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ORIGINAL ARTICLE

Morphological evolution and the behavioral organization of agricultural division of labor in the leafcutter ant *Atta cephalotes*

I. B. Muratore[1] · I. Ilieş[2] · A. K. Huzar[1] · F. H. Zaidi[1] · J. F. A. Traniello[1,3]

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Abstract

A main challenge in the analysis of division of labor in insect societies characterized by worker polymorphism has been identifying the number of physical castes and determining their relationship to task performance. We addressed this question using the extremely polymorphic leafcutter ant *Atta cephalotes* as a model by measuring 22 morphological characters and applying multivariate Gaussian mixture modeling to define worker size-class boundaries. Our statistical approach discriminated five physical worker size classes (subcastes) and found continuous variation in most morphological characters. Some worker size classes showed patterns of covariance, modularity, and integration in head, mandible, and leg traits, suggesting biomechanical functionality in tasks such as leaf cutting. Task repertoires and act frequencies were recorded to identify how fungal gardening, brood care, leaf-harvesting, and other tasks were distributed across worker size groups. Results showed that small and mid-sized media workers performed more diverse and complex tasks, including leaf harvesting, than workers of other size classes. Minims participated in fungal-gardening and nursing tasks, whereas large medias mainly participated in leafcutting and majors made few direct contributions to fungal cultivation. Results of our integrative analyses demonstrate the existence of an unexpectedly large number of worker size classes that can differ significantly in performance of agricultural tasks. However, overlap in task repertoires of workers at size-class boundaries suggests that not all morphologically distinct groups display discrete behavioral profiles. Morphological groups are instead distinguished by a combination of the tasks that each performs and their relative frequencies.

Significance statement

The evolutionary coupling of behavior and morphology is central to understanding division of labor in insect societies. Our work applies robust statistical modelling to offer a novel analysis of worker physical caste categorization based on extensive morphometric sampling. Our method improves upon previous approaches by incorporating measurements of a large number of diverse morphological traits likely involved in task performance to examine worker physical caste evolution. We integrate morphological evolution with behavioral data on relative task performance rates and task repertoire size to describe the morphological and behavioral space of size-variable workers. Our approach provides methods to quantitatively analyze division of labor and behavioral performance, and offers new insights into worker trait modularity and integration and task performance associated with the organization of complex insect societies.

Keywords Polymorphism · Social role · Task specialization · Social complexity · Superorganism · Social insects

Introduction

Many eusocial insect species divide labor among size-variable workers, generating complex social phenotypes. Morphological and demographic analysis of workers is often integrated with behavioral studies to better understand the relationship between body size, shape, and social evolution (e.g., Bertossa 2011; Robson and Traniello 2016; Gordon et al. 2017; Bourke and Franks 2019). Wilson (1953) pioneered the study of the evolution of worker physical subcastes (groups of body size- and task-related workers) through the description of growth patterns, ergonomic analysis, and the adaptive nature of colony demography (Wilson 1968; Oster and Wilson

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1978). The categorization of workers into distinguishable subcastes has historically relied on bivariate analyses of morphological measurements that typically involve visual comparison of regression slopes to identify discontinuities ("breaks") across the worker size range. Morphological size classes were then differentiated, sometimes imprecisely and arbitrarily, according to patterns of diphasic or triphasic allometric growth (Wilson 1953; Wills et al. 2018). Quantitative analysis of worker morphological evolution, however, requires categorizing workers according to scaling variation and covariation in morphological characters that are functionally significant in size-related task performance. Examining the nature of worker subcastes also concerns analyzing size-related behavior in accordance with the system of categorization.

Here we describe an analysis of physical castes and division of labor made robust through extensive sampling of morphological traits and multivariate statistical modeling, including covariance analysis (Magwene 2001), to quantitatively distinguish size classes and identify evolutionary and functional patterns among polymorphic workers (Pie and Traniello 2007; Ilieş et al. 2015; Kamhi et al. 2017). Statistically identifiable boundaries along the worker body-size spectrum can be established to categorize subcastes and facilitate analyses of worker functional morphology, biomechanical optimization of task performance (Roces and Hölldobler 1994; Segre and Taylor 2019; Burd 2000; Anderson et al. 2020; Casadei-Ferreira et al. 2021), and behavior. We use the leafcutter ant *Atta cephalotes*, which has evolved morphologically, behaviorally, and neuroanatomically differentiated worker subcastes to support an agricultural life history (Wetterer 1991, 1999; Hölldobler and Wilson 2010; Mehdiabadi and Schultz 2010; Muratore et al. 2022, 2023), as a model. Our study illustrates how the application of covariance analysis and statistical and graphical modeling, integrated with behavioral studies, can aid in the resolution of patterns of physical caste evolution and division of labor.

To analyze the association of morphological and social evolution in *A. cephalotes*, we estimated the number of worker size classes by statistically determining optimal size boundaries using 22 body measurements. We hypothesized that workers cluster into morphometric groups that differ in task specialization, and that we could identify classes by recording patterns of scaling and covariance among morphological traits. In concert with our morphometric and sociometric analyses, we evaluated size-related task performance patterns by recording differences in act frequency, relative probabilities of task performance, and repertoire breadth among morphologically identified groups.

Methods

Study species

Atta cephalotes forms large colonies of strongly polymorphic workers that cultivate a food crop and maintain colony operations through division of labor. Worker size in *A. cephalotes* (head width 0.5–5.0 mm+) broadly correlates with task performance (reviewed in Muratore et al. 2022). Workers have been historically categorized as minims, medias, and majors according to body size. Tasks include brood care (Hölldobler and Wilson 2010), fungal cultivation (Mehdiabadi and Schultz 2010), foraging trail maintenance, (Evison et al. 2008; Dupuis and Harrison 2017), leaf cutting (Nichols-Orians and Schultz 1989; Segre and Taylor 2019), and waste-management (Hart and Ratnieks 2001), and defense (Powell and Clark 2004).

Colony collection and culturing

Incipient *A. cephalotes* colonies and worker corpses were collected in Trinidad and Peru during 2016–2019. Colonies were cultured in a Harris environmental chamber under a 12:12 hour light-dark cycle at 55% humidity and 25 °C at Boston University. All colonies were housed in large plastic bins (30 cm x 46 cm x 28 cm), whose floors served as a foraging arena and for waste disposal. Smaller plastic boxes (11 cm x 18 cm x 13 cm) interconnected by plastic tubes (2 cm diameter) served as chambers for the fungus. Colonies were provisioned with washed, pesticide-free rhododendron, rose, lilac, andromeda, bramble, oak, sugar maple, willow, and beech leaves when available, as well as organic baby spinach, romaine, arugula, frisée, and oat flakes.

