

Soldier neural architecture is temporarily modality specialized but poorly predicted by repertoire size in the stingless bee *Tetragonisca angustula*

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Abstract

Individual heterogeneity within societies provides opportunities to test hypotheses about adaptive neural investment in the context of group cooperation. Here, we explore neural investment in defense specialist soldiers of the eusocial stingless bee (*Tetragonisca angustula*) which are age subspecialized on distinct defense tasks and have an overall higher lifetime task repertoire than other sterile workers within the colony. Consistent with predicted behavioral demands, soldiers had higher relative visual (optic lobe) investment than nonsoldiers but only during the period when they were performing the most visually demanding defense task (hovering guarding). As soldiers aged into the less visually demanding task of standing guarding this difference disappeared. Neural investment was otherwise similar across all colony members. Despite having larger task repertoires, soldiers had similar absolute brain size and the smaller relative brain size compared to other workers, meaning that lifetime task repertoire size was a poor predictor of brain size. Both high behavioral specialization in stable environmental conditions and reassignment across task groups during a crisis occur in *T. angustula*. The differences in neurobiology we report here are consistent with these specialized but flexible defense strategies. This work broadens our understanding of how neurobiology mediates age and morphological task specialization in highly cooperative societies.

KEY WORDS

abejas angelitas, caste, division of labor, expensive brain hypothesis, group defense, social insects, temporal polyethism

1 | INTRODUCTION

Cognitive investment within highly cooperative social groups can be distributed across individuals (O'Donnell et al., 2015), and the question of how differences in brain investment and architecture among

individuals within social groups relates to adaptive group function is a popular area of study (Godfrey & Gronenberg, 2019; Gordon et al., 2019; Kamhi et al., 2019; Muscедере et al., 2014; Van Nest et al., 2017; Withers et al., 1993). Eusocial insect colonies often have pronounced task and/or morphological differentiation among colony members

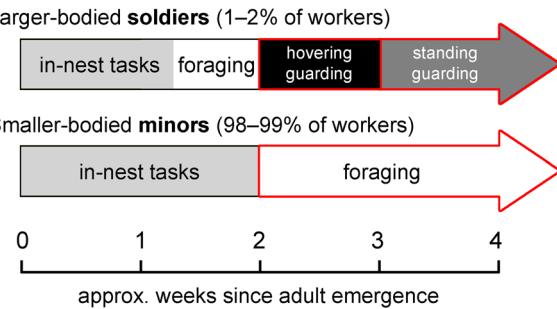


FIGURE 1 Adult age trajectories of soldiers versus minors within colonies of *Tetragonisca angustula*, based on Hammel et al. (2016) and Baudier et al. (2019). Red boxes show sampled focal task groups in this study. Foraging minors were collected according to behavior, not morphotype. This is possible because soldiers typically make up a small percentage of worker forces in general (Grüter et al., 2012; Segers et al., 2016) and likely make up an even smaller portion of foragers due to the shorter time they are believed to perform this task relative to minors (Baudier et al., 2019). As such, the estimated <3% of accidentally collected foraging soldiers among foraging minors was expected to be negligible, as was confirmed by size measurements (Figure 3b)

(Wilson, 1971), making them excellent models for studying the effects of behavioral heterogeneity on the interplay between individual neural investment and socially coordinated function.

Among eusocial insects, the most commonly evolved category of morphologically specialized workers is soldiers: large-bodied non-reproductives performing colony defense behaviors (Oster & Wilson, 1978). The most well-studied soldiers are perhaps those of termites and ants (Gordon, 1996; Haverty, 1977; Oster & Wilson, 1978; Seid, Scheffrahn, et al., 2008; Traniello, 1981), but morphologically distinct soldiers have also evolved multiple times among eusocial stingless bees (Grüter et al., 2017). Unlike ant soldiers whose neurobiology has been relatively better studied (Gordon et al., 2019; O'Donnell et al., 2018), morphologically distinct soldiers of the stingless bee, *Tetragonisca angustula* (Grüter et al., 2012) perform all of the tasks that non-soldier workers (henceforth “minors”) perform but on an accelerated age-trajectory, switching to a repertoire dominated by colony defense tasks in the last 2 weeks of life (Figure 1) (Hammel et al., 2016). Soldiers of *T. angustula* further subspecialize on different defense tasks according to age (Baudier et al., 2019). Younger hovering guards primarily protect against heterospecific invasion using visual and volatile chemical (olfactory) cues while older standing guards on the nest entrance tube also intercept conspecific non-nestmates using close-range olfaction of nonvolatile chemical cues (Baudier et al., 2019; Bowden et al., 1994; Grüter et al., 2011; van Zweden et al., 2011; Wittmann, 1985). These tasks place different demands on visual and olfactory acuity (peripheral brain) and processing (central brain) among these different soldier age groups. There is behavioral evidence for specialization in olfactory invader cue detection between soldier types (hovering versus standing guards) (Baudier et al., 2020) which is not associated with differential antennal sensitivity (Balbuena & Farina, 2020). Neural tissue is energetically costly to produce and maintain, and so neural tissue is expected to be lower in volume (as an indicator of tissue invest-

ment) when lower demands are placed on it (Liao et al., 2016; Luo et al., 2017; Niven & Laughlin, 2008; Safi et al., 2005). We tested how neural investment related to both lifetime task repertoire differences between worker subcastes, and differences in modalities used by soldiers performing discrete defense tasks.

We predicted that younger soldiers in the task of hovering guarding have higher visual investment than older standing guards because hovering guards (unlike standing guards) use visual cues to recognize the invader type on which they specialize. We go on to ask how this visual investment compares to that of age-matched minors, which typically function as foragers. We compared olfactory investment to test whether the ability to discern subtle close-range olfactory differences between conspecific nestmates and non-nestmates (primary responsibility of standing guards) demands different neural investment than the task of recognizing volatile heterospecific invasion cues from a distance (primary responsibility of hovering guards). We also asked how this compares to that of age-matched minors performing foraging tasks.

