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RESEARCH ARTICLE

# Sexual Dimorphism and Phenotypic Plasticity in the Antennal Lobe of a Stingless Bee, *Melipona scutellaris*

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## ABSTRACT

Among social insects, the stingless bees (Apidae, Meliponini), a mainly tropical group of highly eusocial bees, present an intriguing variety of well-described olfactory-dependent behaviors showing both caste- and sex-specific adaptations. By contrast, little is known about the neural structures underlying such behavioral richness or the olfactory detection and processing abilities of this insect group. This study therefore aimed to provide the first detailed description and comparison of the brains and primary olfactory centers, the antennal lobes, of the different members of a colony of the stingless bee *Melipona scutellaris*. Global neutral red staining, confocal laser scanning microscopy, and 3D reconstructions were used to compare the brain structures of males, workers, and virgin queens with a special emphasis on the antennal lobe. We found significant differences between both sexes and castes with regard to the relative volumes of olfactory and visual neuropils in the brain and also in the number and volume of the olfactory glomeruli. In addition, we identified one (workers, queens) and three or four (males) macroglomeruli in the antennal lobe. In both sexes and all castes, the largest glomerulus (G1) was located at a similar position relative to four identified landmark glomeruli, close to the entrance of the antennal nerve. This similarity in position suggests that G1s of workers, virgin queens, and males of *M. scutellaris* may correspond to the same glomerular entity, possibly tuned to queen-emitted volatiles because all colony members need this information. J. Comp. Neurol. 000:000–000, 2015.



**INDEXING TERMS:** stingless bees; worker; queen; male; three-dimensional reconstruction; macroglomerulus; behavior; RRID nif-0000-00262

The ability of social insects to detect and recognize a remarkable number of odorants is probably one of the keys to their ecological success. In particular, chemical signals produced by members of a colony are crucial for the coordination of the society and, thus, for its survival. Within a colony, a plethora of semiochemicals are involved in the organization of social behavior. Pheromones, for instance, facilitate colony homeostasis (primer pheromones: Le Conte and Hefetz, 2008), indicate the fertility status of the reproductive members (queen pheromones: Free, 1987; Winston, 1987), and



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coordinate nest defense (alarm pheromones: Fujiwara-Tsujii et al., 2006; Schorkopf et al., 2009) and the exploitation of food sources (for review see Detrain and Deneubourg, 2009; Jarau, 2009; Reinhard and Srinivasan, 2009).

Among social insects, stingless bees (Apidae, Meliponini) constitute a mainly tropical group of highly eusocial bees comprising more than 500 species (Michener, 2013), which presents an original variety of olfactory-dependent behaviors. Stingless bees display, for instance, a rich diversity of foraging behaviors (Lindauer and Kerr, 1958), many of which are directly mediated by chemical signals and cues (Jarau, 2009). Several species of meliponine bees deposit pheromones from their labial glands at and near valuable food sources (Jarau et al., 2004a; Schorkopf et al., 2007; Jarau, 2009). These pheromones guide nestmates with high efficiency and spatial accuracy to the food sources (Lindauer and Kerr, 1958; Jarau et al., 2004a, 2006, Sánchez et al., 2004; Schorkopf et al., 2007; Jarau, 2009). In addition to these genuine scent marks, stingless bees also leave chemical footprints at food sources, which influence the decisions of food-searching workers (Nieh, 1998; Hrncir et al., 2004; Jarau et al., 2004b; Schmidt et al., 2005; Jarau, 2009; Lichtenberg et al., 2009). Depending on the context previously experienced by the foragers, these olfactory cues will either be treated as attractants (when previously associated with a rewarding food source) or as repellents (when previously associated with a depleted food source; Roselino et al., personal information).

Another prominent example of the importance of chemical signals in meliponine bees, and painfully well known to most researchers who investigate their behavior, is the colonies' coordinated, aggressive nest defense. Pheromones from the mandibular glands of two stingless bee species (*Trigona spinipes* and *Scaptotrigona* aff. *depilis*) can release aggressive behavior not only among nestmates but also among conspecifics of different colonies and even from colonies of different meliponine species (Schorkopf et al., 2009). Thus, mandibular gland secretions contain both pheromones (acting within a species) and allelochemicals (acting between species). Meliponine bees are thus also an interesting model for studying both intra- and interspecific communication (Schorkopf et al., 2009).

As in other insect species, chemical communication is thought to play a central role in the reproduction of stingless bees (Ayasse et al., 2001). So far, however, little is known for this bee group with regard to the pheromones produced by both queens and males and their role in sexual communication. Several studies nevertheless demonstrate differences between virgin queens and mated queens in the production of cephalic volatiles and in their attractiveness for males (Engels et al., 1990, 1997; Fierro et al., 2011; Verdugo-Dardon et al., 2011). In addition, recent studies provide behavioral evidence for chemical communication among queens, demonstrating that mated queens actively search for and invade unrelated, queenless colonies of the same species to reproduce within (Wenseleers et al., 2011; van Oystaeyen et al., 2013).

For these different cases of olfactory communication to be efficient, signal-receiving individuals require an elaborate sensory machinery allowing them to detect and process each specific signal (Masson and Mustaparta, 1990; Sandoz et al., 2007). In insects, odorants are detected at the level of olfactory receptor neurons (ORNs) located in numerous sensilla on the antennae. ORN axons project through the antennal nerve to their corresponding antennal lobe (AL), bilateral primary olfactory centers in the insects' deutocerebrum. Each AL consists of tens to hundreds of spheroid structures, the olfactory glomeruli (Shepherd, 1974; Baumann et al., 1996). Within the glomeruli, many synaptic contacts take place among ORN axon terminals, AL local neurons (LNs), and projection neurons (PNs; Homberg et al., 1988; Winnington et al., 1996; Anton and Homberg, 1999; Kanzaki et al., 2003). After neural processing by local networks, PNs convey the olfactory message to higher-order integration centers in the protocerebrum, the mushroom bodies (MB), and the lateral protocerebral lobe (LPL; Schildberger, 1983; Kanzaki et al., 1989; Malun et al., 1993; Hildebrand, 1996; Anton and Homberg, 1999; Kanzaki et al., 2003; Zube et al., 2008).