Worker morphometrics

Twenty-two morphological character measurements (Table 1) were recorded by two observers using an ocular micrometer. Worker traits were measured in whole optical units using an Olympus SZ30 stereo dissecting microscope at 10x, 20x, or 40x power and converted to millimeters with a conversion factor calibrated to the microscope. The number of optical units (often single digits) recorded for the smallest structures in minims may have decreased measurement precision, and thus affected calculations of trait modularity or integration, due to the small size of these structures. The modularity that we observed in minim petiole height (PL), post-petiole height (PPH), and prothorax width (PW) might have been inflated by this

Table 1 Worker morphological measurements and abbreviations

Trait	Shorter name	Acronym
Scape length	Scape length	SL
Maximum distance across eyes	Eye to eye	HW
Maximum eye diameter	Eye length	EL
Distance from edge of eye to antennal scape in side view	Eye to antenna	EtA
Width of mandible at base with head in side view	Mandible width	MB
Mandible length	Mandible length	ML
Maximum width across widest part of head, excluding the distance across the eyes	Max. head width	MHW
Head length (posterior cephalic margin to labrum)	Head length	HH
Head depth (maximum thickness from front to back of the head in side view	Head depth	HD
Head maximum length in side view (from insertion of the mandible to head dorsum)	Head side length	HL
Alitrunk (mesosoma) length, from anterior prothorax margin to the insertion of petiole	Alitrunk length	AtL
Pronotal dorsal spine length	Pronotal spine length	PnS
Prothorax width	Prothorax width	PW
Distance between anterior base of prothoracic coxa and dorsum of prothorax	Alitrunk height	AtH
Propodeal dorsal spine length	Propodeal spine length	PdS
Petiole length	Petiole length	PL
Petiole height	Petiole height	PH
Post-petiole height	Post-petiole height	PPH
Post-petiole length	Post-petiole length	PPL
Metathoracic leg femur length	Femur length	FL
Metathoracic leg tibia length	Tibia length	TL
Gaster length in side view	Gaster length	GL

factor, although related characters with smaller sizes and ranges such as post-petiole length (PPL) or petiole height (PH) did not show such levels of non-coordination, so the impact of any such accuracy issues must be low.

Measurements were taken from 743 worker corpses collected from the midden piles of two mature laboratory colonies (labelled as Ac16, AcP1; collected as incipient colonies in July, 2014 and August, 2018, respectively). We also sampled the refuse piles of three mature field colonies (as evidenced by the presence of majors, and the large number of corpses in the piles) in the Tambopata National Reserve and the Kawsay Biological Station at Madre de Dios, Peru. We measured traits of carcasses to avoid sacrificing workers. Individuals with gasters that were modified in shape (e.g., compressed) were not measured.

To optimize the characterization of worker size-groups, we attempted to exhaustively measure relevant morphological traits recognizing that some, but not all, are likely involved in the performance of tasks by polymorphic workers. This comprehensive sampling allowed the identification of worker groups and assessment of covariance among traits with virtually no risk of bias due to assumptions of the importance of any particular trait. While all other traits are represented by a single measurements per dimension, two head-size measurements, maximum distance across the eyes (head width, HW) and maximum distance across the widest part of head excluding the distance across the eyes (MHW), were included. This was because the higher investment in mandibular musculature in larger *A. cephalotes* workers produces cephalic variation causing these metrics to diverge in some worker size groups (Wilson 1953). MHW is more reflective of increased mandibular power (Püffel et al. 2021), and HW is commonly used to delineate worker size in *A. cephalotes* (Arganda et al. 2020).

All measurements were uniformly normalized to facilitate different analyses. To identify physical subcastes, trait measurements were normalized to total body length [sum of mandible length (ML), head length (HH), alitrunk (mesosoma) length (AtL), petiole length (PL), post-petiole length (PPL), and gaster length (GL)] to preclude overall body size from dominating cluster analysis outputs. In addition, to ensure covariance matrices were well-defined for groups with fewer observations, normalized values were subjected to dimensionality reduction via principal component analysis (PCA). To validate the robustness of the proposed subcaste detection method, analyses were repeated using natural-log transformed measurements. The log transformation was also used for the analysis of covariance patterns within size groups, to account for non-linear (allometric) trait scaling patterns.

Statistical determination of the number of physical castes and their size boundaries

To our knowledge, our study is the first to apply multivariate methods for morphospace analysis in conjunction with covariance analysis in order to discriminate worker physical castes. Our approach distinguishes from prior studies (e.g., Diniz-Filho et al. 1994; Pie and Traniello 2007; Pie and Tschá 2013) by (i) the large number of morphological characters measured; (ii) extensive data sampling, with emphasis on uncommon size ranges; (iii) normalizing to total length, instead of exclusively log transforming data, to reduce the impact of size on subcaste separation; and (iv) using statistical modeling to identify worker classes, instead of relying on visual separation. We also performed conditional independence analysis to better assess trait modularity and integration in each identified worker class, and to more clearly recognize between-subcaste differences in trait covariance patterns.

We expanded physical caste identification beyond estimating breakpoints in the slope of regression lines for pairs of morphological characters by simultaneously comparing covariance patterns between groups across multiple pairs of traits. To do so, we used Gaussian mixture models (GMM), an unsupervised learning approach that can detect groups with different sizes, means, and covariance matrices (McLachlan and Peel 2000). To find the optimal number of worker size groups (classes), we fitted GMMs consisting of two to 10 classes with full and independent covariances to the top four principal components of the total-length-normalized measurements. As GMMs can be sensitive to initial group means, each attempt was replicated 100 times starting from different solutions generated by k-means++ (Arthur and Vassilvitskii 2006). For each possible number of worker groups, the replicate with the best fit (lowest negative log-likelihood) was selected as the candidate solution. The GMM with the best model-complexity-corrected fit (lowest Bayes information criterion; Schwarz 1978) among these nine candidates was then selected as global solution (Roberts et al. 1998).

To determine size boundaries between worker groups, individuals were assigned to the class with the highest likelihood. Groups with less than five individuals were considered noise and discarded, with affected individuals reassigned to their second-best match. As GMM class assignments are based on probability distributions, which can result in fuzzy class boundaries, size thresholds along individual traits were computed using linear discriminant analysis (LDA) of class memberships using the raw (non-normalized) measurements as predictors.

Correlation analysis of worker morphological traits

To explore patterns of integration versus modularity among morphological traits, including similarities or differences among putative worker subcastes, we employed a correlation-based approach. Integration and modularity in this context were defined, respectively, as the developmental convergence of trait metrics, manifest as highly correlated scaling, versus the dissociation of traits that scale in an uncorrelated manner through mosaic evolution. These definitions are consistent with Magwene (2001) and have been previously applied in ants (Ilieş et al. 2015; Kamhi et al. 2019). To quantify integration and modularity, Pearson and partial correlation coefficients were calculated between each pair of traits within each identified worker size group and used as measures of overall morphological integration (many high values) or modularity (few high values). Pearson correlations are measures of total (direct plus mediated) covariance, whereas partial correlations quantify only the direct portion, but require large samples for accurate estimation and statistical significance assessment.

