Supplementary Materials

Supplementary Methods

Task-specialization. In 17 colonies living on acacia trees with a range of 5 - 113 number of spines (hollow thorns used by the ants for nesting), we marked a total of 2688 ants, with an average of 158 ants marked per colony, and with approximately half of the ants from each task-class per colony (detailed sample sizes in Table S1). We marked ants with a small dot of non-toxic odorless paint, following [1], and we used location-specific colors, that is, different colors for workers found on the leaves versus the trunk base (hereafter called leaf-ants and trunk-ants, respectively). After 24 hours, we revisited the colonies and recorded the number of ants of each type of mark that were found in the two tree locations (trunk base and leaves), by scanning for ants on the trunk base (i.e., from the ground to the first branch of the host-tree), and then scanning for ants on the leaves with Beltian bodies. We only counted ants performing foraging or defense behaviors, and did not count ants that were walking. Counting the same ant twice was unlikely, because trunk ants are usually standing still, and ants collecting Beltian bodies spend some time detaching it from the leaflet and then move to a spine to store the Beltian body. All trees were less than 2 m in height, so all branch tips could be easily observed.

From these worker counts, we calculated measures of foraging and defense specialization as the number of ants with a color-mark found in the same location (trunk, leaves) where they were originally marked, divided by the total number of observed marked ants from that color-mark, then multiplied by 100. This measure is the percentage of marked ants that were found engaged in the same task on two consecutive days (i.e., percentage not switching tasks). To assess the accuracy of these estimates based on two counts, in another four colonies we marked workers in the morning of one day (sample sizes were: 50 leaf-ants and 36 trunk-ants in colony 1; 40 and 30 in colony 2; 50 and 60 in colony 3; 290 and 280 in colony 4), and re-censused them three more times: in the afternoon of the same day (~5 hours later), and during the mornings of the following two days (approximately 24 and 48 hours after marking). We calculated the standard deviation among those three estimates of each specialization for each of the four colonies. Across colonies, we found a mean standard deviation of 5% ± 2% for defense specialization, and of 4% ± 2% for foraging specialization. Estimates of task-specialization percentages reported in this study of the 17

studied colonies (based on a single observation 24 hours after marking) therefore have approximately a 5% margin of error.

Colony size. We used tree diameter and number of occupied spines (spines with entrance hole) as estimations of colony size. Two observers independently counted the number of spines of all trees; if these two counts differed, the count was repeated until both observers agreed. Using the records of marked ants (Table S1), we estimated the number of adult workers working outside the spines with the Petersen method of mark and recapture [2]. Estimates of number of outside workers ranged between 14 and 700 ants. Because data did not fit a normal distribution, we used a non-parametric Spearman correlation-analysis to evaluate the correlations between task-specialization percentages (as defined above) and three proxy-measures of colony size (number of spines on tree, tree diameter, and estimated number of workers outside spines). All Spearman correlations were calculated with the package pspearman [3] for R statistics software [4].

To assess the effect of colony size and task-specialization on brain morphology, we first summarized colony size-related traits in a single variable called "colony size-related traits", defined as the first factor of a principal component analysis (PCA) including: (a) defense specialization; (b) foraging specialization; (c) number of outside workers; and (d) number of tree spines. This first factor of the PCA explained 56% of the variation between colonies (Table S2). We multiplied values of the first factor by minus 1, such that larger numbers indicate larger colonies on larger trees.

Behavioral assays. To test whether differences in behavior between leaf- and trunk-ants increased with colony size, we assessed the reactions of marked ants towards two stimuli: food (sample size: 27 ± 11 SD ants per colony, for a total of 117 leaf-ants and 97 trunk-ants from nine colonies) and intruders (sample size: 22 ± 6 SD ants per colony, for a total of 81 leaf-ants and 53 trunk-ants from seven colonies). We tested the reaction towards food by placing a Beltian body attached to a little fragment of leaf on top of leaves or on spines of the tree trunk and waited for a marked ant to find it, following the methods of [5]. We recorded whether the ant picked up the Beltian body with her mandibles to store it inside a spine (hereafter, "stored"), or whether the ant moved it to the edge of the leaf and dropped it to remove it from the tree (henceforth, "discarded").