Within the brain of an insect, mushroom bodies are associated with higher cognition, as well as multimodal processing of sensory information (Heisenberg, 1998). Soldier ants with smaller lifetime task repertoires than minors have previously been shown to have a lower ratio of total neuropil (henceforth “total brain”) volume to body size and a smaller portion of their brains consisting of mushroom bodies (Gordon et al., 2019; O’Donnell et al., 2018). However, eusocial insect soldiers do not always have smaller lifetime task repertoires than other workers. *Tetragonisca angustula* soldiers work 34 to 41% more than nonsoldier nestmates and have a lifetime task repertoire size, that is, 23 to 34% larger (Hammel et al., 2016). Soldiers of *T. angustula* also specialize and subspecialize on defense tasks while minors do not typically perform colony defense (Hammel et al., 2016) unless in a crisis (Baudier et al., 2019). By comparing brain volume and mushroom-body volume between soldiers and minors of *T. angustula*, we also tested whether the widely observed trends of smaller relative brain and mushroom-body size in eusocial insect soldiers is observed for stingless bee soldiers, despite soldiers in this species having a larger lifetime task repertoire. Such a result would challenge the notion that repertoire size differences drive the relatively smaller size of soldier brains in social insects at large.

We also investigate age-associated, within-soldier neural diversity in terms of discrete defense task. By comparing neural investment in brain regions associated with visual versus olfactory acuity and processing, we investigate the degree of sensory-specific neural specialization which underpins this rapid age progression through discrete defense tasks. Soldier temporal polyethism across distinct defense tasks has only been demonstrated in two eusocial species previously (Baudier et al., 2019; Yanagihara et al., 2018) and is still largely unstudied. To our knowledge, this is the first study to investigate the potential rapid neural investment transitions that underlie age-related shifts in discrete defense tasks.

In this study, we simultaneously explore neuroanatomical correlates of the two most prominently studied forms of division of labor in social insect colonies: morphological castes and temporal polyethism. Together these cross-age and cross-morphotype comparisons of

stingless bee neural investment inform a broader framework for considering underlying neuroanatomical characteristics of finely tuned but flexibly specialized (Baudier et al., 2019) individuals within heterogeneous social groups.

2 | METHODS

2.1 | Field samples

We collected all bees from naturally occurring nests in the town of Gamboa in Colón Province, Panama ($9^{\circ} 7' N$, $79^{\circ} 42' W$). Per established methods (Baudier et al., 2020, 2019; Hammel et al., 2016), at each nest entrance, we collected bees from three discrete task groups (Figure 1): standing guards (typically 2- to 3-week-old soldiers), hovering guards (typically 3- to 4-week-old soldiers), and foragers (predominantly 2- to 4-week-old minors). We selected minors while in the task of foraging to compare to soldiers for total brain investment comparisons because they were of similar age but contrasting morphotype. A bee was deemed a forager if it exited the nest and immediately flew away from the nest entrance (distinguished from waste-removal workers by lack of carried detritus). We observed nest entrance guards for 20 s each. If a bee spent the full duration of that time standing motionless on the nest entrance tube while facing toward the entrance of the tube, we considered it a standing guard. If a bee spent the full 20 s flying in static formation in front of the nest entrance tube while facing inwards toward the flyway, we considered it a hovering guard.

We used traditional resin embedding and slicing histology to test for regional and whole-brain volumetric differences across task groups. However, neuroanatomical features beyond regional brain size alone can also mediate behavior and comparison of volumes alone can paint an incomplete picture (Chittka & Niven, 2009; Healy & Rowe, 2007). As such, we also used immunohistochemistry to ask whether patterns of modality-specific synaptic density differed across task groups. Contrasts in mushroom body synaptic density according to behavior were previously observed for minors and soldiers of the turtle ant *Cephalotes varians* (Gordon et al., 2019). We collected a total of 41 bees from 5 colonies that live in 95% ethanol in February 2018 for use in the assessment of neuroanatomical volumes (14 hovering guards, 13 standing guards, 14 foragers). In February 2019, we collected 50 additional bees (17 hovering guards, 16 standing guards, 17 foragers) from 5 colonies for immunohistochemistry. We anesthetized bees to be used for immunohistochemistry via 5-min exposure to $-40^{\circ}C$ before heads were separated from bodies. We then removed the caudal cuticle and muscle of the head before fixing and storing the brains in 4% paraformaldehyde (PFA) in PBS at $4^{\circ}C$.

2.2 | Volumetric histology and quantification

Following field collection, we photographed the heads of each ethanol specimen using a DSLR mounted camera on a dissecting scope with a micrometer, and quantified head width and height using ImageJ 1.52

digital imaging analysis software (Schneider et al., 2012). We approximated head capsule volume from head width and height, assuming an ellipsoid shape with head depth equal to half of the head height.

We stored heads in Prefer glyoxal fixative (Anatech Ltd) for a minimum of 7 days before dehydrating them in a series of increasing ethanol, acetone, then Embed 812 plastic resin concentrations (Electron Microscopy Sciences). We incubated heads in 0.1 mL resin at $60^{\circ}C$ for 72 h in pyramid molds. We then cut resin-embedded heads along the frontal plane into 10 or 7 μm thick sections using a rotary microtome, mounted them on gelatin-coated microscope slides, stained them with Toluidine blue (stain was cleared in a series of distilled water, increasing ethanol concentrations, and HistoChoice® clearing medium), and coverslipped them under DEPEX transparent mounting medium.