To measure the degree of differentiation among worker groups in the multidimensional space of worker body measurements (morphospace), differences in average and variance of trait correlations were assessed using ANOVA and Levene's test, respectively. Following standard practice, correlations were subjected to Fisher (hyperbolic tangent) transformation prior to analysis for variance stabilization. In addition, to identify directions of divergence, differences between worker groups at trait-pair level were determined using z-tests of differences between the transformed correlation coefficients, separately for each pair of size groups. In each case, standard errors were set to the inverse square root of the degrees of freedom of the two compared correlation coefficients, per standard practice, then pooled across groups. Statistically significant differences ($p < 0.05$, uncorrected) were classified as differences in magnitude (involving two coefficients with the same sign) or in direction (different signs).

To identify the main drivers of differential allometric scaling, we conducted PCA on the Pearson correlation matrix separately for each worker group. To facilitate interpretation and visualization, the two largest principal components (PCs) were variance scaled and then subjected to varimax (variance maximization) rotation (Kaiser 1958), a linear transformation that limits the number of traits associated with each PC while maximizing the corresponding loadings. Traits were assigned to the rotated component (RC) with the largest loading if unambiguously possible, respectively assumed to be associated with both components (equally large loadings) or with other, higher-order principal components (both loadings close to zero). Thus, PCA enabled the definition of as many as four non-overlapping morphological trait clusters within each worker size group.

To confirm or refine trait clusters, partial correlations significantly different from zero at the 0.05 level were used to construct conditional independence (CI) graphs (Magwene 2001). A PCA trait cluster was considered to represent a true morphological module if it was connected in the graph theoretical sense by positive conditional dependences, and if there were no or few such links with traits outside the cluster (Kamhi et al. 2019). Significantly negative partial correlations were assumed to represent system-level constraints, rather than indications of modularity (Ilieş et al. 2015), and were therefore not used to define modules.

Worker size and task performance

Patterns of task performance by *A. cephalotes* polymorphic workers were quantified in a mature lab colony (Ac16) that maintained two large fungal combs. Nest container floors and fungal garden box vertical side outer surfaces were divided into a grid of 7 cm x 7 cm quadrats. Worker HW was recorded, and tasks and/or behaviors performed by each worker in a given quadrat during a 10-min observation period were recorded as an audio file and later transcribed. Each quadrat on the nest box floor and each vertical side of a fungal chamber was observed approximately twice, resulting in 866 individual observations.

Behaviors were divided into task categories of brood care (feeding, grooming, carrying brood), fungal gardening (implanting, removing, transporting hyphae and mulching fungus), hygiene (transporting dead workers and waste), and leaf harvesting (cutting and carrying plant material). Our use of transparent next boxes and the otherwise open design of colony containment allowed us to observe behaviors (including aspects of the leaf-cutting process [Garrett et al. 2016]) normally confined to the nest interior. Defense was not analyzed, as natural predators such as army ants that induce well-documented intense, aggressive responses by majors (Powell and Clark 2004) were not available to provide test stimuli. Majors may also physically maintain foraging trails (Cherrett 1972; Dupuis and Harrison 2017) in nature, although we did not observe this behavior in our lab colony arrangement.

Observers wore surgical masks to prevent disturbance from carbon dioxide and/or air currents. It was not possible for observers to be blind to worker size, as our aim was to record size-related behaviors. We assessed observer accuracy in categorizing worker size class by estimating the ability to visually estimate the HW of 50 corpses viewed from approximately the same distance at which behavioral observations were made. Visually estimated worker HWs recorded were within +/- 0.1 mm of the true value in 82% of cases, and within +/- 0.2 mm in all cases.

Worker size class and task performance analysis

Sampling ranges used to record size-related patterns of behavior reflect worker size-class boundaries established in our morphological analysis (see Results). Workers were classified in HW-delineated groups: minims (one group, $\leq$ 0.81 mm HW), medias (three groups, >0.81–1.57 mm “small medias”, >1.57–2.45 mm “mid-sized medias”, or >2.45–3.50 mm “large medias”), and majors (one group, >3.51 mm) for statistical comparison of task repertoires. We elected to use this categorization because it is in general accordance with most prior literature on *Atta* polymorphism. If our statistically determined boundaries did not correspond with previous descriptions, we retained our nomenclature. Wilson (1980b, 1983), for example, referred to “minors” (HW of 0.6–1.2 mm), which overlapped substantially with his categorization of “minims” (0.6–1.0 mm) in *A. sexdens*. Because our second-smallest group of workers surpassed both these ranges at its upper limit, and because of the behaviors these workers perform, we designated them as small medias rather than minors.

To determine whether the rates at which different worker groups performed different task types deviated from null expectations, a multinomial test was run on the counts of individuals in each worker size group that performed behaviors in each task category, relative to the overall proportions of observations of individuals from the different size classes. Subsequently, to assess if each type of task was performed at a higher-than-expected rate by any specific worker type, two-tailed post hoc comparisons were performed using binomial tests for each worker size group and for any task category with significant differences, as identified by multinomial analysis. Statistical significance values were corrected for multiple comparisons using the Benjamini-Hochberg method, separately for multinomial and binomial tests.

To explore differences and similarities among worker size classes in task performance in detail, we computed Shannon diversity indices (Peet 1975) of task repertoire breadth, with tasks modeled as species and worker groups as sites. Measuring task repertoire breadth through a diversity index indicates to what extent a group of workers is narrowly specialized versus broadly generalized in their task performance, which may create differences in biomechanical requirements. To compare the extent to which the repertoire of each worker group was dominated by one or more particular tasks, we used the relative probability of task performance (RPTP; Seeley 1982), a probability-based metric. RPTP was calculated for each worker size group and task category as:

$$RPTP = r_{ij} = p_{ij}/p_{i_{\max}}$$

where

r_{ij} = the relative probability of task i performance by a member of group j,

p_{ij} = probability of task i performance by a member of group j, calculated as

$$p_{ij} = n_{ij}/\sum_{i=1}^{k} n_{ij}$$

n_{ij} = number of performances of task i observed for members of group j,

k = total number of task categories, and

$p_{i_{\max}}$ = probability of task i performance by a member of the group with the highest p_{i*}.

In addition, we estimated task repertoire similarity separately for each worker group pair using Morisita-Horn similarity indices (Wolda 1981). Comparing the similarity of repertoires among groups allows the assessment of potential similarities in biomechanical demands placed upon different types of workers, which could be linked to similar body scaling.

Statistical software

All analyses of morphological measurements, including data normalization, GMM fitting, correlation analysis, and PCA, were performed in MATLAB (The MathWorks, Inc.) using built-in functions and the Statistics and Machine Learning toolbox. All analyses of task performance were conducted in R using functions from the following packages: *EMT* (multinomial.test; Menzel 2021), *stats* (binom.test and p.adjust), *vegan* (diversity; Oksanen et al. 2020), and *fossil* (morisita.horn; Vavrek 2011). All figs were produced using MATLAB or R. Probability density functions of task performance as a function of worker size were illustrated using kernel smoothing (ggdensity function in the *ggplot2* package; Wickham and Chang 2015).