We tested the reaction toward intruders by placing workers of sympatric leaf-cutter ants (*Atta colombica*) on the focal acacia trees. To transfer the intruders with minimal

disturbance, we picked up these *Atta* workers from their foraging trails by inducing them to walk onto a small stick and from there onto a leaf or the trunk of the acacia tree. When a marked acacia ant found the intruder (i.e., touched it with the antenna), we recorded whether she attacked (by biting) or ignored it by walking away. Because defense and foraging specialization increased with colony size of acacia ants, we expected trunk-ants to be more likely to attack the *Atta* intruders, and leaf-ants more likely to ignore the intruders as colony size increased. Because of the nature of the field behavioral assays, observations could not be done blind to the ant color-mark or colony identity (e.g., size); however, the observers recording the reactions of the ants were naïve to the hypotheses being tested.

To test whether the probability of performing a behavior (discarding food; attacking intruders) changed with colony size, we used generalized estimating equations [6]. The response variables were binary (binomial family, logit link), recoded as 1 for discarding and 0 for storing a food body, and 1 for attacking and 0 for ignoring an intruder; the type of ant was a fixed categorical predictor (trunk-ant was the reference group); the percentage of task-specialization was a continuous predictor, and ant-colony was included as a block or random factor. We performed two separate analyses using percentage of foraging specialization and defense specialization as continuous predictors, with the function geeglm of the package geepack in R [7].

Brain anatomy. To obtain brain measurements of ants from colonies of different size, we collected leaf- and trunk-marked ants from eight colonies, and brought them alive to the laboratory facilities of the Smithsonian Tropical Research Institute (10 minutes from the field site) for histological preparation. Brains of ants were immediately removed from the head capsule in phosphate buffered saline (PBS, pH = 7.4), and preserved in fixative (4% formaldehyde in PBS) overnight at 4°C. Brains were washed twice in buffer and once in water, for one hour each. We stained brains with 1% OsO_4 in the dark for two hours at 4°C, then for 30 min at room temperature, and washed them three times for 30 min with distilled water. We then dehydrated brains with 50% ethanol for 10 min, followed by 20 min of 2, 2 - dimethoxypropane. Fixation in plastic resin was preceded by two washes in 100% acetone for 10 min each, followed by immersion for 6 hrs in 50:50 acetone: Spurr's (Electron Microscopy Sciences RT 14300) low viscosity resin, 8 hrs in 10:90 acetone: resin, and 8 hrs in 100% resin. Brains were embedded in Beem® capsules, and cured at 60°C for 18 hours. Most brains were sectioned at 7 μm, and the smallest brains were sectioned at 6 μm to

obtain about the same number of sections per brain. Sections were arranged in order on the microscope slide, and stained with 1% toluidine blue, and cover slides were attached with Permount®. We photographed each section (Fig. 2a) using a camera (Leica DFC 320) attached to a light microscope with Köhler illumination (Leica DM LB) at the Microscopy and Imaging Facility of the Institute for Cellular and Molecular Biology at The University of Texas at Austin.

We obtained volumetric measurements of brains from digital 3D reconstructions, using Reconstruct software [8]; Fig. 2b). We first aligned section photographs and drew the contours of the brain neuropiles (excluding cell bodies) superimposed on the brain images (Fig. 2a). By coding section-images prior to measurement, brains were aligned and measured blind with respect to colony identity and type of ant. For each brain, we reconstructed and measured the volume of the whole brain, and the volumes of the following neuropiles: three regions of the optic lobe (lobula, medulla, lamina), olfactory lobes, the vertical and medial lobes of the mushroom bodies, and the lip and collar of the medial and lateral calyces (following [9]). The basal ring was indistinguishable from the collar. All volumetric measures were relativized to the total brain volume and the sub-esophageal ganglion, which are fused in insects. Neuropile volumes are a proxy for the density (mass) of synaptic circuits, or the number of axonal and dendritic connections [10]. We also performed analyses of those measurements relativized by "brain volume remainder", i.e., the total brain volume excluding the brain regions of interest, that is: total volume minus volume of optic lobes, antennal lobes and mushroom bodies. Because results relativizing by brain volume remainder were congruent with results relativizing by total brain volume, we only present the latter results.

