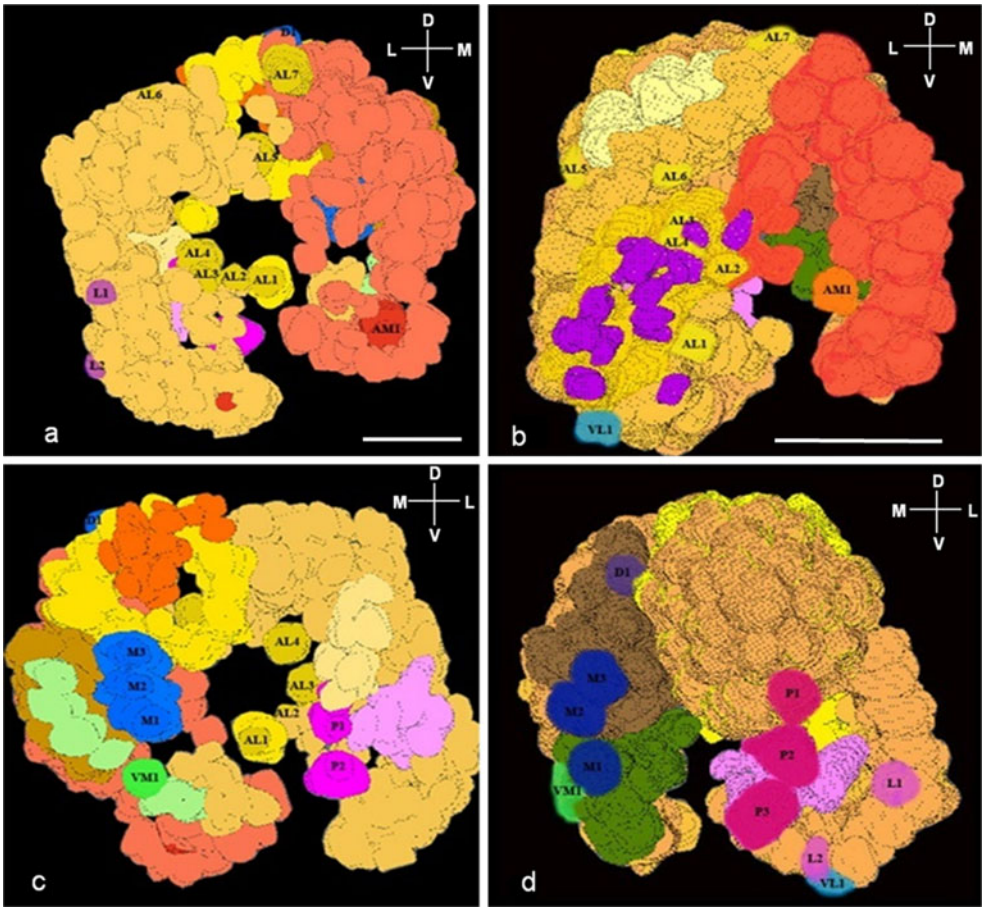


Fig. 4 **a–d** Surface reconstruction 3D models of the antennal lobe of female *M. croceipes* (**a** anterior view, **c** posterior view) and female *C. marginiventris* (**b** anterior view, **d** posterior view). Abbreviations as in Fig. 2. *Bars* 50 μm

parasitoid species was designated as "a putative satellite glomerulus" (Fig. 6). Qualitative inspection of the MG and the associated satellite glomeruli in other individuals revealed a similar spatial arrangement. Interestingly, the antennal lobes of females of both species completely lacked both the MG at the entrance of the antennal nerves and the CMG at the posterior region of the antennal lobe.

Discussion

The results presented here have revealed important differences in the antennal lobe morphology and glomerular organization between *M. croceipes* and *C. marginiventris* and also sexual differences. In *M. croceipes*, the medial half of antennal lobe is larger and encloses a greater number of glomeruli compared with the lateral half. In contrast, the lateral half in *C. marginiventris* is larger with a greater glomeruli number than the medial half. The innervation pattern of the median and lateral tracts in *C. marginiventris* is similar to the patterns described for *Cotesia glomerata* and *C. rubecula* (Smid et al. 2003), suggesting that closely related species have evolved similar antennal lobe architecture. The average volume of the antennal lobe is similar between the sexes within each species but is three times greater in *M. croceipes* compared with *C. marginiventris*. The observed difference in antennal lobe volume is probably related to size: *M. croceipes* is larger overall and has a larger brain and hence greater antennal lobe mass than *C. marginiventris*.

Despite the significant species difference in antennal lobe volume, the number of glomeruli per antennal lobe is only slightly higher in *M. croceipes* (219–224) than in *C. marginiventris* (192–196), and not different between the sexes. Results from a previous study have shown that sensilla placodea, the main antennal olfactory sensilla in both parasitoid species, are significantly more abundant in *M. croceipes* than in *C. marginiventris* (Das et al. 2011).

