Behavioral performance requirements for division of labor influence adaptive brain mosaicism in a socially complex ant

Muratore, I.B.^{1*}, Fandozzi, E.M.¹, J.F.A. Traniello^{1,2}

1. Department of Biology, Boston University

2. Graduate Program in Neuroscience, Boston University

* Corresponding author. imurator@bu.edu, 1-516-477-7714, 5 Cummington Mall, Boston

02215, MA, USA

Keywords: brain evolution, social brain, collective intelligence, cognition, caste evolution, task performance, response threshold, mushroom body, sensory processing, motor control

Abstract

Brain evolution is hypothesized to be driven by neuroarchitectural requirements for behavioral performance. Assessments of such needs should be informed by the nature of sensory and motor processes underpinning behavior. We developed a novel metric to estimate the relative neuroanatomical investments required to perform tasks varying in sensorimotor and processing demands across polymorphic and polyethic workers of the leafcutter ant *Atta cephalotes* and quantified brain size and structure to examine their correspondence with our computational approximations. Investment in multi-sensory integration and motor requirements for task performance was estimated to be greatest for media workers whose leaf-harvesting repertoire involves the most diverse and demanding sensory and motor processes, including plant

discrimination, leaf cutting, and fragment transportation. Volumetric analysis of confocal brain images revealed that absolute brain size increased with worker size and compartmental scaling allometries among functionally specialized brain compartments differed among polymorphic workers. The mushroom bodies, centers of sensory integration and learning, and the antennal lobes, which process olfactory inputs, were significantly larger in medias than in minim workers (fungal gardeners) and major workers (“soldiers”), which had lower estimated task-related neural demands. Minims had a proportionally larger central complex, perhaps to control navigation in subterranean fungal garden chambers. These results indicate that variation in task performance requirements has selected for adaptive variation in brain size and mosaic scaling.

Introduction

Identifying the selective forces that contribute to the evolution of brain size and patterns of investment in functionally specialized brain centers is key to understanding the organization of behavior. Social life – as an agent of selection – has been predicted to both increase (Adolphs, 2003; Dunbar, 1998, 2009) and decrease brain size (Godfrey & Gronenberg, 2019; Gronenberg & Riveros, 2009; Jaffe & Perez, 1989; O’Donnell et al., 2018; Reséndiz-Benhumea et al., 2021; Riveros et al., 2012; Sulger et al., 2014), and/or alter compartmental scaling relationships (DeCasien & Higham, 2019; Muscedere & Traniello 2012; O’Donnell et al., 2015; O’Donnell et al., 2018; Smaers & Soligo, 2013). Factors favoring enlargement of the brain are constantly debated (Aiello & Wheeler, 1995; DeCasien et al., 2017; Lihoreau et al., 2012; Navarrete et al., 2016; Wartel et al., 2019). Assessments of sensory, motor, and processing demands for behavioral performance are required to make predictions about brain size and adaptive scaling given the cost of neural tissue, but the nature and extent of such demands are rarely estimated in analyzes that link behavior and neuroanatomy.

Insects have been proposed as models to examine behavioral and/or cognitive evolution (Lihoreau et al., 2019; Simons & Tibbetts, 2019), and some clades may facilitate the quantification of behavioral demands required to understand the evolution of neural systems (Boogert et al., 2018; Muratore & Traniello, 2020). Ants, as exemplars of insect sociality, form complex societies that may be characterized by striking patterns of task specialization associated with ergonomic division of labor (Beshers & Fewell, 2001; Hölldobler & Wilson, 1990) and worker size-related variation in innate task routines and/or cognitive capabilities. The brains of workers are composed of functionally specialized compartments involved in visual and olfactory processing (optic lobes [OL] and antennal lobes [AL], respectively), higher-order processing, learning, and memory (mushroom bodies [MB]), navigation, orientation, and movement (central complex [CX], also associated with the MBs) (Currier et al., 2020; Green et al., 2019; Kamhi et al., 2020; Le Moël et al., 2019; Pisokas et al., 2020; Sun et al., 2020), and mandibular control and gustation (the suboesophageal zone [SEZ]). The remainder of the central brain (ROCB) is composed of several protocerebral compartments thought to integrate sensory information with the CX (Currier et al., 2020; Green et al., 2019; Strausfeld, 2012). Although brain size may be limited as a predictor of computational power and cognitive capacity (Chittka & Niven, 2009; Muscedere et al., 2014), the volumes and structural elaboration of visual and olfactory neuropils and the MBs in ants are considered to correlate with processing capability and have ethological significance (Farris, 2011; Gronenberg, 2001). Brain size and compartmental allometries (Gordon et al., 2017; Muscedere & Traniello, 2012) and covariance among brain compartments (Ilieș et al., 2015; Kamhi et al., 2019) correlate with variability in worker size and task performance. Task experience affects MB size (Durst et al., 1994; Fahrback, 2006; Gronenberg et al., 1996), and large MBs may be associated with increased behavioral flexibility (Gronenberg

& Riveros, 2009; O'Donnell et al., 2015; Riveros et al., 2012). Complexity in colony organization may therefore involve selection for either smaller, neurally differentiated worker brains (Feinerman & Traniello, 2015; Gronenberg & Riveros, 2009; Lihoreau et al., 2012; O'Donnell et al., 2018; O'Donnell et al., 2015; Riveros et al., 2012; Sulger et al., 2014) or larger brains (Wehner et al., 2007) potentially able to metabolically offset increased production and operation costs (Kamhi et al., 2016).

Social organization in fungus-growing ants is characterized by morphologically differentiated workers in derived species (Hölldobler & Wilson, 2010; Muratore & Traniello, 2020). Colonies of leafcutter ants, *Atta*, have strongly polymorphic workers categorized into size-based groups (subcastes) differing in labor roles and efficiencies of task performance (Figure 1) (Wilson, 1980a, 1980b). Worker size-related specializations include fungal care, nursing immatures, leaf selection, cutting, and transport, waste management and hygienic behavior, and colony defense. These tasks differ in requirements for multimodal sensory perception, integrative processing, and motor functions associated with monitoring the growth of fungus and brood development, decoding plant chemistry, leaf harvesting, navigation, recognizing infectious agents, detecting environmental hazards, and combating predators and parasites (Arenas & Roces, 2016a, 2016b, 2017; Arganda et al., 2020; Goes et al., 2020; Green & Kooij, 2018; Groh et al., 2014; Howard et al., 1996; Saverschek & Roces, 2011). This pattern of division of labor characterizes *Atta cephalotes*, whose workers range in size from 0.5 to more than 4.5mm in head width and are divisible into groups according to task repertoires (Table 1). Brain size and structural variation could reflect differences in behavioral diversity: workers displaying the broadest task repertoire and corresponding behavioral and/or cognitive demands could require higher investment in neural tissue. To examine factors contributing to brain

evolution in *A. cephalotes*, we evaluated the distribution of functional needs for sensory perception, integration, and sensorimotor functions associated with task performance across polymorphic workers. We determined patterns of variation in behavioral demands associated with task performance and work environments to provide a semiquantitative estimate of brain tissue requirements to efficaciously perform tasks.

To identify how brain size, compartmental scaling, and differential task performance demands of division of labor are associated and thus determine correspondence with our estimate, we quantified patterns of sensory, higher-order processing, and motor neuropil investment. This allowed us to link variation in task repertoires and their sensory challenges among workers to size scaling among functionally differentiated brain compartments. Based on an assessment of worker size-related sensory and motor functions, olfaction needs, and higher-order processing, we hypothesized that intermediate-size (media, leaf-harvesting) workers would have higher MB investment than small (minims, fungus-gardening) or large (majors, defensive) workers. We additionally hypothesized that our estimate would accurately describe the pattern of investment in the ALs, with this neuropil proportionally largest in medias due to the need for processing diverse olfactory cues. We also determined the fit of our estimates to the proportional volumes of the CX and OLs.

Methods

Colony collection and culturing

Incipient *A. cephalotes* colonies were collected in Trinidad during 2016. Colonies (Ac09, Ac16, Ac20, Ac21) were cultured in a Harris environmental chamber under a 12-hour light: 12-hour dark cycle at 55% humidity and 25°C at Boston University, and Costa Rican colonies (M1 and M2) were cultured in an environmental chamber under a 12-hour light: 12-hour dark cycle at

20°C at the Boston Museum of Science. All colonies were housed in large plastic bins (30cm x 46cm x 28cm), whose floors served as a foraging arena and for waste disposal. Smaller plastic boxes (11cm x 18cm x 13cm) interconnected by plastic tubes (1cm diameter) served as chambers for the fungus. Colonies were provisioned with washed and pesticide-free leaves from rhododendron, rose, lilac, andromeda, bramble, oak, sugar maple, willow, and beech trees (as available), and organic baby spinach, romaine, arugula, frisée, and oat flakes.