We used generalized linear models to test for homogeneity of slopes for a regression between the colony size-related traits (factor 1 of the PCA described above) and the relative brain-region volume between trunk- and leaf-marked ants. The model included the relative volume of a particular brain region as the response variable, and we tested the interaction between type of ant (leaf- and trunk-ants) as a categorical fixed factor, and colony size as a continuous factor. A significant interaction in this model means that the continuous variable differently affects the two groups of ants (i.e., that the slopes differ). To correct for multiple comparisons for the generalized linear models, we used false discovery rates [11] to calculate a cut-off q-value where the probability of finding a false positive among our significant results was less than one, specifying the bootstrap method in the q-value function of the

package "qvalue" for R [12] . We also report partial omega squared (ω_p^2) with 95% confidence intervals as a measure of effect size, which were calculated with bootstrapping (1000 repetitions) using the package "boot" for R [13]. We used omega-squared estimates because they are less biased than eta squared estimates, although usually yielding lower effect sizes [14].

To evaluate how colony size traits correlated with another non-neural morphological trait, we also measured the head size area of trunk- (n = 51) and leaf-ants (n = 43). We took pictures of the ventral view of heads from which we dissected the brains, using a camera (Leica DFC240) attached to a stereoscope (Leica MZ16). In the calibrated image, we measured the head area as the contour of the head excluding the eyes and the mouthparts, using ImageJ [15]; Fig. 2c). We tested for homogeneity of slopes of trunk- and leaf-ants in the correlation between colony size-related traits and head area, as explained above for brain volume.

Supplementary Results

Descriptive measurements of brain anatomy. Brains of leaf and trunk-ants did not differ in total volume (Table S6; $F_{1,59} = 0.98$, $p = 0.33$; ω^2=0.0003, CI: 0 - 0.10). Optic lobes and mushroom bodies comprised similar proportions of the total brain volume (about 15%), and olfactory lobes comprised 6% of the brain volume (Table S6).

Table S1. Number of acacia ants (*Pseudomyrmex spinicola*) that were color-marked either on leaves (leaf-ants) or on the trunk base (trunk-ants) of the acacia host-tree. Because acacia ants obligatorily nests inside hollow spines on the host tree, the total number of spines per host tree is also listed.

Colony code	Spines on host tree	Leaf-ants	Trunk- ants	Total marked ants
35	5	25	67	92
37	5	44	30	74
31	8	50	45	95
33	8	60	60	120
13	12	51	70	121
38	13	70	70	140
30	15	90	80	170
36	23	60	60	120
42	26	83	70	153
01	27	80	90	170
34	30	161	140	301
45	40	90	72	162
44	41	100	100	200
41	59	135	110	245
40	60	100	100	200
25	61	90	80	170
43	113	75	80	155

Table S2. Loadings of the size-related variables of the acacia ant colonies for the first factor of a principal component analysis, which explained 56% of the variation in the colonies.

Trait	Loadings Factor 1
Estimated number of outside workers	0.593
Number of spines on the tree	0.577
Defense specialization	0.544
Foraging specialization	0.138

Table S3. Estimated parameters of Wald statistic and associated probability (P) for the generalized estimating equation assessing the **effect of defense specialization on the log odds of discarding food** (log odds of discarding food = β_0 (intercept) + β_1 Ant type+ β_2 Defense specialization + β_3 Ant type*Defense specialization). For trunk-ants, the log-odds of discarding changed with task-overlap by β_2, while for leaf-ants they changed by $\beta_2 + \beta_3$.

	Estimate (log-odds)	**SE**	**Wald**	**P-value**
β_0 Intercept	0.28	2.3	0.014	0.90
β_1 Ant type (reference is trunk-ants)	-1.65	3.3	0.242	0.62
β_2 % Defense specialization	-0.008	0.03	0.085	0.77
β_3 Ant type*defense specialization	0.024	0.04	0.372	0.54

Table S4. Estimated parameters of Wald statistic and associated probability for the generalized estimating equation assessing the **effect of foraging specialization on the log odds of attacking intruders** (log odds of attacking intruders = β_0 (intercept) + β_1 Ant type+ β_2 Foraging specialization + β_3 Ant type* Foraging specialization).