Results

Worker size class identification

Analysis of morphological traits indicated a continuum of character covariance patterns across polymorphic workers. PCA of total-length-normalized measurements revealed a curvilinear distribution of individuals within the space defined by the top four principal components (Fig. 1), with position along this curve-like structure broadly associated with total body length (Supplementary Fig. 1A-C). A similar curvilinear distribution was found by applying PCA to non-normalized, log-transformed trait measurements (Supplementary Fig. 1E-F), indicating that worker size and trait covariances are strongly linked. This was confirmed by inspection of selected bivariate log-log plots (Fig. 2), revealing gradual changes in slopes (increasing then decreasing in Fig. 2A, D; decreasing at large values in Fig. 2B, C), as well as an absence of shifts, and minimal breaks (except at low values; Fig. 2A, C). Cluster analysis using GMMs indicated an optimal partitioning of the worker size continuum into five groups characterized by distinct body sizes and trait covariance patterns (Figs. 1 and 2). Subsequent LDA of assigned worker size classes with respect to the underlying metrics indicated group boundaries at HW values of 0.81, 1.57, 2.45, and 3.51 mm (Fig. 2A; Supplementary Table 1).

Trait correlation patterns

Correlation analysis showed that most morphological measurements were strongly correlated with each other in most worker size groups (illustrated by the relative proximity of plotted points near top right in Fig. 3; values listed in Table 2). Pearson correlations were the highest within small- and mid-sized medias (average of 0.88 and 0.84; Fig. 3B, C; Table 2), suggesting strong morphological integration, followed by majors and large medias (average of 0.67 and 0.62; Fig. 3D, E). Minims exhibited the weakest correlations between morphological traits (average of 0.14; Fig. 3A), and the lowest variability across correlations (SD 0.28, compared to 0.35–0.48 in the other groups; Table 2), suggesting extreme modularity. Inspection of partial correlations supported this ranking along the integration-modularity continuum: while there was no significant difference in average correlation levels between the five worker groups (range 0.033–0.041; Table 2; Fig. 3), small- and mid-sized medias showed the highest number of conditional dependences (54–55 conditional dependences), i.e., the strongest level of morphological integration, followed by large-sized medias (45), majors (36), and minims (30).

Morphospace characterization

Detailed analysis at trait level uncovered manifold significant differences between worker classes in magnitude or direction of individual correlations, underlying the observed variation in modularity patterns. Consistent with overall differences in average levels, Pearson correlations showed mainly differences in magnitude: 53.5% of comparisons between worker size classes found trait pairs to be correlated in the same way but to different degrees in one group compared to the other, with only 11.6% of comparisons indicating statistically significant reversals. By contrast, changes in direction were more frequent in partial correlations: 7.3% of comparisons found trait pairs correlated positively in one worker group

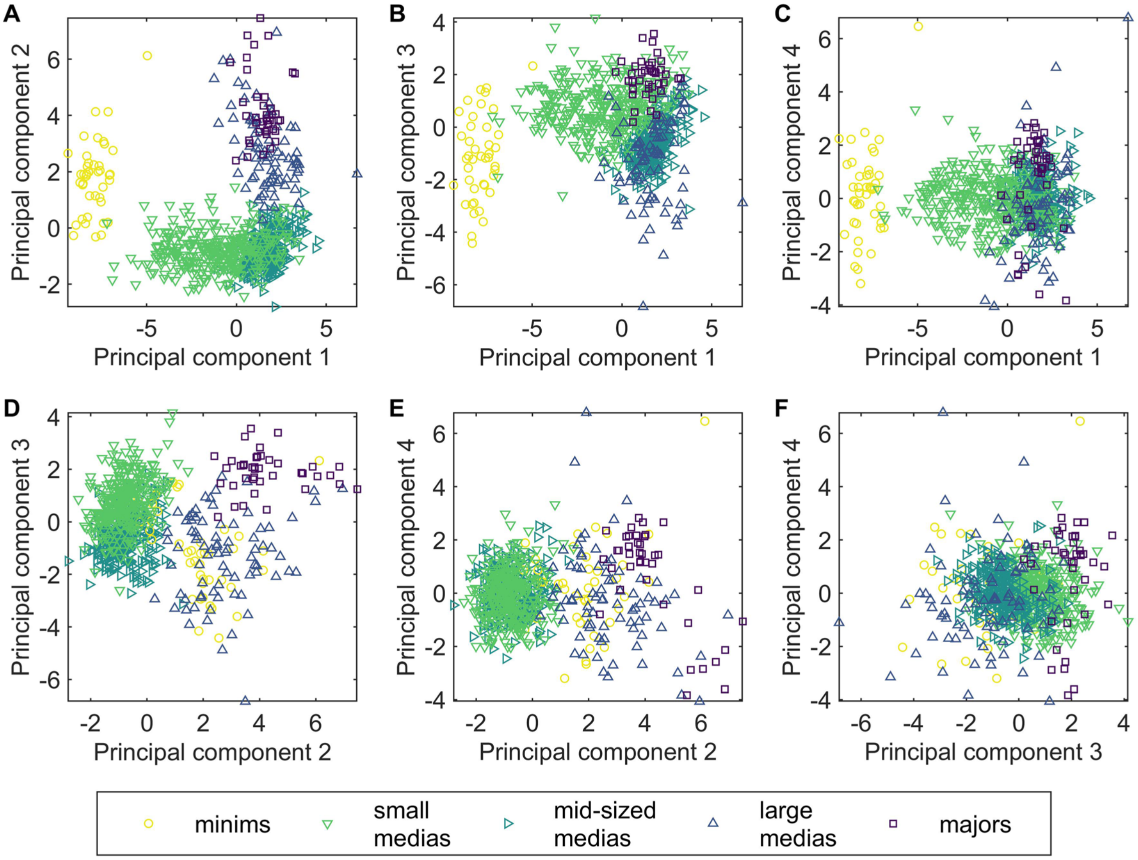

Fig. 1 Covariance-based worker classes. These data illustrate the relative degree of separation in the multidimensional morphospace between identified worker groups. Different panels highlight these relationships along different principal component pairs. **A-B** Principal component analysis of total-length-normalized trait measurements uncovered a curvilinear distribution of individuals. Observations were optimally separated into five groups with increasing body size using Gaussian mixture model fitting. The first principal component helped separate minims from other groups, as well as, to a lower extent, small medias from remaining groups (**A–C**). The second component helped distinguish between small and mid-sized medias on one hand, and minims, large medias, and majors on the other (**A**, **D**, **E**). The third principal component helped discriminate between large medias and majors (**B**, **D**, **F**), as well as between small and mid-sized medias (**B**, **F**). The fourth component further helped separate majors from medias (**E**, **F**). Yellow circles, minims; green downward triangles, small medias; teal right-facing triangles, mid-sized medias; blue upward triangles, large medias; purple squares, majors

and negatively in the other, while 2.4% detected a difference in magnitude, and the majority were not significant (Table 3).