Behavioral performance demands and estimates of required neuroanatomical support

We integrated fungus-growing ant adaptive morphology and behavior (Hölldobler & Wilson, 2010; Wilson, 1980a, 1980b) with personal observations and data from the literature (references in Table 1) to inform our estimate of needs for sensory integration motor control in *A. cephalotes* worker behavior. Based on our results of research on visual system evolution in *A. cephalotes* (Arganda et al., 2020), we assumed greater investment in neuropils such as the MBs and ALs would be required to process more diverse stimulus arrays and coordinate sensorimotor processes. For example, tasks such as leaf selection, cutting, and transport involve olfactory discrimination, proprioception and mechanosensory and muscular systems to control the mandibles, appendages, head position, and direction of movement (Currier et al., 2020; Green et al., 2019; Khalife et al., 2018), whereas other tasks differ substantially in these needs. Estimates for task performance frequency were based on published results of studies of worker size-related behavior (references listed in Table 1) validated by unpublished data (Muratore et al., in prep.). Sensory integration and other cognitive process requirements were based on overlap in the known sensory capacities of ants and documented instances of behaviors being disrupted through manipulations of the brain or sensory pathways (see references in Table 1).

Approximations of neuropil requirements for sensory integration were calculated as a sum of each worker group-task performance process combination from estimates in Table 1 according to the following equation:

$$D_w = \sum_{t=1}^n x_t y_{wt}$$

D_w = Estimated requirement for neuronal substrate to process and integrate sensory and/or sensorimotor inputs for a given worker size group

x_t = Sensory integration-task demand score, the estimated degree of sensory integration required to perform a given task t

y_{wt} = Worker size group task performance score, estimating the tendency of a given worker group w to perform a given task t

n = total number of tasks

This equation integrates contributions from the type of brain investments likely to be necessary for the performance of individual tasks with the frequency with which different types of workers are likely to perform them in order to generate a hypothesis about the likelihood of selection acting to prioritize or deprioritize sensory integration in the brains of different workers. x_t was rated on a 0-3 scale according to the role an input or process is thought to serve in a specific task. 0 = not involved, 1 = possible role, 2 = likely role, and 3 = significant role. y_{wt} was rated on the same scale according to the likelihood of a given worker size group to perform a specific task.

Immunohistochemistry and confocal microscopy

Mature fully sclerotized workers collected from colonies Ac09, Ac16, Ac20, and Ac21 were decapitated immediately prior to brain dissection and fixation. Workers were sampled from five worker size groups identified by head width (HW): minims (0.6mm±0.1mm), medias

(1.2mm±0.1mm, 1.8±0.1mm, or 2.4mm±0.1mm), and majors (3.0mm or larger). Brains (n=30) from workers sampled from Ac09, Ac20, and Ac21 were dissected in ice-cold HEPES Buffered Saline (HBS), placed in 16% zinc-formaldehyde (Ott, 2008) and fixed overnight at room temperature (RT) on a shaker. Whole brains were processed to visualize the presynaptic protein synapsin. Fixed brains were washed in HBS six times, 10 minutes per wash, and fixed in Dent's Fixative (80% MeOH, 20% DMSO) for minimally 1 hour. Brains were then washed in 100% methanol and either stored at -17°C or immediately processed. Brains were washed in 0.1M Tris buffer (pH=7.4) and blocked in PBSTN (5% neutral goat serum, 0.005% sodium azide in 0.2% PBST) at RT for 1 hour before incubation for 3 days at RT in primary antibody (1:30 SYNORF 1 in PBSTN; monoclonal antibody anti-synorf 3C11 obtained from DSHB, University of Iowa, IA, USA; 62). They were washed 6x10 minutes in 0.2% PBST and incubated in the secondary antibody (1:100 AlexaFluor 488 goat anti-mouse in PBSTN) for 4 days at RT. Brains were then washed a final time (6x10 minutes in 0.2% PBST) and dehydrated in an ethanol and PBS series (10 minutes per concentration, 30/50/70/95/100/100% ethanol in 1x PBS), then cleared with and immersed in methyl salicylate, and mounted on stainless steel glass windowed slides for imaging.

Brains were imaged with a Nikon C2 confocal microscope and images were manually annotated using Amira 6.0 software to quantify neuropil volumes (not including cell bodies). We recorded the volumes of OL, AL, MB, CX, SEZ, and ROCB. We also measured substructures of the MB: the medial calyxes (MB-MC), lateral calyxes (MB-LC), and peduncle and lobes (MB-P). These were combined to quantify total MB size (MB-S) across worker size groups. For bilateral structures, one hemisphere was measured, and for compartments located along the brain

midline (SEZ and CX), the whole structure was measured (Supplementary Table 1; Supplementary Table 2).

Volumetric analysis

Statistical *post hoc* comparisons among worker size groups and brain compartment metrics were performed using R (version 3.6.2). Absolute volumes of total measured brain volume, total brain volume scaled to head width, all individual brain compartments, and two normalized brain compartments were not normally distributed (Supplementary Table 3), and the significance of differences among groups was therefore determined using Kruskal-Wallis tests followed by Wilcoxon rank sum *post hoc* pairwise comparison with Bonferroni correction. Relative volumes were calculated by dividing the volume of the compartment of interest by total brain volume. These data were distributed normally. If ANOVA results showed a significant effect of worker size group on the proportional size of a brain compartment, Tukey's *post hoc* tests were performed with a Bonferroni correction for multiple comparisons to determine the significance of compartment size differences among groups.

Linear regression was used to assess correspondence between estimate values of D_w for each worker size group and either proportional volumes of brain compartments or total brain volume scaled to worker size (i.e., the sum of all measured neuropils/HW).

Principal component analysis (PCA) was performed on log-transformed proportional volumes using the `prcomp` function from the base stats package in R. Linear discriminant analysis (LDA) was performed using the `lda` function from the MASS package in R (Liaw & Wiener, 2001). A previously collected data set of brain volume measurements from the same species with measurements taken by a different observer was used as a training set (Supplementary Table 4).

Results

Division of labor and requirements for task performance

Estimated requirements for sensory integration were highest in medias (1.2mm $D_w = 124$, 1.8mm = 127, 2.4mm = 102), with the estimate for minims (0.6mm $D_w = 82$) exceeding that for majors (3mm+ $D_w = 20$) (Figure 2). A linear regression of MB-S volume against D_w showed a significant correspondence between our estimate and the pattern of proportional investments in the MBs (Table 2, Figure 3). Similarly, MB-P volume showed moderate but significant correspondence to D_w estimates. MB-MC, MB-LC, OL, AL, CB, SEZ, and ROCB volumes were also compared to D_w estimates, all showing very low levels of explanation of variance with mixed significance (Table 2).

Division of labor and neural phenotypes

Absolute total brain volume of increased with worker size (Figure 4a; Table 3a; Supplementary Table 5a). Interestingly, no significant change in total brain size occurred in workers in the 0.6 to 1.8mm size range, despite a three-fold increase in body size. Only workers in the 2.4mm and 3mm+ size groups had significantly larger brains. In contrast, the smallest two size groups showed slightly but significantly larger brain volumes in relation to body size (Figure 4b; Table 3a; Supplementary Table 5b). The absolute volumes of all compartments except the CX significantly increased with worker size (Figure 5; Table 3a; Supplementary Table 6). Consistent with the pattern of total brain size, many brain compartments were significantly larger in 2.4mm or 3mm+ workers. There was a significant effect of worker size on the proportional volumes of all brain compartments except the MB-MC, MB-LC, and the SEZ (Table 3b). In contrast to the relatively uniform pattern of increase in absolute volumes, the directions of these

trends differed, indicating neuroanatomical differentiation of social role phenotypes (Figure 6; Table 3b; Supplementary Table 7).

Principal component analysis of log-transformed proportional brain volumes explained a significant portion (PC1 = 15.83%, PC = 66.04%) of the variance observed (Figure 7a). Linear discriminant analysis, using a model that included the proportional volumes of all neuropils except for the ROCB (which was colinear with other variables) and trained on a separate data set of *A. cephalotes* brain volumes, was able to classify samples in the main data set with 95.8% accuracy (Figure 7b). However, this result was found only when 1.2, 1.8, and 2.4mm worker groups were clustered as medias. LDA using five worker size groups (0.6, 1.2, 1.8, and 3mm+) and the same testing and training data sets achieved 54.2% accuracy.

Discussion

We assessed how estimated sensory and motor requirements for task performance underlying complex agricultural division of labor correlate with quantified variation in brain size and mosaic structure among polymorphic workers of *A. cephalotes*. Behavioral performance challenges in social insects have been typically inferred from interspecific and intraspecific variation in neuropil scaling patterns and general assessments of sensory environments and socioecological influences (Amador-Vargas et al., 2015; Gronenberg, 1999; Gronenberg et al., 1996; Muscedere & Traniello, 2012; O'Donnell, Bulova, Barrett, & Fiocca, 2018), casually correlated with sociobiological characteristics such as colony size, foundation strategy and queen/worker differentiation (for example the reduction in MB volume in solitary compared to social wasps; O'Donnell et al., 2015), and/or diet and life history (Sayol et al., 2020). To the best of our knowledge, our study is the first to computationally estimate the need for brain investment in relation to sensorimotor integration requirements for behavioral performance and establish a

correlation with neuroanatomy. The parameters of task performance used to develop our estimate were derived from results of published studies and our own observations rather than subjective or casual assessments of behavior. We evaluated the need for neuropil investment to serve sensory input processing and sensorimotor functions, conservatively evaluated the size-related involvement of polymorphic workers in performing these tasks (Figure 2) and used this metric to generate hypotheses concerning brain evolution. Comparisons of brain compartment volumes indicated significant differences in absolute size and proportional investment across polymorphic task-differentiated workers, supporting the concept that worker neural phenotypes are adaptively designed to meet variation in requirements for task performance. Neuroanatomical scaling patterns broadly correlated with sensory, somatosensory, and integrative information-processing demands associated with the specialized task repertoires of polymorphic workers. Such mosaic scaling is consistent with demands on workers in species with large colonies and worker specialization (Riveros et al., 2012). Notably, our estimate showed a pattern broadly consistent with total MB and MB-P scaling patterns, though the percentage of explained variance was only moderate, lending some support to the idea that selection on brain compartment volume optimizes for task integration requirements though other factors likely influence levels of investment. Such factors may include differences in the amount of tissue required to maintain baseline neural functions across workers that vary in body size and metabolic expenses (Kamhi et al., 2016; Packard, 2020), or other size-related constraints (Finlay & Darlington, 1995; Herculano-Houzel, 2012; Herculano-Houzel et al., 2014; O'Donnell, Bulova, Barrett, & Fiocca, 2018).