	Estimate (log-odds)	**SE**	**Wald**	**P-value**
β_0 Intercept	5.60	2.94	3.6	0.056
β_1 Ant type (reference is trunk-ants)	-3.99	1.78	4.9	**0.026**
β_2 % Foraging specialization	-0.040	0.03	1.5	0.217
β_3 Ant type * % Foraging specialization	0.038	0.02	2.5	0.110

Table S5. Estimated parameters of Wald statistic and associated probability (P) for the generalized estimating equation assessing the **effect of defense specialization on the log odds of attacking intruders** (log odds of attacking intruders = β_0 (intercept) + β_1 Ant type+ β_2 Defense specialization + β_3 Ant type * Defense specialization).

	Estimate log-odds	**SE**	**Wald**	**P**
β_0 Intercept	2.98	3.16	0.89	0.35
β_1 Ant type (reference is trunk-ants)	-4.47	3.16	1.99	0.16
β_2 % Defense specialization	-0.005	0.04	0.02	0.88
β_3 Ant type * % Defense specialization	0.041	0.04	1.24	0.27

Table S6. Brain measures of trunk-ants and leaf-ants inhabiting acacia trees (mean, SD).

Brain region	Trunk-ants	Leaf-ants
Total brain volume (mm^3)	0.10 ± 0.023	0.095± 0.019
Optic Lobes %	0.139 ± 0.02	0.137± 0.02
Olfactory Lobes %	0.064 ± 0.015	0.068 ± 0.013
Mushroom bodies, %	0.16 ± 0.017	0.15 ± 0.017

Figure S1. Monomorphic workers of acacia ants (*Pseudomyrmex spinicola*) (**a**) nest inside the swollen spines (*s*) of acacia trees, and feed on the nectar produced in extrafloral nectaries (*n*), and on Beltian bodies (*bb*) produced at the tip of the leaf folioles. (**b**) Workers specialized in defense of the trunk base of the acacia tree, which provides potential access to the tree for other ants. The worker on the trunk (trunk-ant) is showing the typical guarding posture: standing motionless with the head directed downwards; the particular trunk-ant is holding with the mandibles a worker of the intruding ant *Crematogaster brevispinosa*. (**c**) A worker specialized in foraging on leaves (leaf-ant) is harvesting a Beltian body to feed the brood; this ant is marked with a green dot on the abdomen to identify it as a leaf-ant.

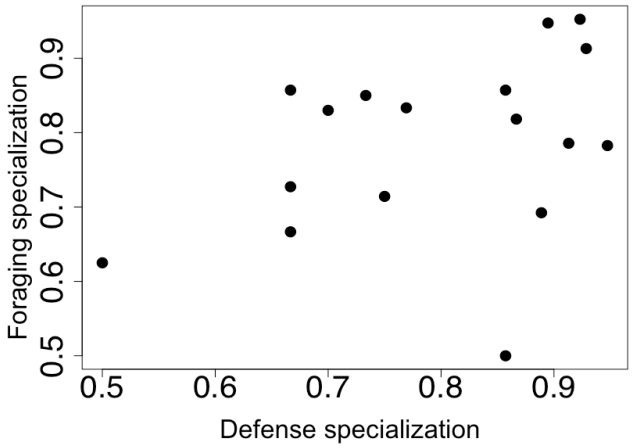

Figure S2. The proportion of ants specialized in foraging tasks (i.e., workers that were marked and re-sighted foraging on leaves) did not correlate with the specialization on defensive tasks (i.e., returning to work at the trunk base of the host tree). Each dot represents a colony of ants inhabiting one acacia tree.