Most Pearson correlation differences were found when comparing minims to small- and mid-sized medias (48% of trait pairs), indicating that these two sets of groups differ the most in morphological integration. At the other end of the size spectrum, large medias and majors were the least different from each other (14% of correlation differences were statistically significant; Table 3), suggesting similar levels of integration/modularity and similar trait covariance patterns. Differences in partial correlations, which are indicative of divergence in the structure of any morphological modules, did not vary as much across worker group pairs, with the highest count (34) found between small- and large-sized medias, and the lowest (16) between minims and mid- or large-sized medias. The morphological traits most frequently involved in diverging correlations included EL and EtA (in both types of correlations); MB, TL, FL, and PdS (in Pearson correlations); and PnS, HH, AtL, and ML (in partial correlations).

PCA results paralleled correlation matrix statistics, with little indication of modularity in small- and mid-sized medias, and strong evidence of divergent evolution in minims (Fig. 4). In all groups except minims, there was only one dominant component, associated with at least 20 of the 22 measured traits. This first component accounted for 82–86% of the variance in small- and mid-sized medias, and for 63–64% in large-sized medias and majors, with no other component exceeding 8% in any of these groups. After varimax rotation (performed to minimize the number of traits associated with each component), its share decreased to

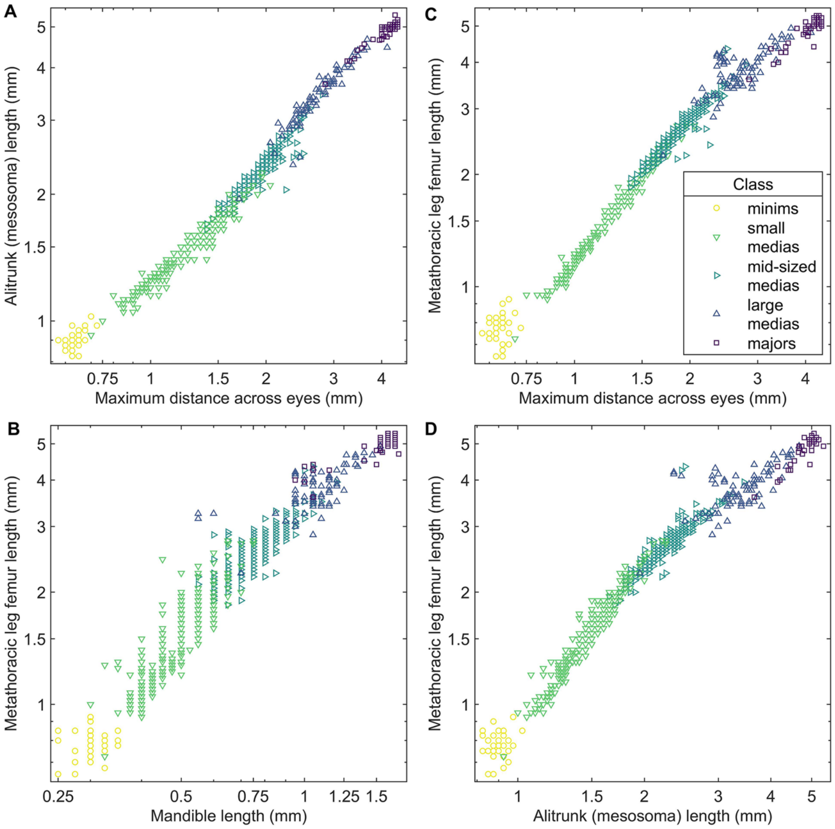

Fig. 2 Four examples of size-related growth allometries in *A. cephalotes* workers. These data illustrate the relationships between key pairs of traits in different worker groups. Log-log plots of alitrunk (mesosoma) length plotted against head width (**A**), and of femur length plotted against head width (**B**), mandible length (**C**), and alitrunk length (**D**). Color coding indicates group identity based on size boundaries determined through linear discriminant analysis of group memberships generated by Gaussian mixture model fitting (see Fig. 1) with respect to all 22 traits. Yellow circles, minims; green downward triangles, small medias; teal right-facing triangles, mid-sized medias; blue upward triangles, large medias; purple square, majors

58–70% of the variance and 18–21 associated traits, including zero to five traits equally attributable to the second component (Fig. 4). Across media and major worker size classes, traits not exclusively associated with the first component included EtA (all four groups); PnS (large medias and majors); ML, AtH, PL, PPL, and PPH (small medias); SL, EL, MB, FL, and TL (large medias); and HD (majors) (Fig. 4B–E; Supplementary Table 2). The distribution of component eigenvalues was less extreme in minims, with the first three components accounting for 25%, 18%, and 10% of the variance, suggesting multiple trait modules. Component loadings were minimally affected by varimax rotation in this worker group and indicated the presence of three or four trait clusters, consisting of two to nine morphological characters each (Fig. 4A; Supplementary Table 3).

CI graphs (Supplementary Fig. 2) generally supported the above inferences. Using positive partial correlations to identify connected components—i.e., sets of traits reachable from each other via conditional dependence relationships—revealed striking differences between media workers on one side, and majors and minims on the other. In each size class of medias, 21 of the 22 traits (except EtA in small-, GL in mid-, and HH in large-sized individuals) belonged to the same connected component, indicating strong morphological integration (Supplementary Table 3). By contrast, analysis of CI graphs constructed for minim and major workers uncovered substantial modularity, with one large weakly connected component (eight to nine traits), several smaller components (two to four traits each), and multiple isolated traits. These results support the notion of modularity in minims and majors but not in large medias. Rather, large medias exhibit a morphological integration pattern intermediate between majors and the other two groups of medias.

Worker task performance

Multinomial tests of counts of worker size-group behavioral performance (Supplementary Table 4) compared to expected proportions based on observed population counts showed significant differences in leaf harvesting ($p < 0.001$, Bonferroni corrected) and fungal- gardening tasks ($p = 0.045$). Post hoc comparisons showed that leaf harvesting by mid-sized medias was significantly different from the expected rate ($p = 0.002$; 0.541 vs. 0.320), with this value being higher than the null expectation. In contrast, mid-sized medias also showed a significant but opposite trend with respect to fungal-gardening performance ($p = 0.004$; 0.140 vs. 0.316), with a lower than expected rate. Brood-care performance and hygiene-related tasks did not significantly differ among groups ($p = 0.172$, $p = 0.132$).