We photographed every section containing brain tissue using a compound light microscope-mounted digital camera at 5x magnification using LAS V4.9 software (Figure 2a). We quantified brain region area in ImageJ, and multiplied area by section thickness to estimate slice volume, summing the volumes across all slices to estimate total brain region volumes. We used Reconstruct Software (Fiala, 2005) to generate 3D reconstructions of the brain regions quantified: mushroom bodies (calyx lip, calyx collar, basal ring, peduncles), antennal lobes, optic lobes (medulla and lobula only), central complex, and the rest of the brain (protocerebrum, protocerebral bridge, subesophageal ganglion) (Figure 2b).

All analyses in this study were conducted in R statistical software version 4.0.0. We tested for differences in relative volumes (region volume/total brain volume) of peripheral visual subregions (optic lobes) (Strausfeld, 2005), peripheral olfactory subregions (antennal lobes) (Sun et al., 1993), central visual processing (collar) (Gronenberg, 2001), and central olfactory processing (lip) (Gronenberg, 2001) across task groups (minors collected while foraging, hovering guard soldiers, and standing guard soldiers) using separate mixed-effect ANOVAs that took into account colony as a random factor. We also explored general neuroanatomy of all measured region-specific volumes across the three sampled groups using Principal Component Analysis.

Total brain volume, as well as relative volume of mushroom bodies (brain regions associated with higher cognition), are commonly used metrics for neural tissue investment in social insects (Groh et al., 2012; Maleszka et al., 2009; O'Donnell et al., 2018, 2015). To test whether investment is better predicted by bee size or lifetime task repertoire size, we compared absolute and relative total brain volume and mushroom-body volume across soldiers (pooled hovering guards and standing guards) and minors. First, to confirm that collected morphotypes (minors versus soldiers) were different in size as expected, we fit a linear mixed-effect model which included colony as a random factor, subcaste (soldier vs. minor) as a fixed factor, and head width as a response variable. We compared absolute brain sizes by fitting a linear mixed-effect model that included colony as a random factor, subcaste as a fixed factor, and absolute total brain volume as a response variable. We compared relative brain volumes by fitting a linear mixed-effect model that included colony as a random factor, subcaste as a fixed factor, and relative brain volume (brain volume/head capsule volume)

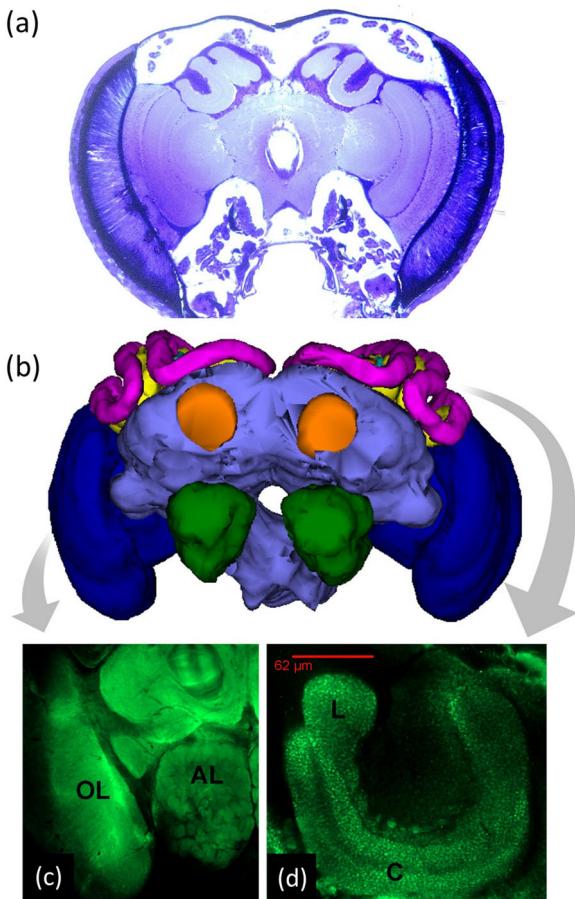


FIGURE 2 (a) A representative brain section from resin-embedded slicing histology done to quantify brain region volumes. (b) A 3-dimensional reconstruction based on volumetric measurements of resin-embedded specimens. Dark blue = optic lobe, green = antennal lobe, magenta = lip, yellow = collar, aqua = basal ring, orange = peduncle, lavender = central brain mass. (c) Synapsin immunostaining of peripheral sensory regions of interest: optic lobe (OL) and antennal lobe (AL). (d) Synapsin immunostaining of sensory regions of interest within the mushroom body calyx: lip (L, olfaction) and collar (C, vision). Note visibly distinct synaptic vesicles

as a response variable. Separate Type II Wald Chi-square tests were used to test for predictive significance of morphological subcaste (soldiers vs. minors) in all three models. We used a mixed-effect ANCOVA (colony was a random factor) to test for differences between morphological subcastes in the relationship between mushroom-body volume and total brain volume.

2.3 | Immunohistochemistry and synaptic quantification

Partially dissected brains were fixed in 4% PFA at 4°C for 7 months, after which they were completely removed from the head capsule and subjected to synaptic staining histology. We used a modification of an existing immunohistochemistry protocol (Ghaninia et al., 2007; Som-

merlandt et al., 2016) with increased incubation times to allow for improved penetration in these stored specimens. Brains were made permeable by washing (4×20 min) with 0.5% Triton-X100 phosphate-buffered saline (PBS-T) while on a shaker. After PBS-T was removed, the samples were blocked with 4% Normal Goat Serum (NGS) and placed on a shaker for 1 h at room temperature. Following the removal of NGS, the primary antibody SYNORF1 (Sigma S193, Sigma-Aldrich, Saint Louis, MO) was added in a 1:10 dilution of 0.5% PBS-T for 7 days at 4 °C. After another PBS-T wash (4×15 min), the brains were incubated with the secondary antibody, 488 anti-mouse 1:50 in 4% NGS, for 7 days at 4°C. Brains were mounted with antennal lobes oriented upward using Vectashield® mounting medium (Maravai Life Sciences, San Diego, CA).