Differentiation of worker neural phenotypes

Principal component and linear discriminant analysis distinguished individual *A. cephalotes* brains from different worker groups on the basis of compartment volumes. LDA demonstrated that the degree to which brains can accurately be classified in terms of worker body size is greater when all media size classes (1.2, 1.8, and 2.4mm) are pooled, but is nevertheless able to distinguish samples belonging to five different groups. These results suggest that medias are readily distinguished from the largest and smallest worker specialists in terms of tissue requirements for task performance but differentiation among size groups of medias is less substantial, and consistent with our estimates (Figure 2).

Antennal lobes

Medias were predicted to have greater sensory integration needs than other worker groups (Figure 2) due to their large task repertoire and collaterally diverse behavioral challenges such as selecting and harvesting plant material and navigating to and from food sources (Arenas & Roces, 2017; Blanton & Ewel, 1985; Falibene et al., 2015; Groh et al., 2014; Howard, 1987; Howard et al., 1988; Hubbell et al., 1983, 1984; Wilson, 1980a, 1980b). Media workers in the 1.8mm and 2.4mm size groups showed increased AL investment compared with minors and majors, as well as 1.2mm medias. Increased input to the AL from antennal odorant receptors is associated with increased olfactory sensitivity and odor perception (Acebes & Ferrús, 2001; Kuebler et al., 2010). Consistent with their social roles, media worker ALs appear to be enlarged to process more diverse olfactory information, similar to the linkage of AL enlargement and olfaction-based tasks in *Atta vollenweideri* (Kelber et al., 2009; Kleineidam et al., 2005; Kuebler et al., 2010). Nonetheless, little variance in AL volume could be explained by our model, indicating that this method may not be a suitable predictor of investment in primary sensory neuropils, even those sensitive to a wide of array of potential stimuli.

Mushroom bodies

Medias had the highest relative total MB volume. The trend was consistent for the MB-P and the MB-MC (although differences in proportional MB-MC volume were not significant) but not the MB-LC, which showed significant differences in 1.2 vs. 3mm+ and 1.8 vs. 3mm+ comparisons but no overall trend of increasing in either minors, medias, or majors. The functions of the MB-MCs and the MB-LCs may differ, and interestingly, the medial calyx has been shown to be influenced by experience in bees (Riveros & Gronenberg, 2010). The significant differences we found in total MB volume were thus primarily driven by MB-P volume, an axonal bundle that relays outputs from the MBs to other brain compartments (Gronenberg, 1999), whose volume is more influenced by olfactory experience than visual experience (Heisenberg, 1998). Our results suggest *A. cephalotes* 1.8mm medias require relatively larger MB due to the nature and diversity of their leaf-harvesting task repertoire. These findings are consistent with the broader pattern of task repertoire size and diversity of sensory inputs across polymorphic *A. cephalotes* workers, as factored in our estimate for neural investment demands. In respect to other insect examples of specialist or generalist habits (Farris & Roberts, 2005), the enlargement of the MBs, a crucial sensory processing center, is consistent with the idea that generalists - in this case medias that have a larger task repertoire - require more neural tissue to fulfil their tasks. *A. vollenweideri* workers show decreasing investment in the MB calyces with increasing worker size (Groh et al., 2014). Our data indicate greater MB calyx investment in *A. cephalotes* medias, perhaps due to their generalist plant tissue harvesting, which contrasts with the specialized grass harvesting of *A. vollenweideri*.

Additional compartmental allometries

We found that CX volume was proportionally largest in minims, and inversely related to worker size. Greater investment in the CX may represent circuitry to enable multisensory navigation within dark three-dimensional labyrinthal fungal comb chambers. Minims mainly perform fungal-gardening tasks that likely rely on non-visual navigational strategies, perhaps involving CX circuitry (Currier et al., 2020; Green et al., 2019; Le Moël et al., 2019; Mamiya et al., 2018; Pisokas et al., 2020; Shiozaki et al., 2020; Sun et al., 2020).

A. cephalotes worker OL proportional volume increases with worker size and is highest in majors, which have greater visual acuity (Arganda et al., 2020). The repertoire of *Atta* majors appears limited to defense (Powell & Clark, 2004a; Wilson, 1980a), mainly an extranidal task that likely involves target detection.

Total brain volume

Total brain volume sharply increased in the largest two worker size groups. When scaled to body size, minims had the largest brains, consistent with Haller's rule (Rensch, 1956), perhaps due to limits on miniaturization of neural circuits (Beutel et al., 2005; Chittka & Niven, 2009; Groh et al., 2014; Niven & Farris, 2012; Seid et al., 2011). However, Haller's rule does not apply to hymenopteran species such as some parasitoid wasps smaller in size than *A. cephalotes* minims (Groothuis & Smid, 2017; van der Woude et al., 2013; van der Woude & Smid, 2016). This suggests that *A. cephalotes* fungal gardening and nursing behaviors may demand more neural tissue than that required for parasitoid behavior. Size-adjusted total brain volume showed very little correspondence to estimates of our model of requirements for task performance. Given the more significant correspondence found in some brain compartments and coupling of scaling pattern of compartments to sensory requirements, total brain volume may be a too general and

imprecise metric to reflect differences in task performance (Chittka & Niven, 2009; Logan et al., 2018; Muscedere et al., 2014; Muscedere & Traniello, 2012) for *A. cephalotes* workers.

Conclusions

Our results provide insight into the association of task specializations, their sensorimotor and higher-order processing requirements, and brain evolution. Adaptive brain mosaics in *A. cephalotes* polymorphic workers appear to neuroanatomically support the social organization of agricultural division of labor. We found MB size allometry in media workers that corresponded to estimated sensory processing and sensorimotor demands of leaf harvesting, their principal task. The proportional enlargement of the MBs in this size group supports the hypothesis that task sets having apparently greater requirements for sensory integration, motor coordination, and higher-order processing are associated with significantly greater investment in brain centers underpinning these functions. The relationship of our investment estimate to both MBS and MB-P volumes suggests that our method effectively characterizes neuropils that integrate diverse stimuli and facilitate higher-order processing, but does not adequately explain variances in investment in specialized primary input neuropils. This is likely due to the emphasis our model places on behavioral diversity, and thus the integration and processing of multimodal stimuli, and act frequency.

Figures and tables

Table 1. Tasks performed by *A. cephalotes* workers, their relative reliance on sensory modalities, sensory input processing, high-order integration, and motor coordination in relation to division of labor. Sensory and sensorimotor inputs of tasks and processing are rated on a 0-3 scale depending on the role an input or process is considered to serve in a given task. 0 = not

361 involved, 1 = possible role, 2 = likely role, and 3 = significant role. The same scale was used to
 362 rate the performance frequency of a given task in the repertoire of different-size workers. The
 363 “proprioception, somatosensory processing and kinesthesia” category refers to requirements
 364 above common body and appendage motor control systems across polymorphic workers that
 365 reflect greater kinesthetic coordination than standing, walking without load carriage, feeding,
 366 mouthpart and appendage movement, self-grooming, and allogrooming. Value assignments are
 367 based on published results and personal observations.