Differences in relative task performance probabilities (RPTP values) for each task and worker size group

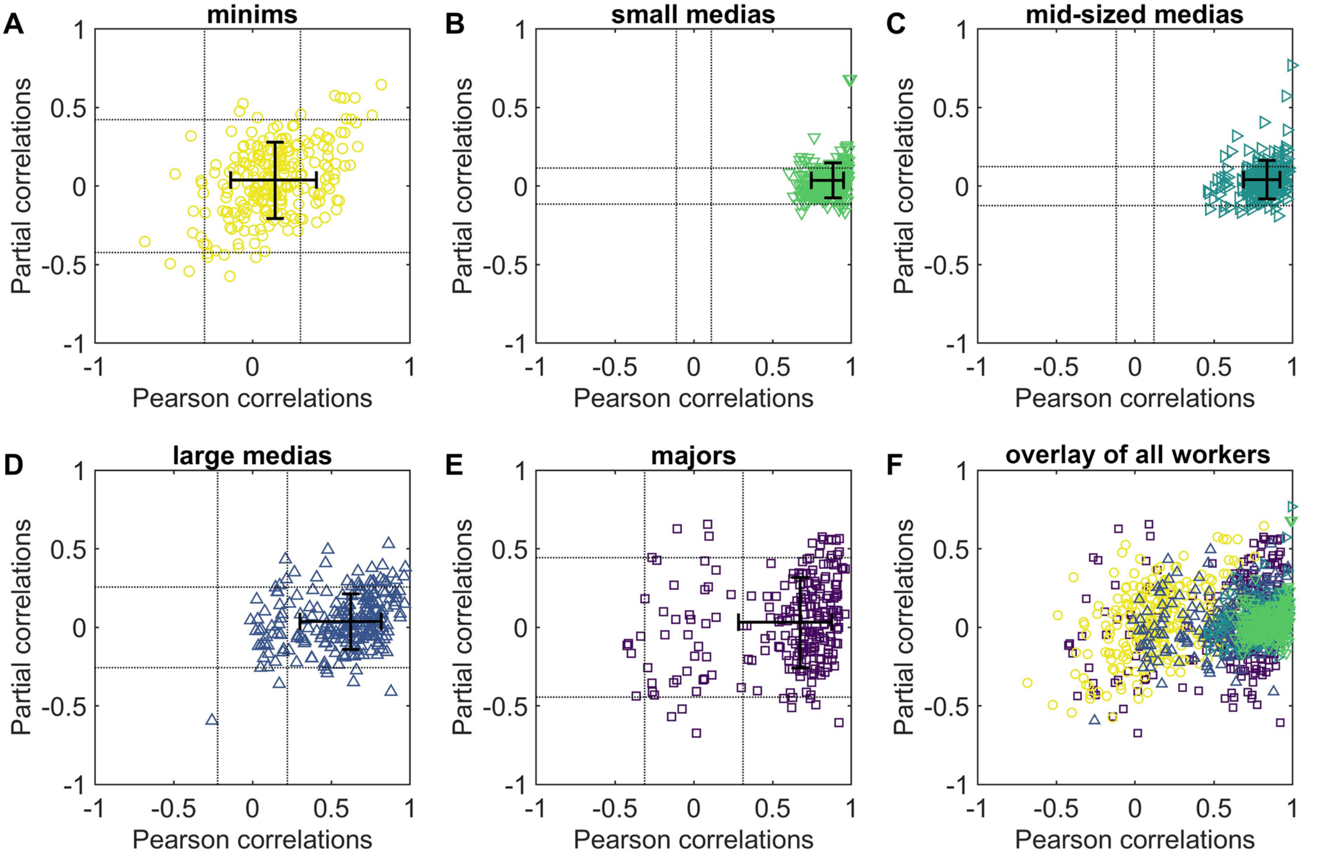

Fig. 3 Distribution of Pearson and partial correlations in *A. cephalotes* worker groups. These data show the extent to which morphological measurements are correlated for each identified worker group. **A** Minims (yellow circles); **B** Small medias (green downward triangles); **C** Mid-sized medias (teal right-facing triangles); **D** Large medias (blue upward triangles); **E** Majors (purple squares); **F** Overlay of all worker groups. Dashed lines in **A–E** mark the 0.05 significance threshold (vertical: Pearson; horizontal: partial). Black crosses denote Fisher-transformation-based average values and corresponding standard deviations

Table 2 Correlation analysis of morphological traits. Fisher-transform-based average Pearson and partial correlations, corresponding standard deviations (SD), and proportions of significantly positive (Pos.; $p < 0.05$) and negative (Neg.) coefficients for the five worker classes identified in the present study. Pairs of groups with non-overlapping superscripts differ significantly ($p < 0.05$, Bonferroni correction, post hoc pairwise comparisons following ANOVA or Levene's test) in the corresponding measure. The last column indicates the pattern of integration or modularity suggested by these findings (high average Pearson correlation and many high partial correlations, versus moderate average Pearson correlation and few high partial correlations)

Group	Pearson correlations				Partial correlations				Pattern
	Mean	SD	Pos.	Neg.	Mean	SD	Pos.	Neg.	
Minims	0.14^{d}	0.28^{c}	10%	5%	0.04^{a}	0.24^{b}	5%	3%	Modularity
Small medias	0.88^{a}	0.41^{a}	100%	0%	0.04^{a}	0.11^{d}	16%	3%	Integration
Mid-sized medias	0.84^{b}	0.35^{bc}	100%	0%	0.04^{a}	0.12^{d}	14%	5%	Integration
Large medias	0.62^{c}	0.40^{ab}	84%	0.5%	0.04^{a}	0.18^{c}	11%	3.5%	Modularity
Majors	0.67^{c}	0.48^{a}	81.5%	2%	0.03^{a}	0.29^{a}	7%	4%	Modularity

largely corresponded to statistical analysis of task performance differences, with some exceptions (Supplementary Table 5). Minims participated in tasks in several categories, reaching the highest RPTP scores for hygiene and fungal gardening acts. Small medias had non-zero RPTP scores for all task categories, and participated most frequently in brood care, corresponding to statistical differences in task-performance frequencies. Mid-sized medias also had a non-zero RPTP for all task categories; their highest RPTP score was for leaf harvesting. Diverging

Table 3 Differences in correlation patterns between identified worker size groups. Percentages of the total number of correlations (231 unique pairs) that were statistically significantly different ($p < 0.05$) in magnitude (above diagonal) or direction (below diagonal) of Pearson and partial correlations between pairs of individual traits. Significance levels were assessed using z-tests between Fisher-transformed coefficients

	Minims		Small medias		Mid-sized medias		Large medias		Majors	
	Pearson	Partial	Pearson	Partial	Pearson	Partial	Pearson	Partial	Pearson	Partial
Minims			35%	1.3%	35%	1.1%	25%	0.6%	26%	0%
Small medias	13%	3.2%			26%	1.1%	39%	1.7%	25%	1.9%
Mid-sized medias	13%	2.4%	0%	4.1%			35%	1.5%	20%	2.8%
Large medias	11%	2.8%	0.4%	5.6%	0.4%	5.0%			13%	0.6%
Majors	10%	5.2%	5.4%	3.2%	5.4%	2.2%	1.5%	4.3%		