We imaged whole-mount preparations using a Zeiss LSM800 confocal microscope (Carl Zeiss Vision Inc., San Diego, CA), using an argon laser at 448 nm. Optical sections had a resolution of 1024×1024 pixels. Five representative section images were taken of each brain: a whole-brain image at 10x taken at a depth approximate to the centroid of the antennal lobes (Figure 2c) for quantifying antennal and optic lobes, and four 20x images, one of each mushroom body calyx, taken at the shallowest depth at which the lip separated and the collar was visible in frame (Figure 2d) for quantifying lip and collar synaptic staining. Gain and digital contrast were adjusted in each image to ensure that none of the focal regions of interest (antennal lobes and optic lobes for whole-brain images, lips and collars for mushroom-body images) were over- or underexposed.

We estimated synaptic density of lip (olfactory) and collar (visual) calyces from representative slice images of each mushroom body calyx, using the Image-based Tool for Counting Nuclei (ITCN) plugin in ImageJ (Byun et al., 2006). Images were inverted and converted to 8-bit before ITCN estimated number of peaks per micrometer square. Based on preliminary image measurements, target peak width was 10 pixels and minimum peak distance was 5 pixels. This was done only for mushroom body calyx images. The ratio of detected synaptic vesicles per micrometer square (lip/collar) was compared across hovering guards, standing guards, and foragers using a mixed-effect ANOVA that took into account Colony ID and Bee ID as nested random factors. Absolute detected synaptic vesicles per micrometer square were compared across hovering guards, standing guards, and foragers using two separate mixed-effect ANOVAs (one for lip, one for collar) that took into account Colony ID and Bee ID as nested random factors. In the majority of cases, four mushroom body calyces (and four lip/collar synaptic density estimates) were imaged and quantified. However, calyces damaged during dissection or that did not stain sufficiently to make imaging criteria possible were omitted from analysis ($N = 166$ imaged).

Another method of quantifying synaptic immunofluorescence is to compare pixel brightness with internal controls that account for non-biologically relevant variation in penetration across individual brains (Waxman et al., 2017). Using a similar approach, we compared the ratio of optic versus olfactory brightness in peripheral and central regions of brains (with brightness ratios calculated from olfactory versus visual regions within the same images). This was measured in addition to synaptic density because it allowed us to quantify synaptic staining at

lower magnification, enabling the comparison of ratios of optic lobe versus antennal lobe as well. We assessed brightness in each slice image using ImageJ by tracing the edges of the lip, collar, antennal lobe, and optic lobe (medulla and lobula), then calculating the mean pixel intensity (brightness) of each region. For antennal and optic lobes, we quantified both right and left sides, but in a minority of cases, a side of the brain that was damaged during dissection was omitted from the study ($N = 86$ imaged). In the majority of cases, all four mushroom body calyces (and four lip/collar synaptic density estimates) were imaged and quantified. However, calyces that were damaged during dissection or did not stain sufficiently to make imaging possible were omitted from analysis ($N = 146$ imaged). Brightness ratios were calculated for olfactory versus visual regions within each slice image (lip versus collar or antennal lobe versus optic lobe, averaged across medulla and lobula). Brightness ratios were log transformed to improve the normality of distributions. Resultant log-transformed brightness data were homoscedastic across task groups (Levene's Test for Homogeneity of Variance, $F_{2,143} = 1.193, p = .306$) and distributions did not differ significantly from normality in two of three task groups according to Shapiro-Wilk normality tests (Foragers: $W = 0.974, p = .336$; Hovering guards: $W = 0.965, p = .139$; Standing guards: $W = 0.938, p = .02$). We compared the \log_{10} of the ratio of lip to collar brightness across mushroom bodies within hovering guards, standing guards, and foragers using a mixed-effect ANOVA that took into account Colony ID and Bee ID as nested random factors. We compared the \log_{10} of the ratio of antennal lobe to optic lobe brightness across sides of the brain in hovering guards, standing guards, and foragers using a mixed-effect ANOVA that took into account Colony ID and Bee ID as random factors.

3 | RESULTS

3.1 | Brain volumes

Bees with larger lifetime task repertoires (soldiers) did not have greater total brain volume or investment in higher-cognition regions (mushroom bodies) compared to those with smaller lifetime task repertoires (minors). Absolute brain volume did not differ between soldiers and minors ($X^2 = 1.44, p = .231$, Figure 3a) despite soldiers having significantly larger heads ($X^2 = 17.27, p < .001$, Figure 3b). The portion of head capsule occupied by brain tissue was, therefore, higher in minors than in soldiers ($X^2 = 5.22, p = .022$, Figure 3c). Although higher cognition regions of the brain (mushroom bodies) increased linearly with total brain size, soldiers and minors did not differ in mushroom-body volume or in the slope of the relationship between mushroom-body volume versus total brain volume (ANCOVA; Table 1, Figure 3d). Head volume was also generally a poor predictor of absolute brain volume (linear regression, $R^2 = 0.07, F_{1,39} = 3.99, p = .053$; Figure 3e).