Behavioral performance/sensory/cognitive process											Size (HW, mm)						
	Vision	Olfaction	Gustation; Contact chemo- reception	Seismic signal perception	Anemotaxis; air current mechanoreception	Magneto- reception	Thermo- reception	Hygro- reception	Proprioception; Somatosensory processing; Kinesthesia	Sensory integration	0.6	1.2	1.8	2.4	≥3	<u>Ref.</u>	
Foraging																	
Trail pheromone deposition	0	3	1	0	0	0	0	0	2	2	3	3	3	3	0	Evison et al., 2008; Wilson, 1980a	
Search	3	3	1	0	0	2	0	0	1	3	0	1	3	3	0	Camargo, 2015	
Trail navigation and orientation	3	3	0	0	0	2	0	0	1	3	1	2	3	3	1	Banks & Srygley, 2003	
Liquid food collection	0	2	3	0	0	0	0	0	1	2	0	1	3	3	0	Kooij et al., 2016; Rytter & Shik, 2016	
Trail clearing	1	2	0	0	0	0	0	0	3	1	0	0	1	2	3	Dupuis & Harrison, 2017	
Leaf harvesting																	
Leaf-quality assessment	0	3	3	0	0	0	0	1	2	3	0	2	3	3	0	Howard et al., 1988; Hubbell et al., 1984; Thiele et al., 2014	
Short-range recruitment	0	3	3	3	0	0	0	0	2	2	0	2	3	3	0	Markl, 1965; Roces et al., 1993	
Leaf cutting	0	2	3	0	0	0	0	0	3	2	0	2	3	3	0	Nichols- Orians & Schultz, 1989	
Leaf fragment size selection	0	0	0	0	1	0	0	0	3	2	0	2	3	3	0	Segre & Taylor, 2019	
Leaf	1	3	0	0	2	2	0	0	3	3	0	2	3	3	0	Segre &	

fragment transport																Taylor, 2019
Leaf fragment load stabilization	0	0	0	0	3	0	0	0	3	3	0	2	3	3	0	Moll et al., 2013
Leaf fragment transfer	0	1	0	0	1	0	0	0	3	1	1	3	3	3	0	Kwaku et al., 2020
Fungal gardening																
Navigating in fungal comb	0	2	0	0	0	1	0	0	2	2	3	2	1	0	0	Farias et al., 2020
Macerating plant material	0	2	3	0	0	0	0	0	2	1	3	2	1	0	0	Hölldobler & Wilson, 2010
Mulching garden	0	2	2	0	0	0	0	0	2	1	3	2	1	0	0	Khadempour, 2018
Monitoring fungal growth	0	3	3	0	0	0	0	0	0	2	3	2	1	0	0	Green & Kooij, 2018; Khadempour et al., 2020; Römer et al., 2017
Licking mycelium	0	2	3	0	0	0	0	0	0	1	3	2	1	0	0	Wilson, 1983
Fertilizing fungus with feces	0	2	3	0	0	0	0	0	0	1	3	2	1	0	0	Kooij et al., 2016; Mueller, 2002
Removing fungal pathogens	0	2	2	0	0	0	0	0	2	1	3	2	1	0	0	Currie & Stuart, 2001; Goes et al., 2020; Rodrigues et al., 2008
Applying metapleural gland secretion	0	2	2	0	0	0	0	0	1	1	3	2	1	0	0	Poulsen et al., 2002
Applying bacterial antibiotics	0	2	2	0	0	0	0	0	1	1	3	2	1	0	0	Poulsen et al., 2002

Transplanting hyphae	0	2	2	0	0	0	0	0	2	1	3	2	1	0	0	Mueller, 2002
Pruning hyphae	0	2	2	0	0	0	0	0	2	1	3	2	1	0	0	Bass & Cherrett, 1996
Brood care																
Brood sorting	0	3	3	0	0	0	0	0	2	2	2	3	2	0	0	Franks & Sendova-Franks, 1992; Schultner & Pulliainen, 2020
Adjusting brood microclimate	0	0	0	0	0	0	3	3	2	2	2	3	1	0	0	Bollazzi & Roces, 2002; Roces & Núñez, 1995; Römer et al., 2018
Grooming brood	0	3	3	0	0	0	0	0	1	1	2	3	1	0	0	Tragust et al., 2013
Covering brood with mycelia	0	2	2	0	0	0	0	0	1	1	2	3	1	0	0	Armitage et al., 2016
Transporting brood	0	2	0	0	0	1	0	0	2	1	2	3	2	0	0	Römer & Roces, 2014
Feeding larvae	0	2	2	0	0	0	0	0	1	1	2	3	1	0	0	Wilson, 1983
Hygiene																
Removing inactive mycelium	1	3	2	0	0	0	0	0	1	1	2	3	2	0	0	Mueller, 2002
Necrophoresis	1	3	1	0	0	0	0	0	1	1	2	3	2	0	0	Hart & Ratnieks, 2001
Policing waste-worker segregation	0	3	1	0	0	0	0	0	0	1	2	2	2	2	0	Hart & Ratnieks, 2001
Colony security																

Nestmate discrimination	0	3	1	0	0	0	0	0	0	2	2	2	2	2	2	Lenoir et al., 2001
Parasitic fly defense	1	1	0	0	0	0	0	0	2	1	3	2	0	0	0	Feener & Moss, 1990; Orr, 1992
Worker rescue	0	2	0	3	0	0	0	0	3	2	0	2	2	2	0	Roces et al., 1993
Secreting alarm pheromone	0	3	0	0	0	0	0	0	1	2	2	3	3	3	2	Norman et al., 2017
Attacking intruders	3	3	0	0	1	0	0	0	3	2	0	0	2	3	3	Powell & Clark, 2004b

Table 2. Fit of D_w estimates to observed patterns of proportional brain compartment investment.

Linear regression statistics for the fit of a subset of categories of behavioral

performance/sensory/cognitive process as predictors for the brain compartments whose function

most closely corresponds to these demands. Degrees of freedom = 1, 48.

Brain compartment	Multiple R-squared	Adjusted R-squared	F statistic	p value
Total neuropil volume/HW	0.012	-0.008	0.597	0.4434
OL	0.351	0.338	25.990	5.770e-06
AL	0.001	-0.020	0.062	0.805
MB-S	0.186	0.169	10.960	0.002
MB-MC	0.087	0.087	0.068	0.037
MB-LC	0.002	-0.019	0.071	0.791
MB-P	0.155	0.138	8.822	0.005
CX	0.104	0.085	5.559	0.023
SEZ	0.079	0.060	4.129	0.048
ROCB	0.025	0.005	1.250	0.269

Table 3. Differences in the significance of comparisons of brain volume and brain compartment

absolute **a.** and proportional volume **b.** among worker size groups. Degrees of freedom = 4.

a. Absolute total brain and compartmental volume

Brain compartment	Kruskal-Wallis chi-squared	p value
Total of neuropils	17.291	0.002
Total neuropil volume/HW	13.600	0.009
OL	21.014	3.000e-04
AL	18.428	0.001
MB-S	17.592	0.002
MB-MC	17.616	0.002
MB-LC	17.217	0.002
MB-P	16.022	0.003
CX	5.987	0.200
SEZ	17.819	0.001
ROCB	17.048	0.002

b. Proportional brain compartment volumes

Brain compartment	ANOVA F statistic	p value
-------------------	-------------------	---------

AL	2.833	0.035
MB-S	66.222	0.013
MB-MC	1.614	0.187
MB-LC	1.120	0.359
CX	8.299	4.208e-05
SEZ	1.921	0.123
ROCB	3.233	0.021
Brain compartment	Kruskal-Wallis chi-squared	p value
OL	41.367	2.257e-08
MB-P	11.363	0.023

Figure 1. Confocal images of *A. cephalotes* polymorphic worker brains (top row). Structural diagrams of brains illustrating color-coded neuropils whose volumes were measured (middle row; compartments not drawn to scale). Blue = OL, green = AL, orange = MB-MC, red = MB-LC, yellow = MB-P, purple = SEZ, pink = CX. Diagram depicting task performance in relation to worker size (bottom row; minim, two images of medias, major attacking antagonistic species).

Figure 2. Calculated estimates (D_w) for neuronal substrate requirements based on tasks performed by *A. cephalotes* worker size groups. Y-axis values are the sum of each sensory integration/sensorimotor function task score multiplied by the corresponding worker group-size task performance score (see Methods). Local regression curve estimates neuronal substrate requirement score as a function of HW plus HW squared (blue line) and 95% confidence interval (grey band). Heads are drawn to scale.

Figure 4. a. Absolute total brain volume (sum of the OLs, ALs, MB-S, CX, SEZ, and ROCB) across worker size groups ($p = 0.002$). **b.** Total brain volume scaled to body size (total volume/HW for each sample across worker size groups ($p = 0.009$). Y axis notation indicates a given number x10 raised to the power of the number indicated to the right of the e.

Figure 5. a. Absolute volume of the OL ($p = 3.000e-04$); b. AL ($p = 0.001$); c. MB-S ($p = 0.002$); d. MB-MC ($p = 0.001$); e. MB-LC ($p = 0.002$); f. MB-P ($p = 0.003$); g. CX ($p = 0.200$), h. SEZ ($p = 0.001$); and i. ROCB ($p = 0.002$) across worker size groups. Y axis notation indicates volume values $\times 10$ raised to the power of the number indicated to the right of e.

Figure 6. Relative investment (compartment volume as a percent of total brain volume) in the a. OL ($p = 2.257e-08$), AL (b., $p = 0.035$), MB-S (c., $p = 0.013$), MB-MC (d., $p = 0.187$), MB-LC (e., $p = 0.359$), MB-P (f., $p = 0.023$), CX (g., $p = 4.208e-05$), SEZ (h., $p = 0.123$), and ROCB (i., $p = 0.021$) across worker size groups. Y axis as in Figure 5.

423

Figure 7. a. Principal component analysis plot of all log-transformed compartmental volumes normalized to total brain volume. **b.** Linear discriminant analysis of samples according to worker size group (minims: 0.5-0.7mm, medias: 1.1-2.5mm, majors: 3mm+) based on compartmental volumes (excluding the ROCB, which was colinear with other variables) normalized to total brain size. Classification accuracy = 95.8%. Color indicates worker size group (red = 0.6mm HW, gold = 1.2mm, green = 1.8mm, blue = 2.4mm, purple = 3mm+).