The glomerular array within each AL constitutes a topographic map of odor quality (Vosshall et al., 2000; Kirschner et al., 2006). Each glomerulus represents a functional unit for processing olfactory information (Hildebrand and Shepherd, 1997; Galizia and Menzel, 2001). Because all ORNs that carry a given olfactory receptor protein converge onto the same glomerulus (Vosshall et al., 2000), the identity of each glomerulus is determined by the odor specificity of these olfactory receptor neurons (Rössler et al., 1998, 1999; Carlsson et al., 2002; Wang et al., 2003). Comparative neuroanatomical studies in insects have demonstrated that the number and size/volume of the glomerular units, as well as their spatial arrangement, are highly stereotyped among individuals of the same species; sex; and, within social insects, caste (Arnold et al., 1985; Rospars, 1988; Flanagan and Mercer, 1989; Galizia et al., 1999; Rospars and Hildebrand, 2000; Huetteroth and Schachtner, 2005; Kleineidam et al., 2005; Masante-Roca et al., 2005; Nishikawa et al., 2008).

In cases in which the detection of a particular odor is crucial for a species, detection and possibly neural processing appear to be optimized through enlargement of the





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functional units that are activated by this odorant. In the AL, this results in enlarged glomeruli, the "macroglomeruli," or groups of glomeruli, the "macroglomerular complexes" (Hildebrand and Shepherd, 1997; Galizia and Menzel, 2001). The best documented examples for the occurrence of such macroglomeruli are the nocturnal moths (Oland and Tolbert, 1996; Hansson and Anton, 2000). In these insects, males possess clusters of macroglomeruli near the antennal nerve, which respond specifically to the females' sex pheromones. In Hymenoptera, the ALs of honeybee drones also contain enlarged glomeruli (Arnold et al., 1985) that process specific components of the sex pheromone produced by queens (Sandoz, 2006). Macroglomeruli, however, are not limited to cases of sexual communication. These structures have been described, for instance, in the sterile worker caste of leaf-cutting ants and are putatively related to the detection of nonsexual odors such as trail pheromones (Kleineidam et al., 2005, Nishikawa et al., 2008; Kelber et al., 2009; Kuebler et al., 2010; Nakanishi et al., 2010; Stieb et al., 2011).

Despite the importance of olfactory information in the life of stingless bees and the existence of original olfactory-based behaviors, rather little is known about the neural structures underlying olfactory detection and processing in these social insects. The aim of the present study was to provide a detailed description and comparison of the ALs of the different members of a colony of the stingless bee *Melipona scutellaris*. This species is one of the best known stingless bees in the Northeast of Brazil. It has a strong weight in the regional meliponiculture for its high-quality honey production (Kerr, 1996; Nogueira-Neto, 1997), and it is responsible for approximately 40–90% of the pollination of native plants (Evangelista-Rodrigues et al., 2008). In addition, it is a choice species for scientific studies because colonies are highly populous and can easily be kept in the laboratory (Nogueira-Neto, 1997). We compared the brains of virgin queens, workers at different developmental stages, and males and asked the following questions: 1) Does the worker AL present specific neuroanatomical adaptations that could be involved in the processing of social pheromones? 2) Does the AL show phenotypic plasticity between queen and worker females? 3) Does the males' AL present macroglomeruli potentially involved in chemical communication?

## MATERIALS AND METHODS

### Animals

Adult workers of forager age, newly emerged workers, virgin queens, and males of *M. scutellaris* Latreille 1811 (Hymenoptera: Apidae: Meliponini) were collected in three colonies at the campus of the University of São Paulo (21°9.4S, 047°51.3W) at Ribeirão Preto, SP, Brazil. Foragers, identified by the presence of pollen on their corbiculae, were caught at the hive entrance with an insect net. Virgin queens and males were collected from within the colony and were identified based on morphological characteristics of the body and the head. Emerging workers were taken directly from the brood comb at emergence. Photographs of the frontal view of the insects' heads were taken with a Leica MZ16 stereomicroscope coupled with a Leica DFC500 digital camera.

### Brain preparation

The bees were decapitated, and the brains in the head capsule were fixed in 4% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.2) for at least 5 days at 4°C. After dissecting them out of the head capsule, the brains were washed in 0.1 M PBS (3 × 10 minutes) and stained in 4% neutral red solution for 3 hours (Neutral Red Solution, Buffered; Sigma-Aldrich, Egham, United Kingdom). The brains were rinsed in 0.1 M PBS (3 × 10 minutes), dehydrated in an ascending ethanol series (50%, 70%, 90%, 95%, and 3 × 100%, 10 minutes each), and cleared in methylsalicylate (Sigma-Aldrich, Steinheim, Germany) for at least 24 hours.

### Confocal laser scanning microscopy and 3D reconstructions

In all preparations, the brains were mounted on aluminum slides with a central hole covered by thin coverslips on both sides. The whole-mount preparations were viewed with a confocal laser scanning microscope (LSM700; Carl Zeiss, Jena, Germany) and a solid-state 555-nm laser for exciting neutral red. Two different objectives were used. For overview scanning of the whole brain, a W N-Achroplan ×10 0.3 (water immersion) objective was used. Brains were scanned in tile-scanning mode (2 × 2 tiles) at 3.0-μm intervals throughout, creating confocal stacks of 1,024 × 1,024 pixels. For detailed scanning of the AL, a W Plan-Apochromat ×20/1.0 W DICIII (water immersion) objective was used. The ALs were scanned at 1-μm intervals, with a 2× line average, creating confocal stacks of 512 × 512 pixels. Three-dimensional reconstruction of the AL glomeruli was performed by outlining glomeruli in three focal planes (xy, yz, xz) and building 3D models with the wrap feature of Amira 5.4.1 (Visualization Science Group, Mérignac, France; RRID nif-0000-00262).

### Whole-brain reconstruction

For the reconstruction of whole brains, particular neuropils were reconstructed by segmentation, followed by interpolation: the AL, medulla, lobula, anterior optic

tubercle, central body, and MBs (calyces, pedicel and lobes together). For paired structures (AL, medulla, lobula, anterior optic tubercle, and MBs), the volumes on both brain sides were added. The total volume of these selected neuropils in each caste and sex was considered as an estimate of its brain volume and was compared by Kruskal-Wallis test, followed by Dunn's test for pairwise comparisons. All volumetric measures of glomeruli and brains were carried out in Amira 5.4.1. In addition to brain size, the relative volumes of individual neuropils (in percentage of brain volume estimate) were compared between castes and sexes by the Kruskal-Wallis test, followed by pairwise multiple comparison (Dunn's test), which include a correction for multiple testing.