The most striking sexual difference observed is the presence of two remarkable glomerular structures in the males of both species, structures that are missing in conspecific females: (1) an enlarged MG at the entrance of the antennal nerve, and (2) a CMG in the posterior region of the antennal lobe. To our knowledge, this is the first report of these structures (MG and CMG) in parasitic wasps. However, similar structures have been reported in other insects such as leaf-cutting ant workers (Kleineidam et al. 2005), fungus-growing ant workers (Kelber et al. 2009), male fruit fly (Laissue et al.

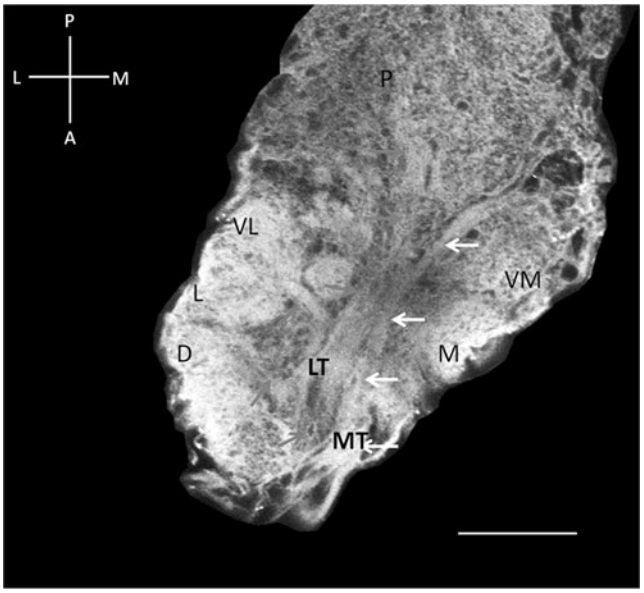


Fig. 5 Optical image section of the antennal lobe of *C. marginiventris*. Dorsal view showing innervation pattern of median (*white arrows*) and lateral (*gray arrows*) tracts that innervate the anterior through the posterior of the medial and lateral halves. The median tract (*MT*, *white arrows*) innervates the median (*M*) and ventro-median (*VM*) glomeruli. The lateral tract (*LT*, *gray arrows*) innervates the dorsal (*D*), lateral (*L*), ventro-lateral (*VL*) and posterior (*P*) glomeruli. Anterior (*A*), posterior (*P*), median (*M*), and lateral (*L*) directions are indicated (*top left*). *Bar* 50 μm

1999), and male moths (Berg et al. 2002; Varela et al. 2011). In many insects such as moths, honey bees, and flies, pheromone is processed in specific glomeruli such as the macroglomerular complex in male moths or by groups of ordinary glomeruli (Galizia et al. 2000; Sachse and Galizia 2002; Varela et al.

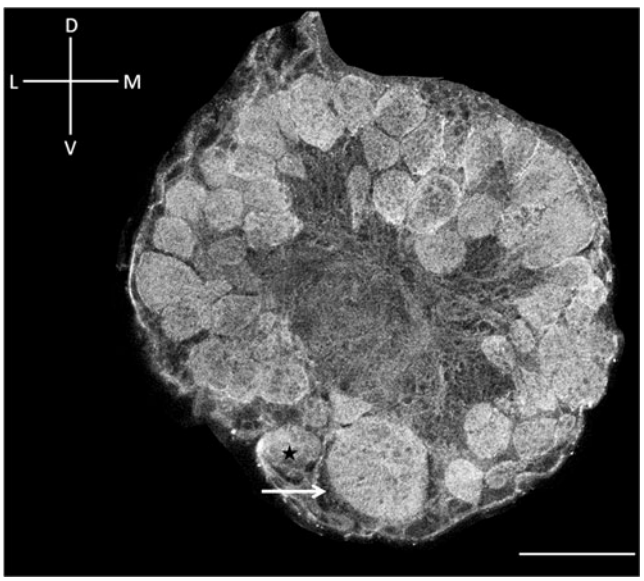


Fig. 6 Frontal optical sections showing a macroglomerulus (*arrow*) at the entrance of the antennal nerve and a putative satellite glomerulus consistently found adjacent to the macroglomerulus (*star*) in a male *M. croceipes*. *Bar* 50 μm