Acknowledgements

We gratefully acknowledge the assistance of Dr. Wulfila Gronenberg, Dr. Sam Beshers and Dr. Sean Mullen (manuscript input), Dr. Todd Blute (confocal microscopy), Dr. Christopher Starr and Ricardo Pillai (field work), Andrew Hoadley (colony collection and brain illustrations), and Zach Coto and Frank Azorsa (animal husbandry and manuscript input). This study was supported by National Science Foundation grants IOS 1354291 and IOS 1953393 to JFAT, an award from the Boston University Undergraduate Research Opportunities Program to EMF, and the Department of Biology Brenton R. Lutz Award and the Belamarich Dissertation Writing Award to IBM. Colonies were collected in Trinidad in compliance with the laws of Trinidad and Tobago and imported to USA in compliance with the conditions of permit USDA APHIS P526P-12-04067.

Author contributions

IBM and JFAT designed the study. IM drafted the manuscript. IM, EMF, and JFAT developed and edited the manuscript. IBM and EMF prepared and imaged brains. EMF measured neuropils and IBM and EMF statistically analyzed volumetric data. JFAT secured funding.

References

- Acebes, A., & Ferrús, A. (2001). Increasing the number of synapses modifies olfactory perception in *Drosophila*. *The Journal of Neuroscience*: *The Official Journal of the Society for Neuroscience*, 21(16), 6264–6273. PubMed. <https://doi.org/10.1523/JNEUROSCI.21-16-06264.2001>
- Adolphs, R. (2003). Cognitive neuroscience of human social behaviour. *Nature Reviews Neuroscience*, 4(3), 165–178. <https://doi.org/10.1038/nrn1056>

- 457 Aiello, L. C., & Wheeler, P. (1995). The expensive-tissue hypothesis: The brain and the digestive system
458 in human and primate evolution. *Current Anthropology*, 36(2), 199–221.
459 <https://doi.org/10.1086/204350>
- 460 Amador-Vargas, S., Gronenberg, W., Wcislo, W. T., & Mueller, U. (2015). Specialization and group size:
461 Brain and behavioural correlates of colony size in ants lacking morphological castes. *Proceedings*
462 *of the Royal Society B: Biological Sciences*, 282(1801), 20142502.
463 <https://doi.org/10.1098/rspb.2014.2502>
- 464 Arenas, A., & Roces, F. (2016a). Gardeners and midden workers in leaf-cutting ants learn to avoid plants
465 unsuitable for the fungus at their worksites. *Animal Behaviour*, 115, 167–174.
466 <https://doi.org/10.1016/j.anbehav.2016.03.016>
- 467 Arenas, A., & Roces, F. (2016b). Learning through the waste: Olfactory cues from the colony refuse
468 influence plant preferences in foraging leaf-cutting ants. *The Journal of Experimental Biology*,
469 219(16), 2490–2496. <https://doi.org/10.1242/jeb.139568>
- 470 Arenas, A., & Roces, F. (2017). Avoidance of plants unsuitable for the symbiotic fungus in leaf-cutting
471 ants: Learning can take place entirely at the colony dump. *PLOS ONE*, 12(3), e0171388.
472 <https://doi.org/10.1371/journal.pone.0171388>
- 473 Arganda, S., Hoadley, A. P., Razdan, E. S., Muratore, I. B., & Traniello, J. F. A. (2020). The
474 neuroplasticity of division of labor: Worker polymorphism, compound eye structure and brain
475 organization in the leafcutter ant *Atta cephalotes*. *Journal of Comparative Physiology A*.
476 <https://doi.org/10.1101/2020.03.04.975110>
- 477 Armitage, S. A. O., Fernández-Marín, H., Boomsma, J. J., & Wcislo, W. T. (2016). Slowing them down
478 will make them lose: A role for attine ant crop fungus in defending pupae against infections? *The*
479 *Journal of Animal Ecology*, 85(5), 1210–1221. <https://doi.org/10.1111/1365-2656.12543>
- 480 Banks, A. N., & Srygley, R. B. (2003). Orientation by magnetic field in leaf-cutter ants, *Atta colombica*
481 (Hymenoptera: Formicidae). *Ethology*, 109(10), 835–846. <https://doi.org/10.1046/j.0179-1613.2003.00927.x>
482

- 483 Bass, M., & Cherrett, J. M. (1996). Leaf-cutting ants (formicidae, attini) prune their fungus to increase
484 and direct its productivity. *Functional Ecology*, 10(1), 55–61. <https://doi.org/10.2307/2390262>
- 485 Beshers, S. N., & Fewell, J. H. (2001). Models of division of labor in social insects. *Annual Review of*
486 *Entomology*, 46(1), 413–440. <https://doi.org/10.1146/annurev.ento.46.1.413>
- 487 Beutel, R. G., Pohl, H., & Hünefeld, F. (2005). Strepsipteran brains and effects of miniaturization
488 (Insecta). *Arthropod Structure & Development*, 34(3), 301–313.
489 <https://doi.org/10.1016/j.asd.2005.03.001>
- 490 Blanton, C. M., & Ewel, J. J. (1985). Leaf-cutting ant herbivory in successional and agricultural tropical
491 ecosystems. *Ecology*, 66(3), 861–869. <https://doi.org/10.2307/1940548>
- 492 Bollazzi, M., & Roces, F. (2002). Thermal preference for fungus culturing and brood location by workers
493 of the thatching grass-cutting ant *Acromyrmex heyeri*. *Insectes Sociaux*, 49(2), 153–157.
494 <https://doi.org/10.1007/s00040-002-8295-x>
- 495 Boogert, N. J., Madden, J. R., Morand-Ferron, J., & Thornton, A. (2018). Measuring and understanding
496 individual differences in cognition. *Philosophical Transactions of the Royal Society B: Biological*
497 *Sciences*, 373(1756), 20170280. <https://doi.org/10.1098/rstb.2017.0280>
- 498 Camargo, R. da S. (2015). Foraging behavior of leaf cutting ants: How do workers search for their food?
499 *Sociobiology*, 62(3), 347–350. <https://doi.org/10.13102/sociobiology.v62i3.714>
- 500 Chittka, L., & Niven, J. (2009). Are bigger brains better? *Current Biology*, 19(21), R995–R1008.
501 <https://doi.org/10.1016/j.cub.2009.08.023>
- 502 Currie, C. R., & Stuart, A. E. (2001). Weeding and grooming of pathogens in agriculture by ants.
503 *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 268(1471), 1033–
504 1039. <https://doi.org/10.1098/rspb.2001.1605>
- 505 Currier, T. A., Matheson, A. M., & Nagel, K. I. (2020). Encoding and control of orientation to airflow by
506 a set of *Drosophila* fan-shaped body neurons. *ELife*, 9, e61510.
507 <https://doi.org/10.7554/eLife.61510>

- DeCasien, A. R., & Higham, J. P. (2019). Primate mosaic brain evolution reflects selection on sensory and cognitive specialization. *Nature Ecology & Evolution*, 3(10), 1483–1493.
<https://doi.org/10.1038/s41559-019-0969-0>
- DeCasien, A. R., Williams, S. A., & Higham, J. P. (2017). Primate brain size is predicted by diet but not sociality. *Nature Ecology & Evolution*, 1(5). <https://doi.org/10.1038/s41559-017-0112>
- Dunbar, R. I. M. (1998). The social brain hypothesis. *Evolutionary Anthropology: Issues, News, and Reviews*, 6(5), 178–190. [https://doi.org/10.1002/\(sici\)1520-6505\(1998\)6:5<178::aid-evan5>3.0.co;2-8](https://doi.org/10.1002/(sici)1520-6505(1998)6:5<178::aid-evan5>3.0.co;2-8)
- Dunbar, R. I. M. (2009). The social brain hypothesis and its implications for social evolution. *Annals of Human Biology*, 36(5), 562–572. <https://doi.org/10.1080/03014460902960289>
- Dupuis, E. C., & Harrison, J. F. (2017). Trunk trail maintenance in leafcutter ants: Caste involvement and effects of obstacle type and size on path clearing in *Atta cephalotes*. *Insectes Sociaux*, 64(2), 189–196. <https://doi.org/10.1007/s00040-016-0530-y>
- Durst, C., Eichmüller, S., & Menzel, R. (1994). Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. *Behavioral and Neural Biology*, 62(3), 259–263. [https://doi.org/10.1016/s0163-1047\(05\)80025-1](https://doi.org/10.1016/s0163-1047(05)80025-1)
- Evison, S. E. F., Hart, A. G., & Jackson, D. E. (2008). Minor workers have a major role in the maintenance of leafcutter ant pheromone trails. *Animal Behaviour*, 75(3), 963–969.
<https://doi.org/10.1016/j.anbehav.2007.07.013>
- Fahrbach, S. E. (2006). Structure of the mushroom bodies of the insect brain. *Annual Review of Entomology*, 51(1), 209–232. <https://doi.org/10.1146/annurev.ento.51.110104.150954>
- Falibene, A., Roces, F., & Rössler, W. (2015). Long-term avoidance memory formation is associated with a transient increase in mushroom body synaptic complexes in leaf-cutting ants. *Frontiers in Behavioral Neuroscience*, 9, 84–84. PubMed. <https://doi.org/10.3389/fnbeh.2015.00084>