Relative optic lobe volume significantly differed across task groups ($X^2 = 5.99, df = 2, p = .049$; Figure 4a). Compared to minors, soldiers had a larger portion of their brains dedicated to optic lobes but only while in the visually demanding task of hovering guarding

TABLE 1 Statistical output of mixed-effect ANCOVA, with a model of structure: Mushroom_body_volume ~ Brain_volume * Morphotype + (1|Colony), where morphotype is a categorical variable with two levels (soldier and minor)

Fixed factors	χ^2	df	p
Brain volume	385.91	1	<.001
Morphotype	0.58	1	.446
Brain volume : Morphotype	1.11	1	.291

($z = 2.42, p = .041$). Bees that had already aged into the task of standing guarding had similar relative optic lobe volumes as minors ($z = 0.86, p = .667$) but also did not significantly differ from hovering guards ($z = -1.545, p = .270$). Standing guards, hovering guards, and foraging minors had the same percentage of brain volume dedicated to antennal lobe ($\chi^2 = 4.83, df = 2, p = .089$; Figure 4b), mushroom-body collar ($\chi^2 = 0.844, df = 2, p = .656$; Figure 4c), and mushroom-body lip ($\chi^2 = 0.69, df = 2, p = .708$; Figure 4d). Proportions of regional brain volumes were otherwise quite similar across task groups, as indicated by Principal Component Analysis (Figure 5).

3.2 | Synaptic brightness and density

Small-bodied foragers and large-bodied hovering and standing guards did not significantly differ in the proportionate synaptic density of olfactory regions (lips) versus visual regions (collars) of the mushroom bodies ($\chi^2 = 0.23, df = 2, p = .889$; Figure 6a). These three groups also had the same ratio of synaptic brightness between lip and collar ($\chi^2 = 1.52, df = 2, p = .468$; Figure 6b) and between peripheral olfactory regions (antennal lobes) and peripheral visual regions (optic lobes) ($\chi^2 = 1.92, df = 2, p = .382$; Figure 6c). There were also no significant differences among these three groups in absolute synaptic density within the lip ($\chi^2 = 4.56, df = 2, p = .102$) or collar ($\chi^2 = 5.33, df = 2, p = .070$). The untransformed ratio of antennal/optic lobes was 0.579 ± 0.022 SE, and for lip/collar was 0.652 ± 0.045 SE, suggesting consistently more synapses in optic compared to olfactory regions.

4 | DISCUSSION

4.1 | Total brain investment

Soldier bees with larger lifetime task repertoires had similar absolute brain volumes, and smaller ratios of brain volume to head volume, compared to similarly aged minors with smaller lifetime task repertoires. Soldiers and minors did not have different relative mushroom-body volumes, regions that are associated with high cognition (Durst et al., 1994; O'Donnell et al., 2015; Sommerlandt et al., 2016). Overall, these results do not support the hypothesis that contrasts in lifetime task repertoire drive differences in brain size between soldiers and nonsoldiers. However, we did observe that stingless bee soldiers had smaller relative brain size to head size, which is similar to patterns observed in

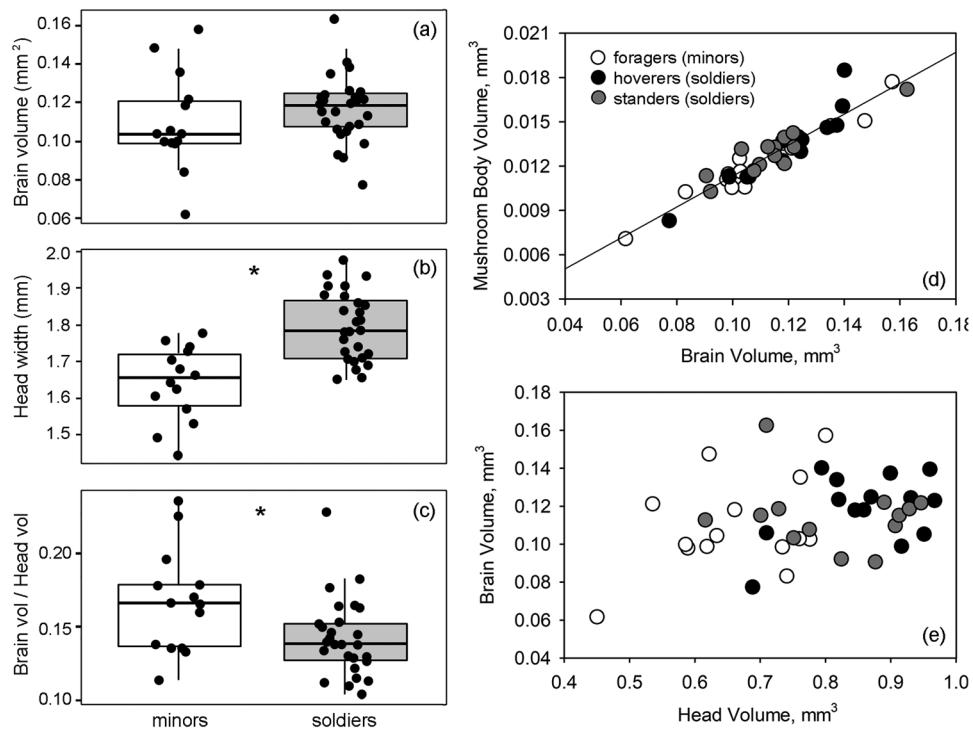


FIGURE 3 External and internal morphometric differences between distinct worker subcastes (minors versus soldiers) of specimens sampled for volumetric data collection ($N = 41$). *Indicates significant difference between soldiers and minors ($\alpha = 0.05$). (a) Total brain volume of soldiers versus minors. (b) Difference in head width between soldiers and minors. (c) Difference in portion of the head capsule composed of brain tissue between soldiers and minors. (d) Mushroom body (lip + collar) volume increasing linearly with total brain volume, regardless of morphotype or specific soldier task group (Table 1). (e) Lack of a relationship between brain volume and head capsule volume, with point color representing morphotype and task group.

army ants (O'Donnell et al., 2018) and turtle ants (Gordon et al., 2019). Together, this suggests that factors other than lifetime task repertoire size likely explain contrasts in relative brain size between soldiers and other workers within social insect colonies.