### Neuroanatomical organization of the antennal lobes

To compare the neuroanatomical organization of the ALs of the different castes and sexes of *M. scutellaris*, both the left and the right ALs of workers, queens, and males were scanned and three-dimensionally reconstructed, and the volumes of all glomeruli were measured. To evaluate possible differences in the organization of the antennal lobes between sexes, castes, and developmental stages, we compared the number of observed glomeruli and the total glomerular volume with a nonparametric Kruskal-Wallis test, followed by pairwise multiple comparisons (Dunn's test). The whole glomerular volume was defined as the sum of the volumes of all reconstructed glomeruli within each AL.

To identify putative macroglomeruli, we searched in individual scans for prominent glomerular structures and noted their locations. The volume of these conspicuous glomeruli was then compared with the distribution of all glomerular volumes by using a nonparametric statistical measure recently applied in ants and bees (Kuebler et al., 2010; Streinzer et al., 2013). For each putative macroglomerulus, we determined the relative distance of its volume from the main distribution of 80% of the data by calculating the index $K$:

$$K = \frac{V_G - V_U}{V_U - V_L}$$

where $V_G$ is the volume of the putative macroglomerulus, $V_L$ is the lower percentile (10%), and $V_U$ is the upper percentile (90%) of the glomerular distribution of this AL. The value ($V_U - V_L$) is called the interpercentile range and represents the range over which 80% of the data are distributed. Following common statistical practices, we used two criteria for defining mild and extreme outliers (NIST/SEMATECH, 2012). Mild outliers were glomerular volumes above the *upper inner fence* with $K \geq 1.5$, whereas extreme outliers were above the *upper outer fence* with $K \geq 3.0$. In accordance with other studies (Kuebler et al., 2010; Streinzer et al., 2013), we classified only extreme outliers as macroglomeruli but still considered mild outliers as putative macroglomeruli. Differences in $K$ values were tested by a nonparametric Kruskal-Wallis test, followed by pairwise multiple comparisons (Dunn's test).

Graphs were plotted using SigmaPlot (SYSTAT; SPSS Inc., Chicago, IL). Statistical tests were performed using SigmaStat 3.5 (SYSTAT; SPSS Inc.) and Statistica 7.0 (Statsoft Inc., Tulsa, OK).

## RESULTS

### Whole-brain reconstruction

*M. scutellaris* presents both sex-specific and caste-specific differences in head morphology (Fig. 1A): virgin queens have smaller heads and smaller compound eyes than workers. Males have typical triangular heads with prominent compound eyes. Even though males and females have the same number of antennal segments, the males' antennae have a shorter scape and a longer flagellum than those of females. In line with these differences in external head morphology, clear differences in brain morphology between sexes and castes were found (Figs. 1B, 2). The total volume of the selected neuropils of workers (brain volume estimate) was significantly larger than that of virgin queens, males falling between (medians: workers $211.8 \times 10^6$ $\mu m^3$, queens $118.9 \times 10^6$, males $143.1 \times 10^6$ $\mu m^3$; N = 4 each; Kruskal-Wallis test: H = 9.8, $P < 0.01$, 2 df; Dunn's test: workers × queens, $P < 0.01$, other comparisons $P > 0.05$).

With regard to the relative volume of visual and olfactory neuropils, we found a clear dimorphism between the female castes (virgin queens and workers; Fig. 2). On the one hand, at the level of the optic lobes, queens had a relatively smaller medulla than workers (Dunn's test: $P < 0.05$). The queens' ALs, their MBs, and their central bodies, on the other hand, were relatively larger than those of workers. In addition to this caste dimorphism, we found a clear sexual dimorphism in the relative volume of some brain structures (Fig. 2). Males presented a relatively larger medulla and smaller MBs than females. The relative volume of the males' ALs was similar to that of workers and, thus, smaller than that of virgin queens. Two of the analyzed brain structures, the lobula and the anterior optic tubercle, did not show any differences between castes or sexes (Fig. 2).

### Antennal lobes

#### *Number of glomeruli and total glomerular volume*

The limits between individual glomeruli of the ALs of *M. scutellaris* were clearly discernible (Fig. 3), allowing the





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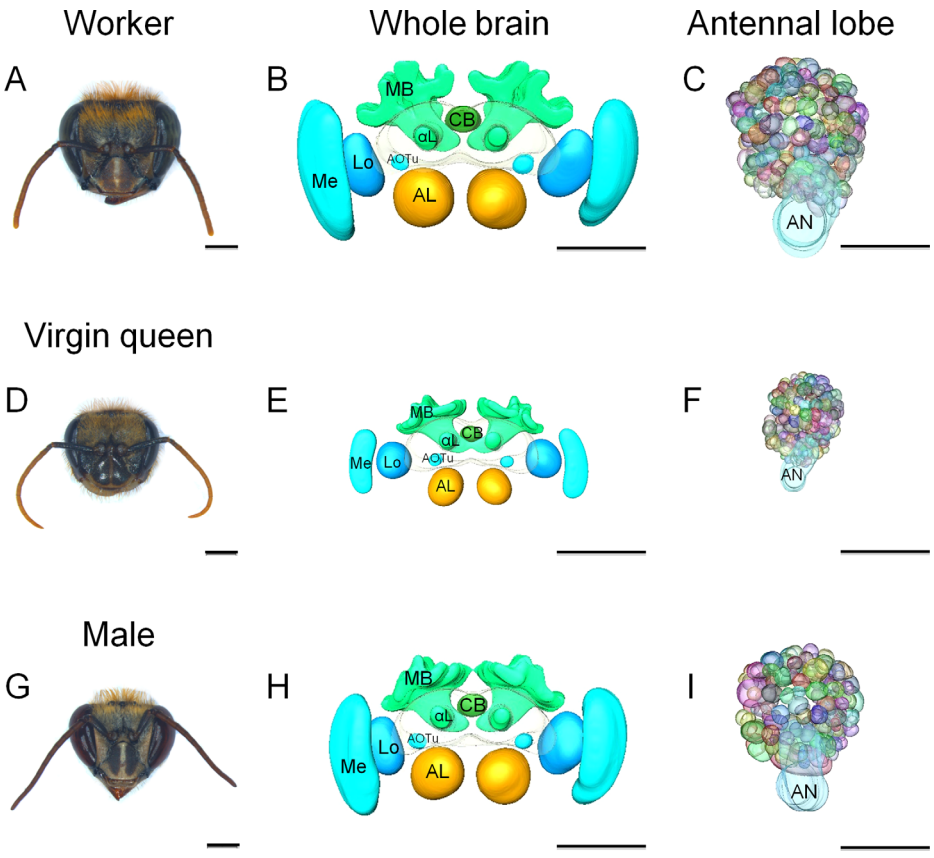