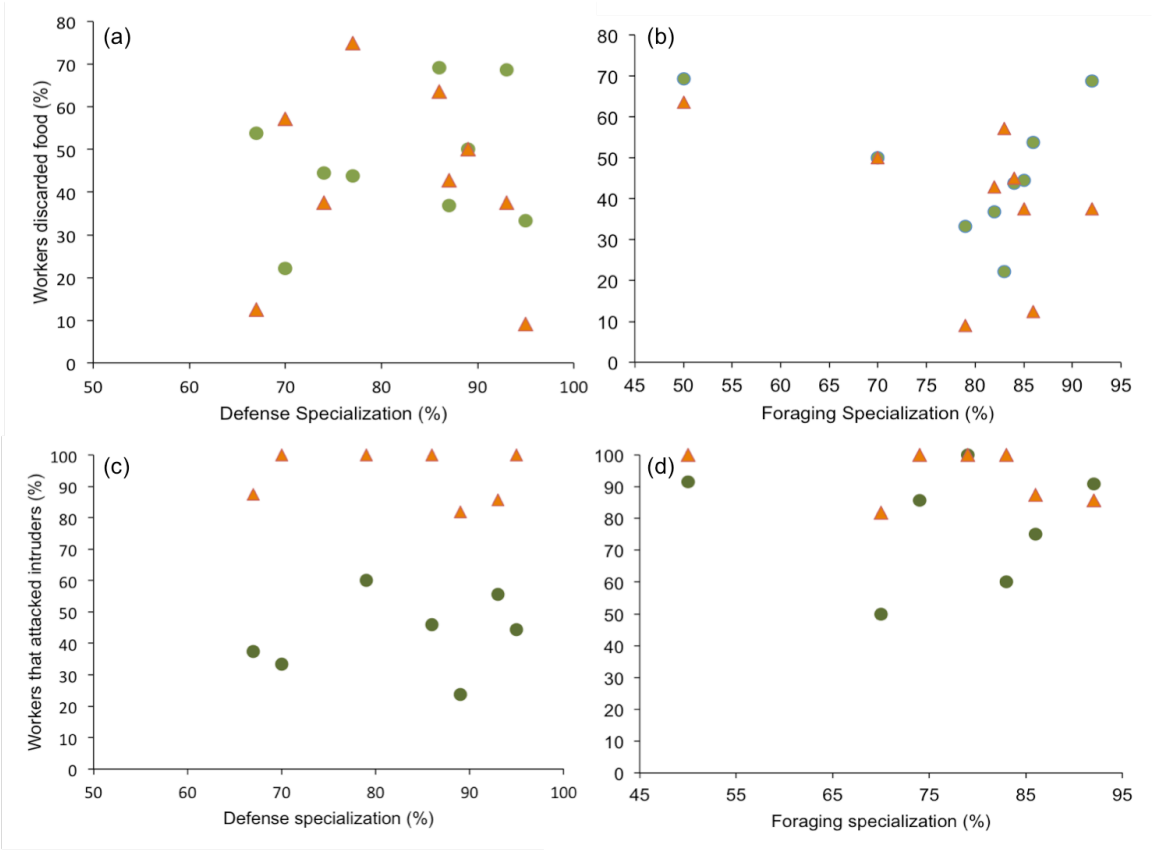

Figure S3. Percentage of trunk-marked (triangles) or leaf-marked (circles) acacia ants that discarded offered Beltian bodies instead of storing them inside the swollen spines of the acacia tree as a function of the percentage of workers specialized in (**a**) defense (i.e., percentage of trunk-marked ants that returned to defense-related tasks after a day, see Methods) or (**b**) foraging specialization (i.e., percentage of leaf-marked ants that returned to foraging-related tasks after a day, see Methods); or that attacked intruders instead of ignoring them as a function of (**c**) defense or (**d**) foraging specialization in the colony . Trunk-ants were less likely to discard food as colony size increased, while the odds of discarding almost did not change for leaf-ants (Table 1). The chances of attacking an intruder did not change with colony size or task-specialization.