- 532 Farias, A. P., Camargo, R. da S., Andrade Sousa, K. K., Caldato, N., & Forti, L. C. (2020). Nest
533 architecture and colony growth of *Atta bisphaerica* grass-cutting ants. *Insects*, 11(11), 741.
534 <https://doi.org/10.3390/insects11110741>
- 535 Farris, S. M. (2011). Are mushroom bodies cerebellum-like structures? *Arthropod Structure &*
536 *Development*, 40(4), 368–379. <https://doi.org/10.1016/j.asd.2011.02.004>
- 537 Farris, S. M., & Roberts, N. S. (2005). Coevolution of generalist feeding ecologies and gyrencephalic
538 mushroom bodies in insects. *Proceedings of the National Academy of Sciences*, 102(48), 17394–
539 17399. <https://doi.org/10.1073/pnas.0508430102>
- 540 Feener, D. H., & Moss, K. A. G. (1990). Defense against parasites by hitchhikers in leaf-cutting ants: A
541 quantitative assessment. *Behavioral Ecology and Sociobiology*, 26(1), 17–29.
542 <https://doi.org/10.1007/bf00174021>
- 543 Feinerman, O., & Traniello, J. F. A. (2015). Social complexity, diet, and brain evolution: Modeling the
544 effects of colony size, worker size, brain size, and foraging behavior on colony fitness in ants.
545 *Behavioral Ecology and Sociobiology*, 70(7), 1063–1074. [https://doi.org/10.1007/s00265-015-](https://doi.org/10.1007/s00265-015-2035-5)
546 2035-5
- 547 Finlay, B., & Darlington, R. (1995). Linked regularities in the development and evolution of mammalian
548 brains. *Science*, 268(5217), 1578–1584. <https://doi.org/10.1126/science.7777856>
- 549 Franks, N. R., & Sendova-Franks, A. B. (1992). Brood sorting by ants: Distributing the workload over the
550 work-surface. *Behavioral Ecology and Sociobiology*, 30(2), 109–123.
551 <https://doi.org/10.1007/BF00173947>
- 552 Godfrey, R. K., & Gronenberg, W. (2019). Brain evolution in social insects: Advocating for the
553 comparative approach. *Journal of Comparative Physiology A*, 205(1), 13–32.
554 <https://doi.org/10.1007/s00359-019-01315-7>
- 555 Goes, A. C., Barcoto, M. O., Kooij, P. W., Bueno, O. C., & Rodrigues, A. (2020). How do leaf-cutting
556 ants recognize antagonistic microbes in their fungal crops? *Frontiers in Ecology and Evolution*, 8.
557 <https://doi.org/10.3389/fevo.2020.00095>

- Gordon, D. G., Ilieş, I., & Traniello, J. F. A. (2017). Behavior, brain, and morphology in a complex insect society: Trait integration and social evolution in the exceptionally polymorphic ant *Pheidole rhea*. *Behavioral Ecology and Sociobiology*, 71(11), 166. <https://doi.org/10.1007/s00265-017-2396-z>
- Green, J., Vijayan, V., Mussells Pires, P., Adachi, A., & Maimon, G. (2019). A neural heading estimate is compared with an internal goal to guide oriented navigation. *Nature Neuroscience*, 22(9), 1460–1468. <https://doi.org/10.1038/s41593-019-0444-x>
- Green, P. W. C., & Kooij, P. W. (2018). The role of chemical signalling in maintenance of the fungus garden by leaf-cutting ants. *Chemoecology*, 28(3), 101–107. <https://doi.org/10.1007/s00049-018-0260-x>
- Groh, C., Kelber, C., Grübel, K., & Rössler, W. (2014). Density of mushroom body synaptic complexes limits intraspecies brain miniaturization in highly polymorphic leaf-cutting ant workers. *Proceedings of the Royal Society B: Biological Sciences*, 281(1785), 20140432–20140432. PubMed. <https://doi.org/10.1098/rspb.2014.0432>
- Gronenberg, W. (1999). Modality-specific segregation of input to ant mushroom bodies. *Brain, Behavior and Evolution*, 54(2), 85–95. <https://doi.org/10.1159/000006615>
- Gronenberg, W. (2001). Subdivisions of hymenopteran mushroom body calyces by their afferent supply. *Journal of Comparative Neurology*, 435(4), 474–489. <https://doi.org/10.1002/cne.1045>
- Gronenberg, W., Heeren, S., & Hölldobler, B. (1996). Age-dependent and task-related morphological changes in the brain and the mushroom bodies of the ant *Camponotus floridanus*. *Journal of Experimental Biology*, 199(9), 2011–2019.
- Gronenberg, W., & Riveros, A. J. (2009). Social brains and behavior: Past and present. *Organization of Insect Societies From Genome to Sociocomplexity*.
- Groothuis, J., & Smid, H. M. (2017). Nasonia Parasitic Wasps Escape from Haller’s Rule by Diphasic, Partially Isometric Brain-Body Size Scaling and Selective Neuropil Adaptations. *Brain, Behavior and Evolution*, 90(3), 243–254. <https://doi.org/10.1159/000480421>

Hart, A. G., & Ratnieks, F. L. W. (2001). Task partitioning, division of labour and nest compartmentalisation collectively isolate hazardous waste in the leafcutting ant *Atta cephalotes*. *Behavioral Ecology and Sociobiology*, 49(5), 387–392. <https://doi.org/10.1007/s002650000312>

Heisenberg, M. (1998). What do the mushroom bodies do for the insect brain? An introduction. *Learning & Memory*, 5(1), 1–10. <https://doi.org/10.1101/lm.8.1.1>

Herculano-Houzel, S. (2012). The remarkable, yet not extraordinary, human brain as a scaled-up primate brain and its associated cost. *Proceedings of the National Academy of Sciences*, 109(Supplement 1), 10661–10668.

Herculano-Houzel, S., Manger, P. R., & Kaas, J. H. (2014). Brain scaling in mammalian evolution as a consequence of concerted and mosaic changes in numbers of neurons and average neuronal cell size. *Frontiers in Neuroanatomy*, 8. <https://doi.org/10.3389/fnana.2014.00077>

Hölldobler, B., & Wilson, E. O. (1990). *The Ants*. Harvard University Press.

Hölldobler, B., & Wilson, E. O. (2010). *The leafcutter ants: Civilization by instinct*. W. W. Norton & Company.

Howard, J. J. (1987). Leafcutting ant diet selection: The role of nutrients, water, and secondary chemistry. *Ecology*, 68(3), 503–515. <https://doi.org/10.2307/1938455>

Howard, J. J., Cazin, J., & Wiemer, D. F. (1988). Toxicity of terpenoid deterrents to the leafcutting ant *Atta cephalotes* and its mutualistic fungus. *Journal of Chemical Ecology*, 14(1), 59–69. <https://doi.org/10.1007/bf01022531>

Howard, J. J., Henneman, L. M., Cronin, G., Fox, J. A., & Hormiga, G. (1996). Conditioning of scouts and recruits during foraging by a leaf-cutting ant, *Atta colombica*. *Animal Behaviour*, 52(2), 299–306. <https://doi.org/10.1006/anbe.1996.0175>

Hubbell, S. P., Howard, J. J., & Wiemer, D. F. (1984). Chemical leaf repellency to an attine ant: Seasonal distribution among potential host plant species. *Ecology*, 65(4), 1067–1076. <https://doi.org/10.2307/1938314>

- Hubbell, S. P., Wiemer, D. F., & Adejare, A. (1983). An antifungal terpenoid defends a neotropical tree (Hymenaea) against attack by fungus-growing ants (*Atta*). *Oecologia*, 60(3), 321–327.
<https://doi.org/10.1007/bf00376846>
- Ilies, I., Muscedere, M. L., & Traniello, J. F. A. (2015). Neuroanatomical and morphological trait clusters in the ant genus *Pheidole*: Evidence for modularity and integration in brain structure. *Brain, Behavior and Evolution*, 85(1), 63–76. <https://doi.org/10.1159/000370100>
- Jaffe, K., & Perez, E. (1989). Comparative study of brain morphology in ants. *Brain, Behavior and Evolution*, 33(1), 25–33. <https://doi.org/10.1159/000115895>
- Kamhi, J. F., Barron, A. B., & Narendra, A. (2020). Vertical lobes of the mushroom bodies are essential for view-based navigation in australian *Myrmecia* ants. *Current Biology*, 30(17), 3432-3437.e3.
<https://doi.org/10.1016/j.cub.2020.06.030>
- Kamhi, J. F., Gronenberg, W., Robson, S. K. A., & Traniello, J. F. A. (2016). Social complexity influences brain investment and neural operation costs in ants. *Proceedings of the Royal Society B: Biological Sciences*, 283(1841), 20161949. PubMed. <https://doi.org/10.1098/rspb.2016.1949>
- Kamhi, J. F., Ilies, I., & Traniello, J. F. A. (2019). Social complexity and brain evolution: Comparative analysis of modularity and integration in ant brain organization. *Brain, Behavior and Evolution*, 93(1), 4–18. <https://doi.org/10.1159/000497267>
- Kelber, C., Rössler, W., & Kleineidam, C. J. (2009). Phenotypic plasticity in number of glomeruli and sensory innervation of the antennal lobe in leaf-cutting ant workers (*A. vollenweideri*). *Developmental Neurobiology*, 70(4), 222–234. <https://doi.org/10.1002/dneu.20782>
- Khadempour, L. (2018). *Microbial mediation of herbivory in leaf-cutter ant fungus gardens* [Ph.D.]. The University of Wisconsin - Madison.
- Khadempour, L., Kyle, J. E., Webb-Robertson, B.-J. M., Nicora, C. D., Smith, F. B., Smith, R. D., Lipton, M. S., Currie, C. R., Baker, E. S., & Burnum-Johnson, K. E. (2020). From plants to ants: Fungal modification of leaf lipids for nutrition and communication in the leaf-cutter ant fungal garden ecosystem. *BioRxiv*, 2020.07.28.224139. <https://doi.org/10.1101/2020.07.28.224139>