These results raise the important question of which timescales of task repertoire size are most relevant to total neural investment. Previous studies of soldier brain size used species for which both instantaneous and lifetime task repertoire size are generally smaller in soldiers than in other workers. However, task repertoire size is complex and dynamic for *T. angustula* soldiers. *Tetragonisca angustula* soldiers work 34 to 41% more than nonsoldier nestmates and have a lifetime task repertoire size that is 23 to 34% larger, but much of this task diversity occurs early in a soldier's adult life (Hammel et al., 2016). Whether and to what extent the shift to primarily defense tasks toward the end of a soldier's life coincides with a reduction in neural investment remains an interesting and relevant question. Such a reduction in total brain size coinciding with a decrease in task repertoire size has been reported in harvester ant queens following nest founding (Julian & Gronenberg, 2002) and in *Harpegnathos saltator* as they reversibly shift from foraging to reproductive (gamergate) tasks (Penick et al., 2021). However, based on our observations that a very large portion of the space within minors' head capsules was occupied by brain tissue, the possibility that young soldiers have an even greater proportion of head capsule occupied by brain tissue than minors (the prediction

if brain size increases with instantaneous task repertoire size) seems unlikely.

If not task repertoire size, then what other factors might help explain the pattern of soldiers having smaller relative brain size than minors across various eusocial insects, including *T. angustula*? One possibility is that higher soldier muscular investment, to enable improved combat capabilities, results in a tradeoff between muscle volume and relative brain size (O'Donnell et al. 2018). In leaf-cutting ants, bite force allometrically scales upward with increasing head size, resulting in soldiers whose head capsules primarily contain musculature (Püffel et al., 2021). Similar to many ants, stingless bee soldiers defend the colony from intruders using biting, with higher defensive bite efficacy exhibited by larger bees with more muscle (Grütter et al., 2012; Shackleton et al., 2015).

It is also possible that the broadly observed trend for relatively larger brains among smaller colony members in eusocial Hymenoptera is due to the physiological scaling constraints of neural tissue more than either task repertoire or muscular investment. Haller's rule predicts larger relative brain size in smaller-bodied animals via negative allometry between body and brain volumes (Haller, 1762; Rensch, 1948). Consistent with Haller's rule, we did find that smaller bees within the colony had relatively larger brains, however, the relationship between head size and brain size was not significant, and so we did not find evidence of negative allometry.

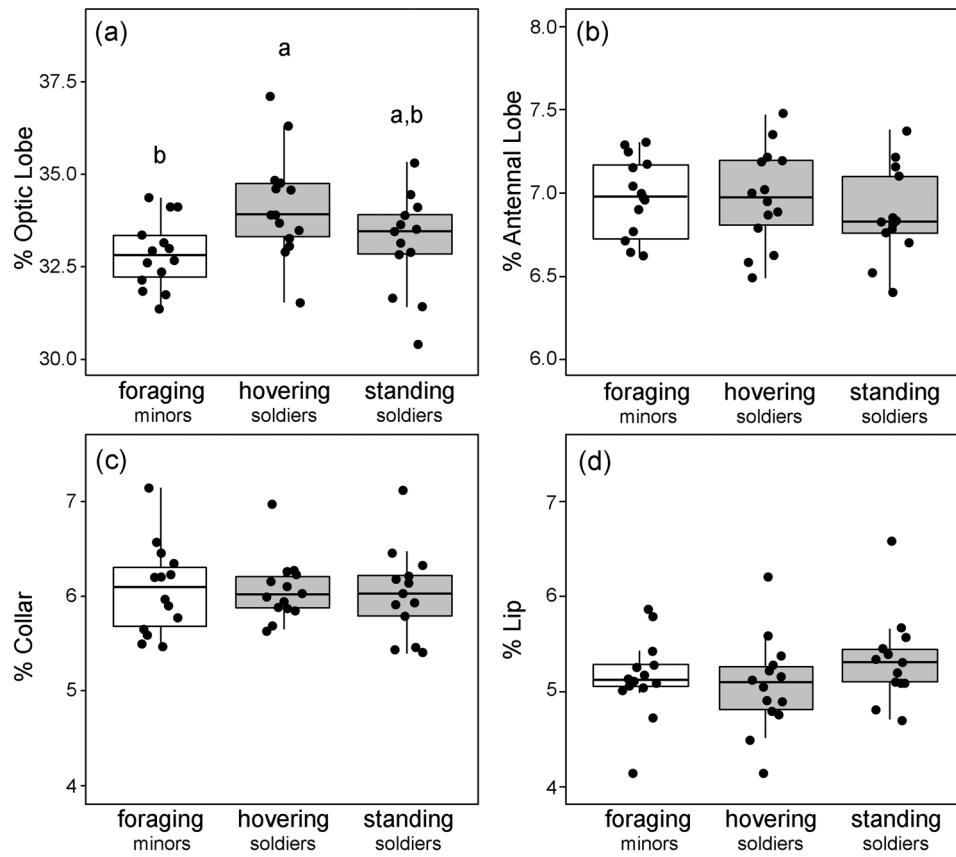


FIGURE 4 Comparisons of relative investment (percent of brain volume) comprised by peripheral brain regions associated with vision (optic lobe, a) and olfaction (antennal lobe, b), as well as sensory processing regions associated with vision (Collar, c) and olfaction (Lip, d). (f) Grey boxes denote defense tasks performed by morphologically larger soldier bees. White boxes denote a task (foraging) performed predominantly by small-bodied minors (see Figure 3b). Lowercase letters denote results of Tukey HSD post-hoc tests performed for regions that differed in relative volume according to linear mixed-effect analyses (colony = random factor)