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Figure 1. Morphological and neuroanatomical differences among members of an *M. scutellaris* colony: worker forager, virgin queen, and male. **A,D,G:** Frontal view of the heads of a forager worker (A), a virgin queen (D), and a male (G). Whole-brain 3D reconstruction showing main neuropil areas in a forager (**B**), a virgin queen (**E**), and a male (**H**). Antennal lobe 3D reconstruction in a forager (**C**), a virgin queen (**F**), and a male (**I**). AL, antennal lobe; Me, medulla; Lo, lobula; AOTu, anterior optic tubercle; CB, central body; MB, mushroom bodies; αL, alpha lobe; AN, antennal nerve. Scale bars = 1 mm in A,D,G; 500 μm in B,E,H; 100 μm in C,F,I.

segmentation and 3D reconstruction of all glomeruli within each lobe with high fidelity. From these reconstructions, volume calculations provided the distribution of all glomerular volumes within each sex and caste (Fig. 4). In all analyzed ALs (foragers N = 22, recently emerged workers N = 7, virgin queens N = 8, males N = 7), the glomeruli were located mostly at the periphery of the lobe in a single layer (Fig. 3C,G,K). In all castes and sexes, the AL was divided into two main glomerular clusters, resulting in a superior caudal and an inferior rostral arrangement of glomeruli (Fig. 3B,F,J). These clusters appeared to correspond to different ORN tracts, but the neutral red staining did not allow a precise description of these tracts.

We found sex-specific and caste-specific differences in the number of glomeruli (Fig. 5A;′ Kruskal-Wallis test: $H = 37.8$, $P < 0.001$, 3 df). The ALs of males contained significantly fewer glomeruli than those of workers, and there was no difference in the number of glomeruli between the ALs of virgin queens and males (medians: males 159, workers 206, queens 194; Dunn's test: male–worker, $P < 0.001$; male–queen, $P > 0.05$). The AL of queens contained significantly fewer glomeruli than that of newly emerged workers ($P < 0.05$) and marginally fewer than that of forager workers ($P = 0.052$). Newly emerged workers (median 210) had numbers of glomeruli similar to those of foragers ($P > 0.05$).

Additionally, we found caste-specific differences and differences between different developmental stages (newly emerged workers vs. foragers) with regard to the total glomerular volume of the ALs (Fig. 5B; Kruskal-Wallis test: $H = 31.6$, $P < 0.001$, 3 df; see also volume distributions in Fig. 4). Males and foragers had a similar total glomerular volume (medians: foragers $4.97 \times 10^6$ $\mu m^3$, males $4.81 \pm 5 \times 10^6$ $\mu m^3$; Dunn's test: $P > 0.05$), which was significantly larger than that of virgin queens (median: virgin queens $3.07 \times 10^6$ $\mu m^3$; Dunn's test: males × queens, $P < 0.05$; foragers × queens, $P < 0.05$). Newly emerged workers had the smallest total glomerular volume, approximately half of that measured in foragers (median: $2.36 \times 10^6$ $\mu m^3$; Dunn's test: foragers × newly emerged, $P < 0.05$).



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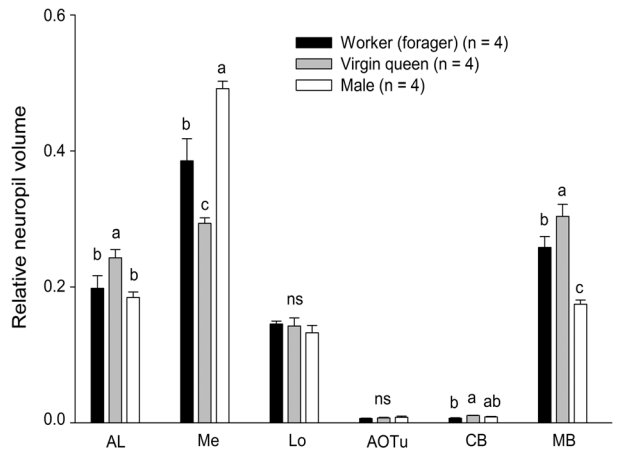


**Figure 2.** Comparison of relative neuropil volume in *M. scutellaris* castes and sexes (worker forager, virgin queen, and male). All selected neuropil volumes were normalized with regard to the brain volume estimate (sum of all selected neuropils). AL, antennal lobes; Me, medulla; Lo, lobula; AOTu, anterior optic tubercle; CB, central body; MB, mushroom bodies. Bars represent mean ± SE (n = 4 each). Relative volumes were compared between castes and sexes by Kruskal-Wallis test, followed by pairwise multiple comparisons (Dunn's test, $P < 0.05$).

### *Macroglomeruli*

In the dorsocaudal region of the AL of *M. scutellaris* workers, close to the antennal nerve entrance on the AL medial side, we detected a particularly conspicuous glomerulus. In all reconstructed ALs (all workers, males, and queens), this glomerulus outmatched the others in size and was, consequently, numbered as G1 (Fig. 3). In foragers, the volume of G1 was on average 7.3 times larger than the median glomerular volume and accounted for 3.1% of total glomerular volume (Fig. 4A). This glomerulus was an extreme outlier ($K = 3.8$) and, thus, considered a macroglomerulus (Fig. 6A). In newly emerged workers, in which the distribution of glomerular volumes was shifted toward lower values, G1 was on average 11.0 times larger than the median glomerular volume, amounting to 4.2% of the total glomerular volume (Fig. 4A). Similar to that in foragers, G1 was an extreme outlier with $K = 5.7$, qualifying it as macroglomerulus (Fig. 6A; forager × newly emerged, Dunn's test: $P = 0.006$).