- Khalife, A., Keller, R. A., Billen, J., Hita Garcia, F., Economo, E. P., & Peeters, C. (2018). Skeletomuscular adaptations of head and legs of *Melissotarsus* ants for tunnelling through living wood. *Frontiers in Zoology*, 15(1), 30. <https://doi.org/10.1186/s12983-018-0277-6>
- Kleineidam, C. J., Obermayer, M., Halbach, W., & Rössler, W. (2005). A macroglomerulus in the antennal lobe of leaf-cutting ant workers and its possible functional significance. *Chemical Senses*, 30(5), 383–392. <https://doi.org/10.1093/chemse/bji033>
- Kooij, P. W., Pullens, J. W. M., Boomsma, J. J., & Schiøtt, M. (2016). Ant mediated redistribution of a xyloglucanase enzyme in fungus gardens of *Acromyrmex echinator*. *BMC Microbiology*, 16(1), 81. <https://doi.org/10.1186/s12866-016-0697-4>
- Kuebler, L. S., Kelber, C., & Kleineidam, C. J. (2010). Distinct antennal lobe phenotypes in the leaf-cutting ant (*Atta vollenweideri*). *The Journal of Comparative Neurology*, 518(3), 352–365. <https://doi.org/10.1002/cne.22217>
- Kwaku, K. M., Gonick, E. A., Ostapovich, E. M., & Weinberg, I. P. (2020). The frequency of leaf transfer in *Atta cephalotes* along horizontal and vertical surfaces near the bases of trees. *Insectes Sociaux*, 67(4), 481–486. <https://doi.org/10.1007/s00040-020-00784-3>
- Le Moël, F., Stone, T., Lihoreau, M., Wystrach, A., & Webb, B. (2019). The central complex as a potential substrate for vector based navigation. *Frontiers in Psychology*, 10. <https://doi.org/10.3389/fpsyg.2019.00690>
- Lenoir, A., D’Ettorre, P., Errard, C., & Hefetz, A. (2001). Chemical ecology and social parasitism in ants. *Annual Review of Entomology*, 46(1), 573–599. <https://doi.org/10.1146/annurev.ento.46.1.573>
- Liaw, A., & Wiener, M. (2001). Classification and regression by randomforest. *Forest*, 23.
- Lihoreau, M., Dubois, T., Gomez-Moracho, T., Kraus, S., Monchanin, C., & Pasquaretta, C. (2019). Putting the ecology back into insect cognition research. *Advances in Insect Physiology*, 1–25. <https://doi.org/10.1016/bs.aiip.2019.08.002>
- Lihoreau, M., Latty, T., & Chittka, L. (2012). An exploration of the social brain hypothesis in insects. *Frontiers in Physiology*, 3, 442–442. PubMed. <https://doi.org/10.3389/fphys.2012.00442>

- Logan, C., Avin, S., Boogert, N., Buskell, A., Cross, F. R., Currie, A., Jelbert, S., Lukas, D., Mares, R., Navarrete, A. F., Shigeno, S., & Montgomery, S. (2018). *Beyond brain size: Uncovering the neural correlates of behavioral and cognitive specialization*. <https://doi.org/10.17863/CAM.25916>
- Markl, H. (1965). Stridulation in leaf-cutting ants. *Science (New York, N.Y.)*, 149(3690), 1392–1393. <https://doi.org/10.1126/science.149.3690.1392>
- Moll, K., Roces, F., & Federle, W. (2013). How load-carrying ants avoid falling over: Mechanical stability during foraging in *Atta vollenweideri* grass-cutting ants. *PloS One*, 8(1), e52816. <https://doi.org/10.1371/journal.pone.0052816>
- Mueller, U. G. (2002). Ant versus fungus versus mutualism: Ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. *The American Naturalist*, 160 Suppl 4, S67-98. <https://doi.org/10.1086/342084>
- Muratore, I. B., Ilieș, I., Huzar, A. K., Zaidi, F. H., & Traniello, J. F. A. (in prep.). *Morphological scaling and division of labor in a socially complex agricultural ant*.
- Muratore, I. B., & Traniello, J. F. A. (2020). Fungus-growing ants: Models for the integrative analysis of cognition and brain evolution. *Frontiers in Behavioral Neuroscience*, 14, 599234–599234. PubMed. <https://doi.org/10.3389/fnbeh.2020.599234>
- Muscedere, M. L., Gronenberg, W., Moreau, C. S., & Traniello, J. F. A. (2014). Investment in higher order central processing regions is not constrained by brain size in social insects. *Proceedings of the Royal Society B: Biological Sciences*, 281(1784), 20140217. <https://doi.org/10.1098/rspb.2014.0217>
- Muscedere, M. L., & Traniello, J. F. A. (2012). Division of labor in the hyperdiverse ant genus *Pheidole* is associated with distinct subcaste- and age-related patterns of worker brain organization. *PloS One*, 7(2), e31618–e31618. PubMed. <https://doi.org/10.1371/journal.pone.0031618>
- Navarrete, A. F., Reader, S. M., Street, S. E., Whalen, A., & Laland, K. N. (2016). The coevolution of innovation and technical intelligence in primates. *Philosophical Transactions of the Royal Society*

- of London. *Series B, Biological Sciences*, 371(1690), 20150186. PubMed.
<https://doi.org/10.1098/rstb.2015.0186>
- Nichols-Orians, C. M., & Schultz, J. C. (1989). Leaf toughness affects leaf harvesting by the leaf cutter ant, *Atta cephalotes* (L.) (Hymenoptera: Formicidae). *Biotropica*, 21(1), 80–83.
<https://doi.org/10.2307/2388446>
- Niven, J. E., & Farris, S. M. (2012). Miniaturization of nervous systems and neurons. *Current Biology*, 22(9), R323–R329. <https://doi.org/10.1016/j.cub.2012.04.002>
- Norman, V. C., Butterfield, T., Drijfhout, F., Tasman, K., & Hughes, W. O. H. (2017). Alarm pheromone composition and behavioral activity in fungus-growing ants. *Journal of Chemical Ecology*, 43(3), 225–235. <https://doi.org/10.1007/s10886-017-0821-4>
- O'Donnell, S., Bulova, S., Barrett, M., & von Beeren, C. (2018). Brain investment under colony-level selection: Soldier specialization in *Eciton* army ants (Formicidae: Dorylinae). *BMC Zoology*, 3(1), 3. <https://doi.org/10.1186/s40850-018-0028-3>
- O'Donnell, S., Bulova, S. J., Barrett, M., & Fiocca, K. (2018). Size constraints and sensory adaptations affect mosaic brain evolution in paper wasps (Vespidae: Epiponini). *Biological Journal of the Linnean Society*, 123(2), 302–310. <https://doi.org/10.1093/biolinnean/blx150>
- O'Donnell, S., Bulova, S. J., DeLeon, S., Khodak, P., Miller, S., & Sulger, E. (2015). Distributed cognition and social brains: Reductions in mushroom body investment accompanied the origins of sociality in wasps (Hymenoptera: Vespidae). *Proceedings of the Royal Society B: Biological Sciences*, 282(1810), 20150791. PubMed. <https://doi.org/10.1098/rspb.2015.0791>
- Orr, M. R. (1992). Parasitic flies (Diptera: Phoridae) influence foraging rhythms and caste division of labor in the leaf-cutter ant, *Atta cephalotes* (Hymenoptera: Formicidae). *Behavioral Ecology and Sociobiology*, 30(6), 395–402. <https://doi.org/10.1007/BF00176174>
- Packard, G. C. (2020). Rethinking the metabolic allometry of ants. *Evolutionary Ecology*, 34(2), 149–161. <https://doi.org/10.1007/s10682-020-10033-5>