4.2 | Modality-specific volume contrasts

Soldiers had different modality-specific neural investment than minors but only at certain ages and according to task demands on that age group. Compared to smaller-bodied nestmates of similar age, soldiers had significantly greater relative optic lobe size, but only while engaged in the more visually demanding defensive task of hovering guarding. As soldiers aged into the less visually demanding task of standing guarding, this difference in peripheral visual investment between soldiers and minors disappeared. Notably, these modality-specific differences seemed to be primarily in peripheral brain regions associated with visual acuity (optic lobe) and not visual information processing (mushroom body collar) (Gronenberg, 2001). Similar peripheral brain investment contrasts have been observed across dimorphic male bees employing alternative reproductive tactics in *Centris pallida* and *Amegilla dawsoni* (Barrett et al., 2021) and between males and females of the sexually dimorphic longhorn bee *Eucera berlandi* (Streinzer et al., 2013). Here, we report similar patterns across age-differentiated soldier subcastes in a eusocial bee. The transition from hovering guarding to standing guarding in *T. angustula* soldiers occurs over approximately 7 days (Baudier et al., 2019). Although the magnitude of observed optic lobe volumetric change is small, and only significant relative to optic

lobe volume of minors, the rapid pace of this transition is striking and suggests the importance of future investigations of the neurobiology of soldier subspecialization in other eusocial taxa.

We also know relatively little about how external investment in sensory structures relates to behaviorally driven neural investment, and how both adaptively scale across individuals within insect societies. Recent work has been done on this topic in leaf-cutting ants by Arganda et al. (2020), who reported similar patterns as we find here, namely, that larger-bodied soldiers performing visually demanding tasks had larger optic lobes. There was also evidence of intracaste neural and eye specialization corresponding to the different light levels of the working environments of different worker subcastes (Arganda et al., 2020). Given their unique task repertoires and added soldier age-subspecialization on different defense tasks, *T. angustula* may be a useful alternative model system for exploring the interplay among such allometric, behavioral, and ecological factors in the future.

We also report no differences in volumetric olfactory investment across minors versus soldiers or hovering versus standing soldiers. Although standing and hovering guards differ in which odors elicit responses (Baudier et al., 2020), both hovering and standing guarding are tasks that demand some degree of olfactory intruder detection (Bowden et al., 1994; Kärcher & Ratnieks, 2009).

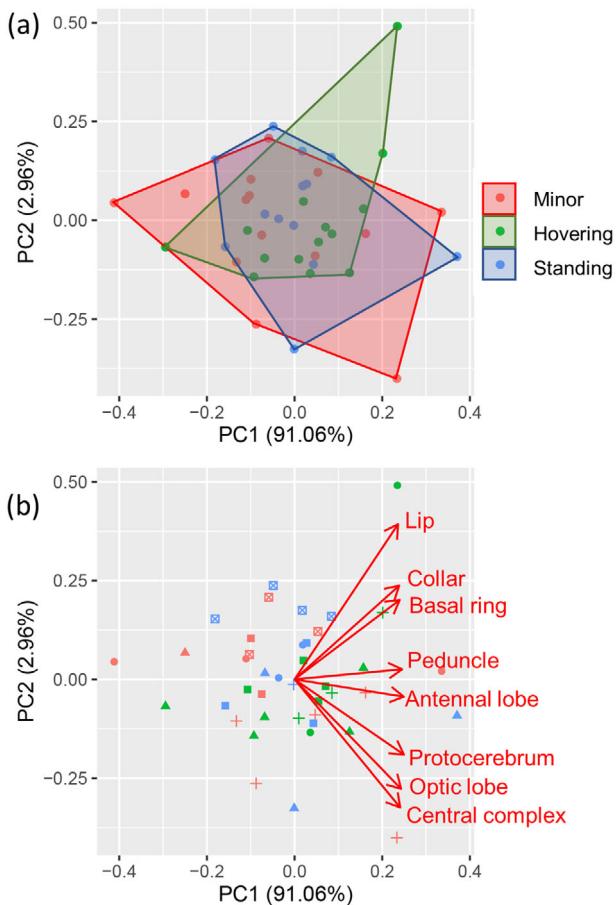


FIGURE 5 Results of principal component analysis of all measured brain region volumes showed a high degree of similarity in neural architecture among morphological/behavioral task groups, despite slight differences in modality-specific investment (as shown in Figure 4a). Colors in (a) and (b) correspond to focal task groups compared in this study. In (b) different symbols (marker types) correspond to the five different colonies sampled.

Our findings of similar gross olfactory investment among hovering guards, standing guards, and foraging minors suggest that using close-range olfaction to identify conspecific threats, using volatile detection to discern heterospecific threats, and the use of olfaction in foraging place similar, but difficult to compare, demands on olfactory acuity and processing investment.

Because hovering guards and standing guards differ not only in tasks performed but also in age (ca. 7 days), whether and how experience versus experience-independent ontogeny mediates the subtle neural transition (decrease) in optic lobe remains an open question. Contrary to our results, honeybee and carpenter ant mushroom bodies typically increase in size with age and experience (Fahrbach et al., 1998; Gronenberg et al., 1996). Also contrary to the patterns we report, optic investment increases with both age and visual experience in young adult *Drosophila melanogaster* (Barth et al., 1997) and *Polistes fuscatus* (Jernigan et al., 2021). However, soldiers typically perform standing guarding tasks in their final week of life (Figure 1), and so whether this volumetric shift is associated senescence is an interesting question for future