In virgin queens, the most voluminous glomerulus (G1; Fig. 3) was, on average, 9.8 times larger than the median glomerular volume, occupying 4.4% of the total glomerular volume (Fig. 4B). As in workers, G1 was an extreme outlier, with $K = 5.2$, qualifying it as macroglomerulus (Fig. 6B; forager × virgin queen, Dunn's test: $P = 0.009$).

The male AL presented four prominently enlarged glomeruli, G1–G4 (Fig. 3). G1 and G2, the two largest glomeruli, occupied most of the dorsocaudal region of the male AL close to the entrance of the antennal nerve. G3 and G4 were located rostrally (distally from the nerve entrance), with G3 positioned ventrolaterally and G4 dorsomedially. G1, G2, and G3 were extreme outliers ($K_{G1} = 9.8$, $K_{G2} = 6.9$, $K_{G3} = 3.6$; Fig. 6C), which qualified them as macroglomeruli. These glomeruli were, on average, 21.5 (G1), 15.9 (G2), and 8.8 (G3) times larger than the median glomerular volume, accounting for 9.2% (G1), 6.7% (G2), and 3.7% (G3) of whole glomerular volume, respectively (Fig. 4C). G4 was a mild outlier ($K = 2.6$; Fig. 6C) and was, consequently, considered only a putative macroglomerulus. This glomerulus was, on average, 7.2 times larger than the median glomerular volume, occupying 3.0% of whole glomerular volume (Fig. 4C).

Three characteristics of G1 in workers, virgin queens, and males point to the possibility that this glomerulus

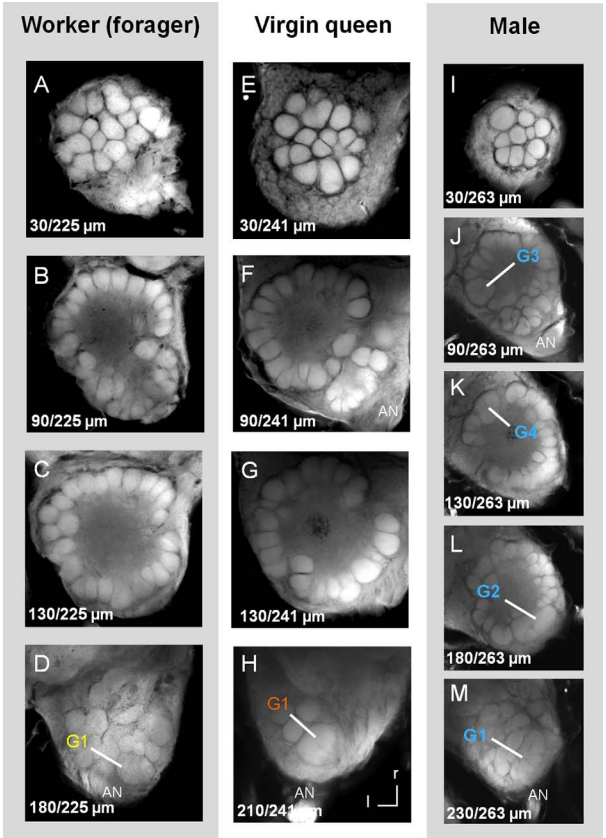


**Figure 3.** Confocal sections through the antennal lobes of the different castes and sexes of *M. scutellaris*. Sections are presented from the frontal surface (top) to the most dorsal side (bottom), for a forager worker (**A–D**), a virgin queen (**E–H**), and a male (**I–M**). AN, antennal nerve entrance; G1–G4, macroglomeruli. In each image, the lower left corner indicates the depth of the section from the frontal surface, relative to the whole depth of the antennal lobe (r, rostral, l, lateral). Scale bars = 100 µm.

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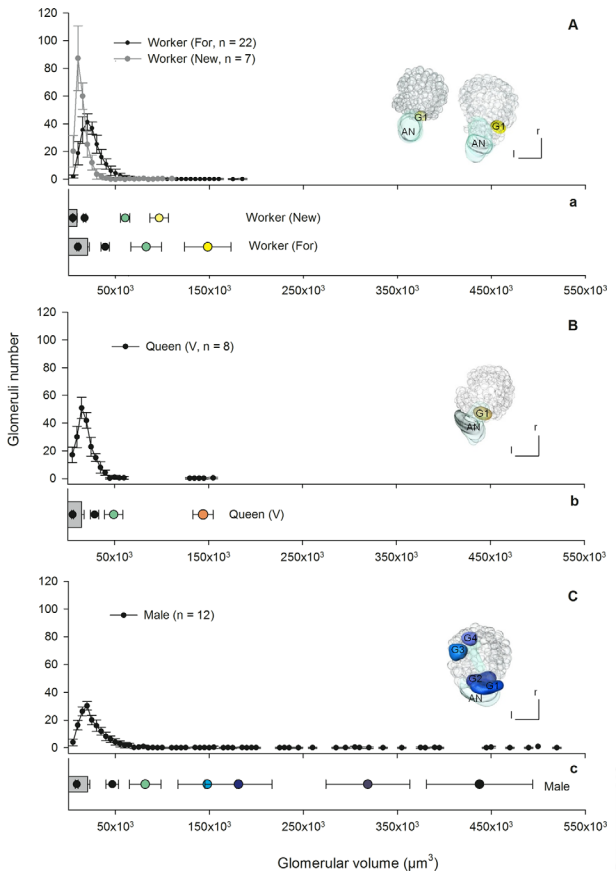


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**Figure 4.** Histogram of glomerular volumes in the antennal lobe of different *M. scutellaris* castes and sexes. The number of glomeruli belonging to each volume range (5 × $10^3$ μm$^3$ interval) was counted in each animal in each group and plotted as mean ± SD; N represents the number of individual lobes. All curves show a skewed positive distribution toward lower volumes. **A:** Forager worker and newly emerged worker. **B:** Virgin queen. **C:** Male. Below each histogram, graphs a–c show the volume of conspicuous glomeruli in the different samples: the gray bar and black dots show the position of the median and of the 10% and 90% percentiles of the volume data (average ± SE of the different samples); the dots in yellow, orange, or blue represent the mean volume ± SE of the macroglomeruli G1 in females and G1–G4 in males, as represented in the 3D reconstruction at right. The green dots represent in each sample the mean volume ± SE of the next most volumetric glomerulus (not qualifying as a macroglomerulus).