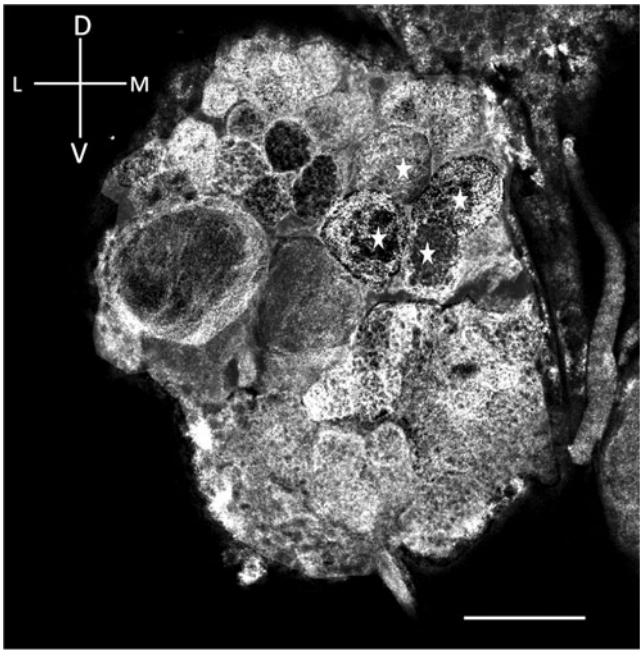


Fig. 7 Posterior optical section showing a complex of four macroglomeruli (*stars*) in a male *M. croceipes*. Note the enlarged fibrous core corresponding to the posterior group of glomeruli. *Bar* 50 μm

2011). Although sex pheromones have not been reported in *M. croceipes* and *C. marginiventris*, the MG and CMG might function in the detection of female odors, such as pheromones. Future identification of sex pheromones for both parasitoid species will aid the determination of the function of the MG and CMG.

The slight variation observed in this study in the number of glomeruli per antennal lobe among individuals of the same species is not uncommon. Several authors have also recorded individual variations in the number of glomeruli in many insect species (Galizia et al. 1999; Laissue et al. 1999; Berg et al. 2002; Smid et al. 2003; Couton et al. 2009). In many cases, this variation is attributable to the difficulty in separating apart fused glomeruli from two different layers (Galizia et al. 1999; Laissue et al. 1999; Berg et al. 2002; Smid et al. 2003; Couton et al. 2009). In the present study, the glomeruli identified in both parasitoid species range from small to large glomeruli, as also reported for the carpenter ant, *Camponotus floridanus* (Zube et al. 2008), fungus-growing ant, *Apterostigma cf. mayri* (Kelber et al. 2009), and several species of stink bugs (Kristoffersen et al. 2008). The small glomeruli are sometimes difficult to separate and differentiate. This variability represents a challenge in glomeruli identification and separation, because of the unclear boundaries between the various glomerular layers and subgroups.

Among the hymenopterans, the highest number of glomeruli per antennal lobe (630 glomeruli) has been reported in the fungus-growing ant, *A. cf. mayri* (Kelber et al. 2009), which is

approximately 2.8 and 3.3 times greater than those recorded in the present study for *M. croceipes* and *C. marginiventris*, respectively, and also approximately 3.3 times greater than those in *C. glomerata* and *C. rubecula* (Smid et al. 2003). Clearly, size cannot account for the above differences in number of glomeruli, since the head width of the fungus-growing ant, *A. cf. mayri*, is only slightly larger than that of the above parasitoid species. In a comparative study of antennal lobe morphology of several species of fungus-growing (Attini) ants, Kelber et al. (2009) have reported a correlation between head width of the ants and antennal lobe volume; however, the number of glomeruli is not positively correlated with head width. Similarly, the number of glomeruli in the antennal lobe of moths ranges between 60 and 66 (Berg et al. 2002; Løfaldli et al. 2010). This is lower than the number of glomeruli in the fungus-growing ant and the two parasitoids species investigated in the present study, but antennal lobe size is greater in moths than in the two parasitoid species investigated here. Thus, differences in the number of glomeruli between insect families or taxa might be related to phylogeny or have functional significance.

This study presents a 3D map comparing the antennal lobe morphology in the two parasitoids, *M. croceipes* (specialist) and *C. marginiventris* (generalist). Despite the marked species difference in antennal lobe architecture, only a slight difference in the number of glomeruli has been recorded between the two species. The most significant finding is the presence of an enlarged glomerulus (MG) and a complex of 3–4 macroglomeruli (CMG) in the males of both species, structures that are lacking in the females. Future studies are necessary to functionally characterize the glomerular activity patterns of additional types of behaviorally relevant odor compounds and mixtures in the two parasitoid species.

Acknowledgment The authors thank Dr. Dawn Olson (USDA-ARS, Tifton, Georgia) and Dr. John Ruberson (University of Georgia) for providing the parent cultures of the parasitoids, and Esther Ngumbi and David Appel for assistance with insect rearing. We also thank Dr. Michael Miller (Auburn University Research Instrumentation Facility) and Dr. John Dennis (Auburn University) for technical assistance with the confocal microscopy.

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