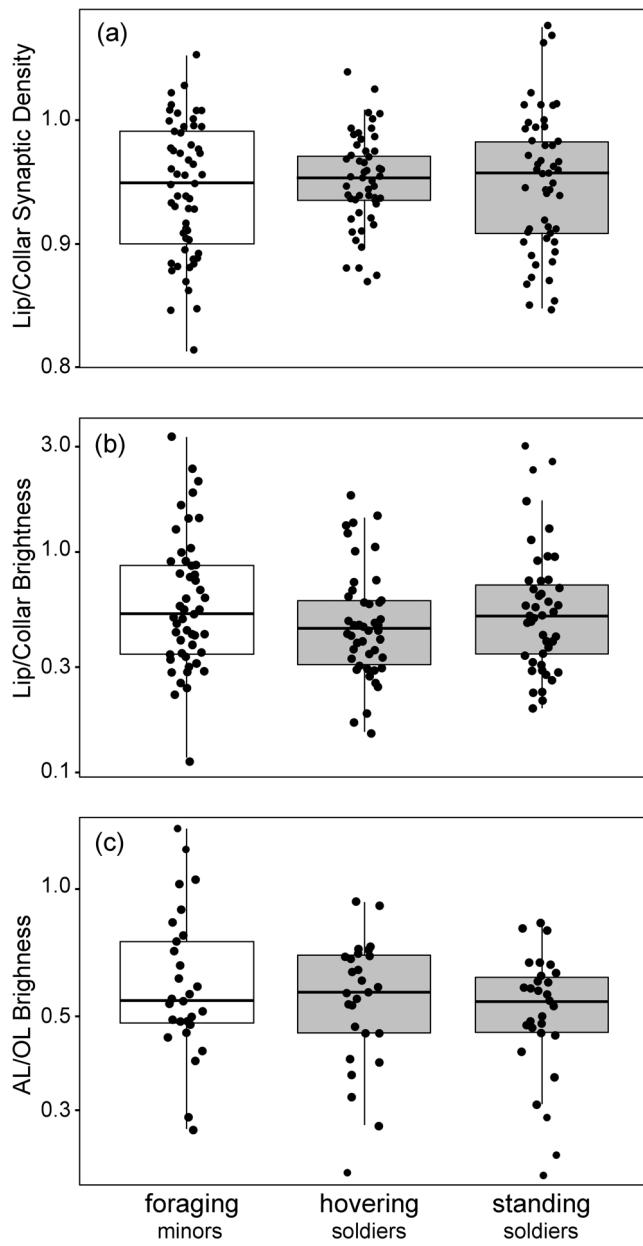


FIGURE 6 Quantified synapsin in brain regions associated with olfactory versus visual sensing and processing. Grey are defensive task groups performed by morphologically larger-bodied soldiers. Meanwhile foraging is performed predominantly by smaller-bodied minors of similar age (a). Ratio of lip (olfactory processing) to collar (visual processing) estimated synaptic density did not differ across groups; N = 166. (b) Log-transformed ratio of lip to collar brightness (pixel density) did not differ across groups; N = 146. (c) Log-transformed ratio of antennal lobe (AL) brightness to optic lobe (OL) brightness did not differ across groups; N = 82

investigation. Optic lobes of the European honeybee (*Apis mellifera*) are especially susceptible to oxidative stress associated with senescence (Seehuus et al., 2006) and numbers of serotonergic cell bodies in the optic lobe change with age in soldiers of the ant *Pheidole dentata* (Seid, Goode, et al., 2008). Whether similar processes relate to the patterns in brain volumetrics, we report here, remains an open question.

Though we report interesting contrasts in optic investment, overall neural architecture was strikingly similar across all ages and morphotypes explored in this study (Figure 5). This stands in stark contrast to studies of minors and soldiers in ants of genus *Pheidole*, where both age and morphotype predicted discrete groups in neuroanatomical space with little overlap (Muscedere & Traniello, 2012).

4.3 | Modality-specific synaptic density and brightness

Synaptic activity is remarkably plastic and can change in social hymenopteran brains throughout adulthood due to age or life experiences, independent of or dependent on shifts in neuropil volume (Fahrbach & Van Nest, 2016; Seid et al., 2005). Indeed, despite observing contrasts among task groups in total and modality-specific brain volume, we found no evidence of differences in the ratio of modality-specific synaptic density or brightness across the three measured task groups, although absolute synaptic density within the collar was marginally nonsignificant. In honey bees, increases in synaptic (microglomerular) density within mushroom bodies have been associated with the formation of long-term modality-specific memory (Hourcade et al., 2010). Among *T. angustula*, standing guards are, in a sense, older and more experienced hovering guards with potentially greater accumulated long-term memories. However, we report no evidence of synaptic density differences between standing and hovering guards in either lip or collar using these methods. These results are contrary to our prediction and may indicate that brain volume but not synaptic activity drives differences in these focal behaviors. Other mechanisms involved in energy metabolism that were not measured in this study could also help explain differences in behavioral phenotypes. Beyond ATP production, mitochondria play a role in maintaining intracellular calcium homeostasis, which is critical for cell signaling (Detmer & Chan, 2007). Whether and how these task groups differ in neural mitochondrial density and activity remains an open question.

5 | CONCLUSIONS

In exploring the neurobiology of stingless bee soldiers, we report both contrasts and similarity in neural architecture across highly sub-specialized but behaviorally flexible stingless bee workers. Although both age and morphological worker subcaste were predictive of some aspects of regional and total brain investment, we also observed striking overlap in brain region volumes at large (Figure 5) and similarity in synaptic/microglomelular density (Figure 6). The neural architecture of worker castes of *T. angustula* is quite similar, though mildly specialized across discrete task groups, and is consistent with this species' simultaneous strategies for specialization but also flexibility in defensive task allocation (Baudier et al., 2019). Although the vast majority of guarding tasks are performed by soldiers under normal circumstances, minors are capable of performing some degree of nest defense when soldiers are removed (Baudier et al., 2019). Neural architecture that is

similar but slightly specialized likely enables both defense specialization, and flexible defensive reallocation in a crisis. As the first study of neural architecture of morphologically distinct soldier brains in a bee, these results broaden our understanding of the neurological mechanisms underlying defense specialization in highly coordinated societies.

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DATA AVAILABILITY STATEMENT

All data used in this study can be found in the supplementary information.

PEER REVIEW

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