might be the same functional unit: First, in both males and females, G1 presents a similar, kidney-like shape (Fig. 7). Second, in all three studied colony members, G1 is located at the same position relative to the entrance of the antennal nerve. Third, independently of its macroglomerular volume, which differed between castes and sexes (volume of G1, males > workers > virgin queens), G1 always had a similar position relative to four identified landmark glomeruli (a–d in Fig. 7). These landmark glomeruli were easily recognizable in males and females, forming a conspicuous cross with two glomeruli at the AL surface (a and b, as most glomeruli) and two, orthogonally arranged glomeruli toward the interior of the AL (c and d). Glomerulus c in particular harbored the smallest volume in this area. These findings suggest that G1 might be a homologous structure in all members of the stingless bee colony.

## DISCUSSION

We investigated the brain structures of males, workers, and virgin queens of the stingless bee *Melipona scutellaris,* with a special emphasis on the AL. We found significant differences between sexes and castes with regard to the relative volumes of olfactory and visual neuropils in the brain but also in the number and the volume of AL glomeruli. In addition, we identified one (workers, queens) and three or four (males) macroglomeruli in the AL of *M. scutellaris*.

### Sex and caste differences in the volume of different neuropils

The 3D reconstructions of the brains of *M. scutellaris* showed clear differences between sexes and among female castes with regard to the relative volume of olfactory, visual, and integration neuropils (Figs. (1 and 3)). These anatomical differences should be seen as the product of differing genetically based developmental programs (males vs. females) as well as developmental effects resulting from the environment (queen vs. workers) and with a possible influence of experience (Ehmer and Gronenberg, 2004; Groh and Rössler, 2008; Farris et al., 2001; Hourcade et al., 2009). The most conspicuous differences were found in two structures. Males presented a larger medulla than females, suggesting differences in visual processing between sexes. As shown in Figure 1, *Melipona* males have larger eyes than females. Larger eyes in Hymenoptera males are usually related to higher ommatidia numbers along with other specializations in ommatidia structure or size (Ribi et al., 1989; Menzel et al., 1991; Baker and Ma, 2006; Streinzer et al., 2013). Males strongly rely on vision during mating flights to detect and pursue virgin queens, a task that requires high spatial resolution and fast information processing and presumably large optic lobes (Winston, 1987; Ehmer and Gronenberg, 2004).

Conversely, the relative volume of the MBs was higher in females than in males. As in honeybees, stingless bee workers are thought to rely most strongly on multisensory integration and learning and memory processes during foraging (Menzel et al., 1993; Hammer and Menzel, 1995; Giurfa, 2007; Roselino and Hrncir, 2012). In addition, they perform a rich repertoire of

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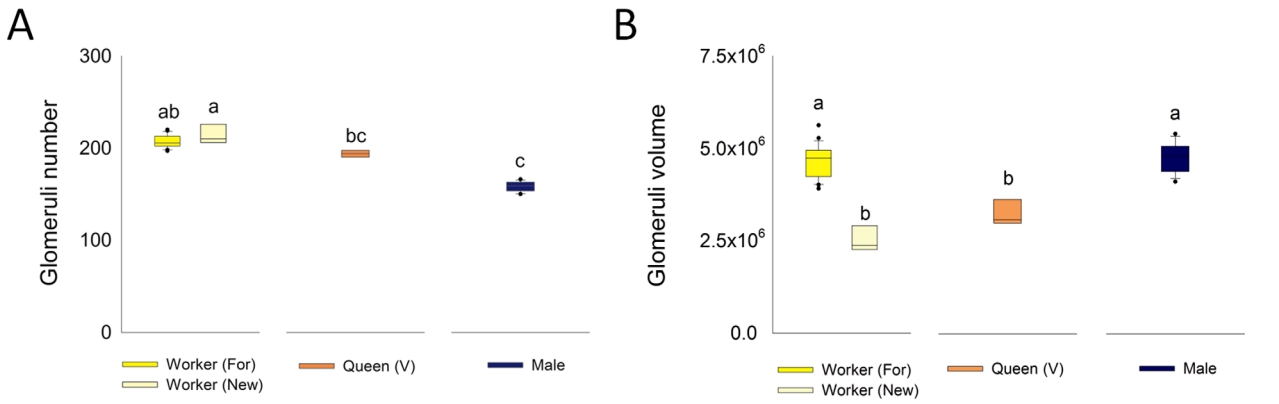


**Figure 5.** Glomeruli number and total glomerular volume in the different sexes and castes of *M. scutellaris*. Boxplots show minimum, first quartile, median value, third quartile, for workers (forager and newly emerged), queens (virgin), and males. Whiskers show 10–90% percentiles (only shown when N $\geq$ 9). Lower case letters indicate statistical differences in pairwise multiple comparisons (Dunn's test) after a Kruskal-Wallis test.

intricate behaviors inside the hive (Seeley, 1982; Withers et al., 1993; Waibel et al., 2006). In agreement with previous studies, the relative size of modality-specific neuropils in stingless bees appears to reflect the importance of the corresponding sensory modality for these sexes' and castes' behaviors (Ehmer and Gronenberg, 2004; Kuebler et al., 2010).