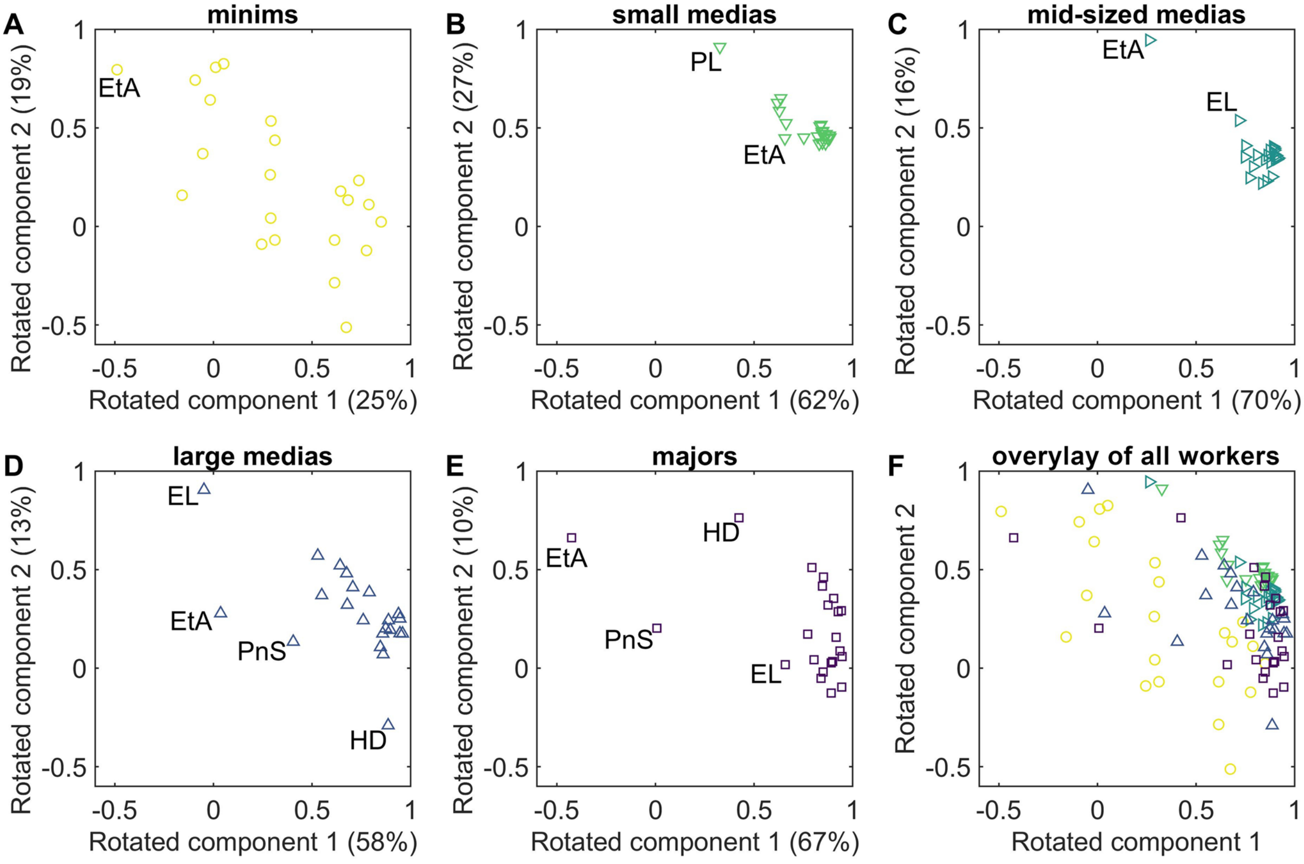

Fig. 4 Principal component analysis of morphological traits in identified *A. cephalotes* worker groups. These plots illustrate the degree of clustering vs. independence between morphological traits in different worker groups. Distance between trait labels is approximately proportional to the correlation between measurements of those traits in individuals from that worker size group. **A** Minims (yellow circles); **B** Small medias (green downward triangles); **C** Mid-sized medias (teal right-facing triangles); **D** Large medias (blue upward triangles); **E** Majors (purple squares); **F** Overlay of all worker groups. In each case, traits are plotted within the morphospace defined by the first two principal components after varimax (variance maximization) rotation. Values in parentheses indicate the covariance proportions explained by each principal component. Traits separated from major trait clusters in **B–D** are labeled in the corresponding graph, as well as in the remaining graphs if feasible. EtA, distance from eye to antenna; PL, petiole length; EL, maximum eye diameter; PnS, pronotal dorsal spine length; HD, head depth

from the statistical analysis result, large medias had the highest overall leaf-harvesting RPTP relative to all other worker groups. However, this size class typically did not participate in other tasks. The total number of task categories performed at least once by each worker size class was 3, 4, 4, 1, and 0 for minims, small-, mid-, large-sized medias, and majors, respectively, serving as an indicator of the relative breadth of their task repertoires. Majors performed relatively few tasks, a result that was not unanticipated given that their defensive role could not be critically examined in the lab.

Comparisons of Shannon diversity indices of behavioral repertoires among worker size classes showed that small medias performed the broadest array of tasks (1.310 diversity index score; larger values are more diverse), whereas mid-sized medias and minims performed similarly broad task arrays (0.964, 0.930). All these groups showed greater behavioral flexibility than large medias (0) and majors (0). These patterns correspond with the overlap in task performance frequency distributions in the media size range (Fig. 5A), and the non-zero RPTP scores for mid-sized medias for all task categories analyzed (Fig. 5B). Estimation of pairwise task repertoire Morisita-Horn similarity indices identified strong overlap between minims and small medias (0.832), between small- and mid-sized medias (0.735), and between mid- and large-sized medias (0.886). There was little overlap between minims and large medias (0.128), and moderate overlap between small and large medias (0.443), and between minims and mid-sized medias (0449).

Discussion

Multivariate analyses of worker size and shape variation (Diniz-Filho et al. 1994; Pie and Traniello 2007; Pie and Tschá 2013) can improve the resolution of physical caste evolution in ants. Our statistical modelling indicated that *A. cephalotes* workers can be divided into five distinguishable size groups, supporting the evolution of a fine-grained physical caste system previously undescribed in *Atta*. Correlation analyses indicated statistically significant between-group differences in trait covariance patterns. Minims showed the least coordination and greatest modularity among morphological characters and the highest degree of integration was found in small- and mid-sized medias. Interestingly, the trait covariance patterns of large- and mid-sized medias reflected transitional states between neighboring size groups, although mid-sized medias were more similar to large- than small-sized medias. These similarities in trait covariance patterns may reflect the evolutionary expansion and gradual morphological divergence of worker size in fungus-growing ants, with the most generalized workers (small- and mid-sized medias in *A. cephalotes*), likely most closely resembling an ancestral worker phenotype. Applying our morphological analyses across fungus-growing species with lower