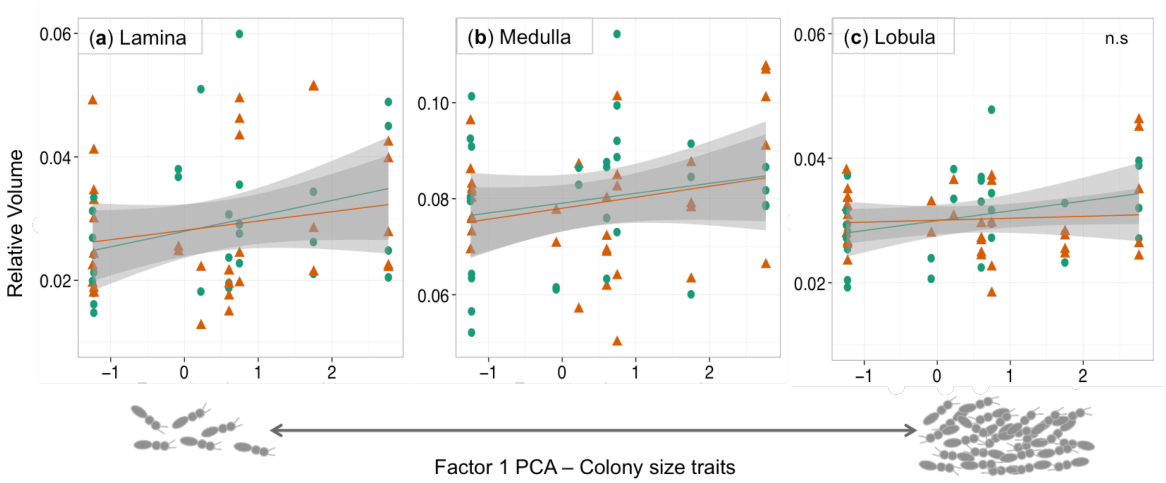

Figure S4. Relative volume of the subregions comprising the optic lobes, as a function of colony size-related traits. The lamina and medulla of both trunk-ants (triangles) and leaf-ants (circles) increased in relative size with colony size-related traits. Shaded areas denote 95% confidence intervals.

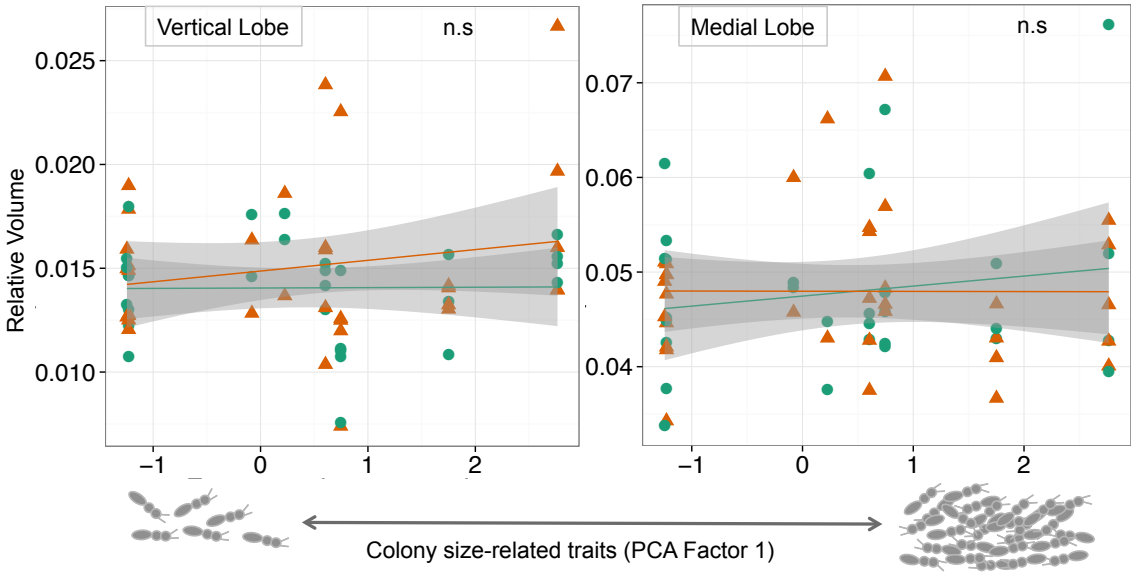

Figure S5. The relative volume of the mushroom body lobes (former terminology: vertical lobe = alpha lobe, medial lobe = beta lobe) was not affected by colony size-related traits. Brains of trunk-ants (guarding and defending) are represented by triangles, and brains of leaf-ants (foraging) are represented by circles.

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