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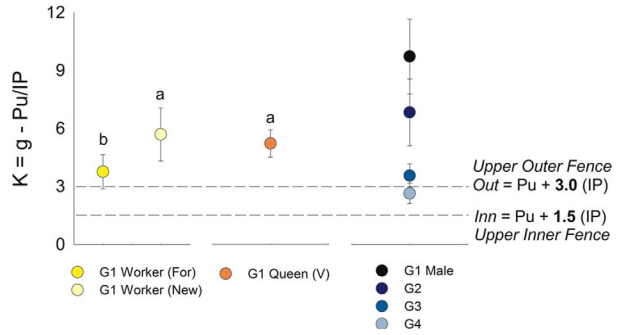


**Figure 6.** Classification of macroglomeruli as volume outliers. Classification of glomeruli as *mild* or *extreme* outliers with regard to the distribution of all glomeruli in the AL. The volume was considered as $V = P_U + K (P_U - P_L)$, where $P_L$ is the lower percentile range (10%) and $P_U$ is the upper percentile range (90%). The difference $(P_U - P_L)$ in called the interpercentile range (IP), and $K = (\text{glomerular volume} - P_U/\text{IP})$ is the outlier criterion. A glomerulus is considered as a *mild outlier* if its volume is above the upper inner fence ($K = 1.5$) and an *extreme outlier* if its volume is above the upper outer fence ($K = 3$). The graph represents the average $\pm$ SE of the $K$ value for G1 in forager and newly emerged workers (**A**) and virgin queens (**B**) and for G1–G4 in males (**C**). All these glomeruli were classified as extreme outliers, except for G4 in males, which was a mild outlier. $K$ values were compared between foragers and newly emerged workers by Kruskal-Wallis test, followed by pairwise multiple comparisons (Dunn's test, $P < 0.05$).

## Number and volume of olfactory glomeruli

Species-specific differences in the number of AL glomeruli are thought to relate to each species' necessity to identify and discriminate among relevant odorants (Hansson and Anton, 2000). In insects, as known so far, the number of glomeruli varies from 32 in *Aedes aegypti* (Bausenwein and Nick, 1998) to 630 in *Apterostigma* cf. *mayri* (Kelber et al., 2009). For *M. scutellaris* workers, we counted ~210 glomeruli (Fig. 5), which exceeds the numbers of glomeruli described for honeybees (*Apis mellifera* ~160; Flanagan and Mercer, 1989; Arnold et al., 1985), despite similar social organization and complexity (Michener, 1974). Such differences might be related to differences in the necessity to process scent information, for instance, during the coordination of foraging. In *Melipona* sp., the principal source of information used by inexperienced food collectors is the floral scents brought back to the colony by successful foragers (Jarau et al., 2000; Jarau, 2009; Roselino and Hrncir, 2012). In honeybees, by contrast, food search by inexperienced bees is based primarily on vector information on the food source's position provided by the successful forager's waggle dance (von Frisch, 1967; Riley et al., 2005). Odors brought back by foragers would be only secondary elements, at least for navigating toward the food source reported by a dancer (Farina et al., 2007).

The observed differences between females and males of *M. scutellaris* regarding the number of glomeruli (Fig. 5A) highlight the structure–function relationship linked to sexual dimorphism (Strausfeld and Reisenman, 2009). Male *M. scutellaris* presented fewer glomeruli than the females (–23%, Fig. 5); this is similar to the situation in honeybees (103 vs. 160 glomeruli, –36%;



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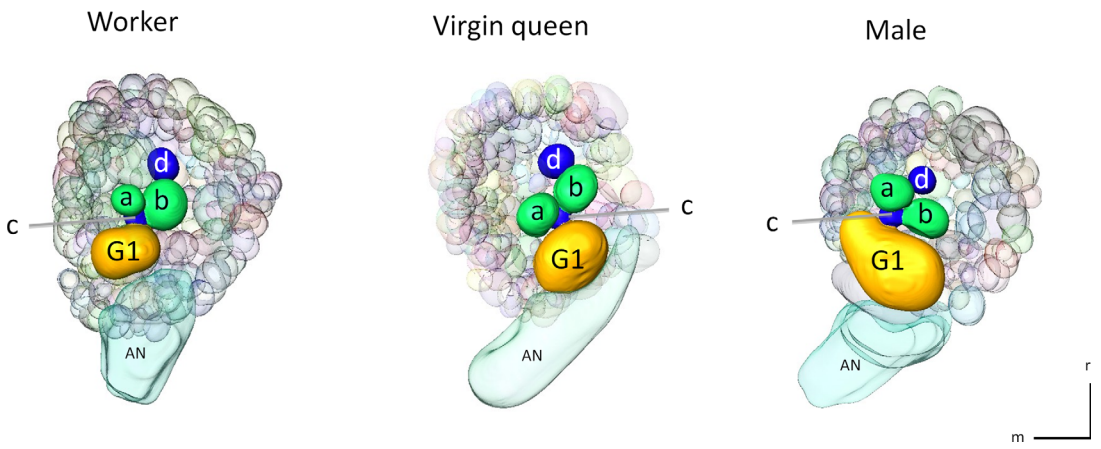


**Figure 7.** Location of macroglomerulus G1 in relation to landmark glomeruli. Three-dimensional reconstructions of the ALs of *M. scutellaris* (as seen from the dorsal side) indicating the most enlarged glomerulus (G1) in worker, virgin queen, and male. G1 is in the same dorsal position relative to the entrance of the antennal nerve. Four landmark glomeruli were identified in the different lobes and are represented by the letters a–d. Despite differences in size/volume, G1 showed a clear kidney shape in both females and males. The letters in the axes indicate orientation; r, rostral; m, medial.

Arnold et al., 1985; Brockmann and Bruckner, 2001). As described above, such difference is attributable to different constraints in the two sexes with regard to general olfactory processing. Although foragers strongly rely on olfactory cues both in a foraging and in a social context, the use of olfactory cues is limited in drones; their principal task is to find and copulate with a virgin queen. Such behaviors involve a small number of enlarged glomeruli devoted to the processing of queen sex pheromone components (Arnold et al., 1985; Masson and Mustaparta, 1990; Sandoz, 2006). The other olfactory glomeruli of males may process odors that are not related to reproduction, such as floral odorants and social pheromones (Sandoz, 2006). This sexual dimorphism in the number of olfactory glomeruli is commonly found in eusocial Hymenoptera (Nishikawa et al., 2008; Zube and Rössler, 2008; Mysore et al., 2009; Kuebler et al., 2010; Stieb et al., 2011) and is observed even in solitary bees such as *Eucera berlandi* (–24%; Streinzer et al., 2013). In some species, such as the ant *Harpegnathos saltator*, this difference reaches –60% (Hoyer et al., 2005).