- 712 Pisokas, I., Heinze, S., & Webb, B. (2020). The head direction circuit of two insect species. *ELife*, 9,
713 e53985. PubMed. <https://doi.org/10.7554/eLife.53985>
- 714 Poulsen, M., Bot, A. N., Nielsen, M. G., & Boomsma, J. J. (2002). Experimental evidence for the costs
715 and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants.
716 *Behavioral Ecology and Sociobiology*, 52(2), 151–157. [https://doi.org/10.1007/s00265-002-0489-](https://doi.org/10.1007/s00265-002-0489-8)
717 8
- 718 Powell, S., & Clark, E. (2004a). Combat between large derived societies: A subterranean army ant
719 established as a predator of mature leaf-cutting ant colonies. *Insectes Sociaux*, 4(51), 342–351.
720 <https://doi.org/10.1007/s00040-004-0752-2>
- 721 Powell, S., & Clark, E. (2004b). Combat between large derived societies: A subterranean army ant
722 established as a predator of mature leaf-cutting ant colonies. *Insectes Sociaux*, 51(4), 342–351.
723 <https://doi.org/10.1007/s00040-004-0752-2>
- 724 Rensch, B. (1956). Increase of learning capability with increase of brain-size. *The American Naturalist*,
725 90(851), 81–95. <https://doi.org/10.1086/281911>
- 726 Reséndiz-Benhumea, G. M., Sangati, E., Sangati, F., Keshmiri, S., & Froese, T. (2021). Shrunk Social
727 Brains? A Minimal Model of the Role of Social Interaction in Neural Complexity. *Frontiers in*
728 *Neurorobotics*, 15. <https://doi.org/10.3389/fnbot.2021.634085>
- 729 Riveros, A. J., & Gronenberg, W. (2010). Brain allometry and neural plasticity in the bumblebee *Bombus*
730 *occidentalis*. *Brain, Behavior and Evolution*, 75(2), 138–148. PubMed.
731 <https://doi.org/10.1159/000306506>
- 732 Riveros, A. J., Seid, M. A., & Wcislo, W. T. (2012). Evolution of brain size in class-based societies of
733 fungus-growing ants (Attini). *Animal Behaviour*, 83(4), 1043–1049.
734 <https://doi.org/10.1016/j.anbehav.2012.01.032>
- 735 Rocas, F., & Núñez, JosuéA. (1995). Thermal sensitivity during brood care in workers of two
736 *Camponotus* ant species: Circadian variation and its ecological correlates. *Journal of Insect*
737 *Physiology*, 41(8), 659–669. [https://doi.org/10.1016/0022-1910\(95\)00019-Q](https://doi.org/10.1016/0022-1910(95)00019-Q)

- Roces, F., Tautz, J., & Hölldobler, B. (1993). Stridulation in leaf-cutting ants. *Naturwissenschaften*, 80(11), 521–524. <https://doi.org/10.1007/BF01140810>
- Rodrigues, A., Bacci Jr, M., Mueller, U. G., Ortiz, A., & Pagnocca, F. C. (2008). Microfungal “weeds” in the leafcutter ant symbiosis. *Microbial Ecology*, 56(4), 604–614. <https://doi.org/10.1007/s00248-008-9380-0>
- Römer, D., Bollazzi, M., & Roces, F. (2017). Carbon dioxide sensing in an obligate insect-fungus symbiosis: CO₂ preferences of leaf-cutting ants to rear their mutualistic fungus. *PLOS ONE*, 12(4), e0174597. <https://doi.org/10.1371/journal.pone.0174597>
- Römer, D., Bollazzi, M., & Roces, F. (2018). Carbon dioxide sensing in the social context: Leaf-cutting ants prefer elevated CO₂ levels to tend their brood. *Journal of Insect Physiology*, 108, 40–47. <https://doi.org/10.1016/j.jinsphys.2018.05.007>
- Römer, D., & Roces, F. (2014). Nest enlargement in leaf-cutting ants: Relocated brood and fungus trigger the excavation of new chambers. *PLoS One*, 9(5), e97872. <https://doi.org/10.1371/journal.pone.0097872>
- Rytter, W., & Shik, J. Z. (2016). Liquid foraging behaviour in leafcutting ants: The lunchbox hypothesis. *Animal Behaviour*, 117, 179–186. <https://doi.org/10.1016/j.anbehav.2016.04.022>
- Saverschek, N., & Roces, F. (2011). Foraging leafcutter ants: Olfactory memory underlies delayed avoidance of plants unsuitable for the symbiotic fungus. *Animal Behaviour*, 82(3), 453–458. <https://doi.org/10.1016/j.anbehav.2011.05.015>
- Sayol, F., Collado, M. Á., Garcia-Porta, J., Seid, M. A., Gibbs, J., Agorreta, A., San Mauro, D., Raemakers, I., Sol, D., & Bartomeus, I. (2020). Feeding specialization and longer generation time are associated with relatively larger brains in bees. *Proceedings of the Royal Society B: Biological Sciences*, 287(1935), 20200762–20200762. PubMed. <https://doi.org/10.1098/rspb.2020.0762>
- Schultner, E., & Pulliainen, U. (2020). Brood recognition and discrimination in ants. *Insectes Sociaux*, 67(1), 11–34. <https://doi.org/10.1007/s00040-019-00747-3>

- Segre, P. S., & Taylor, E. D. (2019). Large ants do not carry their fair share: Maximal load-carrying performance of leaf-cutter ants (*Atta cephalotes*). *Journal of Experimental Biology*, 222(12). <https://doi.org/10.1242/jeb.199240>
- Seid, M. A., Castillo, A., & Wcislo, W. T. (2011). The allometry of brain miniaturization in ants. *Brain, Behavior and Evolution*, 77(1), 5–13. <https://doi.org/10.1159/000322530>
- Shiozaki, H. M., Ohta, K., & Kazama, H. (2020). A Multi-regional Network Encoding Heading and Steering Maneuvers in *Drosophila*. *Neuron*, 106(1), 126-141.e5. <https://doi.org/10.1016/j.neuron.2020.01.009>
- Simons, M., & Tibbetts, E. (2019). Insects as models for studying the evolution of animal cognition. *Current Opinion in Insect Science*, 34, 117–122. <https://doi.org/10.1016/j.cois.2019.05.009>
- Smaers, J. B., & Soligo, C. (2013). Brain reorganization, not relative brain size, primarily characterizes anthropoid brain evolution. *Proceedings of the Royal Society B: Biological Sciences*, 280(1759), 20130269. <https://doi.org/10.1098/rspb.2013.0269>
- Strausfeld, N. J. (2012). *Arthropod Brains*.
- Sulger, E., McAloon, N., Bulova, S. J., Sapp, J., & O'Donnell, S. (2014). Evidence for adaptive brain tissue reduction in obligate social parasites (*Polyergus mexicanus*) relative to their hosts (*Formica fusca*). *Biological Journal of the Linnean Society*, 113(2), 415–422. <https://doi.org/10.1111/bij.12375>
- Sun, X., Yue, S., & Mangan, M. (2020). *Author response: A decentralised neural model explaining optimal integration of navigational strategies in insects*. <https://doi.org/10.7554/elife.54026.sa2>
- Thiele, T., Kost, C., Roces, F., & Wirth, R. (2014). Foraging leaf-cutting ants learn to reject *Vitis vinifera* ssp. *Vinifera* plants that emit herbivore-induced volatiles. *Journal of Chemical Ecology*, 40(6), 617–620. <https://doi.org/10.1007/s10886-014-0460-y>
- Tragust, S., Ugelvig, L. V., Chapuisat, M., Heinze, J., & Cremer, S. (2013). Pupal cocoons affect sanitary brood care and limit fungal infections in ant colonies. *BMC Evolutionary Biology*, 13, 225. <https://doi.org/10.1186/1471-2148-13-225>

- van der Woude, E., & Smid, H. M. (2016). How to escape from haller's rule: Olfactory system complexity in small and large *Trichogramma evanescens* parasitic wasps. *Journal of Comparative Neurology*, 524(9), 1876–1891. <https://doi.org/10.1002/cne.23927>
- van der Woude, E., Smid, H. M., Chittka, L., & Huigens, M. E. (2013). Breaking Haller's Rule: Brain-Body Size Isometry in a Minute Parasitic Wasp. *Brain, Behavior and Evolution*, 81(2), 86–92. <https://doi.org/10.1159/000345945>
- Wartel, A., Lindenfors, P., & Lind, J. (2019). Whatever you want: Inconsistent results are the rule, not the exception, in the study of primate brain evolution. *PLOS ONE*, 14(7), e0218655. <https://doi.org/10.1371/journal.pone.0218655>
- Wehner, R., Fukushima, T., & Isler, K. (2007). On being small: Brain allometry in ants. *Brain, Behavior and Evolution*, 69(3), 220–228. <https://doi.org/10.1159/000097057>
- Wilson, E. O. (1980a). Caste and division of labor in leaf-cutter ants (Hymenoptera, Formicidae, *Atta*). 1. The overall pattern in *Atta-Sexdens*. *Behavioral Ecology and Sociobiology*, 7(2), 143–156. <https://doi.org/10.1007/BF00299520>
- Wilson, E. O. (1980b). Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*) II. The ergonomic optimization of leaf cutting. *Behavioral Ecology and Sociobiology*, 7(2), 157–165. <https://doi.org/10.1007/bf00299521>
- Wilson, E. O. (1983). Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). *Behavioral Ecology and Sociobiology*, 14(1), 55–60. <https://doi.org/10.1007/BF00366656>