Another important neuroanatomical characteristic related to the capacity to process olfactory information is the size of the glomeruli. Total glomerular volume was higher in *M. scutellaris* foragers compared with newly emerged workers (Fig. 5). This result may be related to the combined effects of ongoing neural development and of experience-induced plasticity linked to age polyethism. Both effects have been demonstrated in the honeybee, in which the olfactory system is still not mature at emergence and neural maturation continues during the first 4–8 days of adult life (Masson et al., 1993). In addition, glomerulus size in honeybee workers changes with the experience accumulated by the individual during its lifetime, both within and outside of the hive (Winnington et al., 1996; Brown et al., 2004). Especially olfactory learning and memory formation, which are crucial during foraging, can induce increases in glomerular volume (Hourcade et al., 2009; Arenas et al., 2012). Such volumetric increases can be related to increases in the numbers of synapses within the glomeruli, but not exclusively (Brown et al., 2002, 2004).

The reduced volume of glomeruli in virgin queens compared with workers might also be due to differences in experience (Fig. 5); queens do not forage. However, it could be a more direct consequence of the heterochrony in development. Differences in emergence time after larval and pupal development have been reported for *M. compressipes fasciculata* (worker 45 days and queen 40 days), *M. quadrifasciata* (worker 39.5 days and queen 36.8 days), and *M. rufiventris* (worker 42 days and queen 39.4 days; Kerr, 1996). In *Apis mellifera*, the development is substantially accelerated in queens, lasting 16 days compared with 21 days in workers (Winston, 1987). The functional differences produced during heterochronic development may affect the olfactory centers generating caste-specific differences in olfactory structures (Groh and Rössler, 2008).

## Biological significance of macroglomeruli in *M. scutellaris*

For *M. scutellaris* males, we identified three explicit macroglomeruli (extreme outliers, Fig. 6) and one



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potential macroglomerulus (mild outlier). Given that the principal function of stingless bee males is reproduction (van Veen et al., 1997), the presence of these macroglomeruli can be associated with the necessity to find and copulate with a virgin queen. In stingless bees, queens are fertilized at an age of ~3–8 days after emergence, by a single male (Kerr et al., 1962, Page and Kerr, 1990). Males lose their genitalia and, consequently, their lives during copulation (Camargo, 1972; Michener, 1974), so fruitless copulation efforts with already mated queens should be avoided. Using electroantennogram recordings (EAG), Verdugo-Dardon et al. (2011) showed that the antennae of males of the stingless bee *Scaptotrigona mexicana* respond more strongly to the odor of virgin queens compared with physiogastric queens. Strong EAG responses correlate with a high number of responsive ORNs (Mayer et al., 1984), which in turn would correlate with voluminous glomeruli. Thus, we hypothesize that the macroglomeruli of *M. scutellaris* males may be involved in the processing of the pheromone compounds produced by virgin queens. Each AL glomerulus receives information from a unique class of ORNs expressing only one type of olfactory receptor, so the four macroglomeruli of males are probably tuned to four different odor molecules. One possibility is that the sex pheromone of *M. scutellaris* females contains four key components, which remain to be identified. Alternately, some of the macroglomeruli may detect male-produced pheromones. Widespread among stingless bees are accumulations of males at so-called drone congregation areas (van Veen et al., 1997; López and Kraus, 2009; Fierro et al., 2011). Here, the drones aggregate in tight clusters, usually close to conspecific colonies (van Veen et al., 1997). Depending on the species, these male aggregations may consist of several hundred drones from more than 100 colonies (Michener, 1974; Paxton, 2000; Cameron et al., 2004; Kraus et al., 2008; Mueller et al., 2012) and persist from as short as a single day to several weeks (López and Kraus, 2009). Because these drone clusters are independent of the presence of a virgin queen (Sommeijer and de Bruijn, 1995; López and Kraus, 2009), a male-produced pheromone may be involved (López and Kraus, 2009). For honeybees, such a pheromone has also been hypothesized (Gerig, 1972; Lensky et al., 1985), and recent data tend to confirm its existence (Brandstaetter et al., 2014).

We also found a macroglomerulus in female castes, both virgin queens and workers. Recent studies on the "parasitic" behavior of *M. scutellaris* queens have highlighted the strong biological significance for reproductive females to identify whether or not a colony is orphan (absence/presence of mated queen). Because of the constantly elevated number of new queens in a colony, selective pressure on the virgin queens is high to seek alternative opportunities for reproduction, such as by taking over conspecific nests (Wenseleers et al., 2011). Thus, as in males, the macroglomeruli of females could be involved in the processing of a queen signal. A further observation strengthens this hypothesis. In both sexes and all castes, the largest glomerulus (G1) was found in a similar position, close to the entrance of the antennal nerve and relative to four identified landmark glomeruli (Fig. 7). The G1 of workers, virgin queens, and males may thus correspond to the same glomerular entity. One possibility is that this macroglomerulus detects and processes the same odorant in the different castes/sexes. As mentioned above, chemical messages with a biological relevance for all colony members of stingless bees are scents related to the presence/absence of a queen in the nest (Ayasse et al., 2001; Grajales-Conesa et al., 2007; Jarau et al., 2009; Fierro et al., 2011; Verdugo-Dardon et al., 2011). However, apparently homologous macroglomeruli in the different sexes may be tuned to different odorants altogether. This is the case for the macroglomerular complex in male moths and the female-specific glomeruli, which rather respond to host plant odors (Rössler et al., 1998; Masante-Roca et al., 2005). Neurophysiological experiments (optical imaging or electrophysiology) are needed to identify whether the G1 macroglomerulus is a specialized neural unit dedicated to the processing of olfactory signals from mated queens. This will be the goal of our future studies.



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## CONFLICT OF INTEREST STATEMENT

All authors have confirmed that there is no identified conflict of interest, including any financial, personal or other relationships with other people or organizations within 3 years of beginning this work that could inappropriately influence, or be perceived to influence, the work.

## ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the

data and the accuracy of the data analysis. Study concept and design: ACR, MH, CCL, MG, J-CS. Acquisition of data: ACR. Analysis and interpretation of data: ACR, J-CS. Drafting of the manuscript: ACR, MH, J-CS. Critical revision of the manuscript for important intellectual content: ACR, MH, CCL, MG, J-CS. Statistical analysis: ACR, J-CS. Obtained funding: ACR, J-CS. Administrative, technical, and material support: ACR, CCL, MG, J-CS. Study supervision: CCL, MG, J-CS.

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