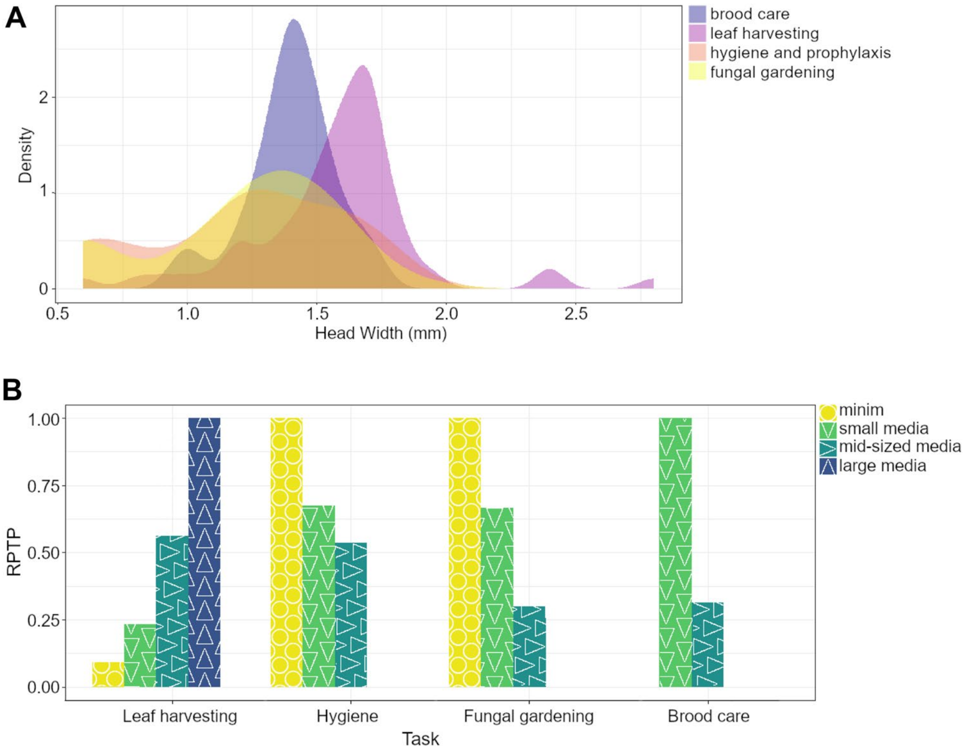

Fig. 5 **A** Task performance likelihood as a function of worker size for each task category. These data illustrate both the spectrum of worker sizes most commonly performing different tasks, and which tasks were most frequently performed by a given worker group. Plots show probability density functions based on total counts of workers of all sizes performing task sets. Blue, brood care; purple, leaf-harvesting; orange, hygiene and prophylaxis, yellow, fungal gardening. **B** Relative probability of task performance (RPTP) for each task category and each worker size group. Yellow circles, minims; green downward triangles, small medias; teal right-facing triangles, mid-sized medias; blue upward triangles, large medias. Majors are not shown, as they were not observed performing any of the examined tasks

degrees of polymorphism (the paleoattines) can determine whether covariance patterns similarly reflect the divergence of worker groups from an ancestral caste (Mehdiabadi and Schultz 2010).

In *A. cephalotes,* morphological characters that diverged across worker size groups involved the head, mandibles, and legs and thus could functionality correlate with the performance of tasks such as leaf cutting and fungal gardening. Smaller media workers showed high levels of morphological integration, but as medias increase in size, a degree of modularity that further increased in majors was found. This trend of reduced integration with size was exemplified by a decrease in the percentage of variance explained by the top PC in majors. For example, there was a weakening of the correlation between leg traits (FL and TL) in larger medias. This trend towards modularity in the leg may relate to biomechanical requirements of leaf cutting and leaf transport. The compass-like leaf cutting motion in which metathoracic legs anchor the body while rotating to cut a circular arc (Roces and Hölldobler 1994) may be performed with greater efficiency due to this appendage allometry. Indeed, leg length scales allometrically among fungus-growing ant workers (Feener et al. 1988) and body size is important in leaf harvesting (Wilson 1980b; Roces and Hölldobler 1994; Burd 2000). Similarly, the correlation between HW and MHW that is strong in minims and small- and mid-sized medias was not found in large medias and majors. MHW reflects the extent of mandibular muscle development (Püffel et al. 2021). The allometric increase in MHW in larger workers indicates disproportionate investment in these muscles to produce greater bite force (Gronenberg et al. 1997; Paul and Gronenberg 1999), which is likely important for their defensive roles (Powell and Clark 2004).

Minims similarly showed a high degree of modularity, specifically with respect to PL, PPH, and PW. These small body-segment dimensions may not be coordinated with the scaling of other morphological traits in minims due to selection favoring miniaturization to enhance movement through the fungal comb during gardening. However, whether these differences offer biomechanical benefits or were subject to independent selection is unclear.

The high degree of modularity in minims relative to other workers groups could alternatively reflect evolutionary constraints on *A. cephalotes* worker polymorphism or developmental pathways (Molet et al. 2012; Trible and Kronauer 2017, 2021; Friedman et al. 2020; Abouheif 2021), although the history of this process in minims is unknown. The diversity of related fungus-growing ant species that also produce minims enables analysis of the degree of modularity in these workers. Applying our methods phylogenetically could generate hypotheses that may identify the evolutionary and developmental processes governing polymorphism in fungus-growing ants as a clade.

Worker size and morphology can determine worker task performance and efficacy in strongly polymorphic species, although monomorphic worker behavior may correlate with age or other factors (Mertl and Traniello 2009; Waddington and Hughes 2010; Grüter et al. 2012; Gordon et al. 2017; Josens et al. 2018; Honorio et al. 2020; Powell et al. 2020; West and Purcell 2020; Wagner et al. 2020). Significant differences in worker size-related task frequencies provided evidence of strong subcaste division of labor in *A. cephalotes*. Differences in behavioral performance did not consistently conform to morphologically discernable groups, but instead varied on a task-by-task basis. For example, leaf harvesting was performed by four size classes and fungal gardening and hygiene tasks were performed by three size classes, although group-specific performance of leaf-harvesting and fungus-gardening tasks significantly differed from the expected rates, reflecting either an over- or under-emphasis on such behavior in certain workers. Brood care was also performed by two size classes, without significant differences in act frequency. The number of morphologically defined groups may therefore diverge from the number of groups characterized by specialized behavioral repertoires, if task frequency is not considered. This is supported by the Morisita-Horn similarity index overlap among adjacent pairs of worker groups.

In *A. sexdens*, "fixed" tasks are performed exclusively by a specialized set of workers of a given size range, whereas "flexible" tasks are performed by workers of many different sizes or ages depending on the context of the task (Wilson 1980a). In our study of *A. cephalotes*, rates of fungal gardening and leaf harvesting differed significantly with worker size. Nursing did not significantly differ among workers groups that participated in this task but was only performed by small- and mid-sized medias. This result, and evidence that several tasks were performed by multiple size groups to various extents even if one size group predominantly performed a given task, indicates that *A. cephalotes* division of labor can be flexible. This may relate to the low number of clearly identifiable gaps in the worker size frequency distribution, despite the ability to robustly and quantitatively distinguish workers morphologically by leveraging trait covariance patterns. Workers of body sizes distributed at the boundaries of defined size classes may overlap in task repertories, even if dissimilar in allometric scaling patterns.

In summary, we found the association between worker size class and task performance was clear in terms of act frequency, but not all tasks were performed exclusively by only one size class, and morphological groups do not all have distinct behavioral repertoires. Workers in the large-media group showed the highest RPTP score for leaf harvesting, although they did not perform this task with the highest frequency. Instead, mid-sized medias performed leaf harvesting at a rate significantly differing from would be expected based on their relative demography, and at a higher rate than other worker groups.

Quantification of size-related task performance patterns showed that small- and mid-sized medias have the broadest task repertoires, and mid- and large-sized medias showed more leaf-harvesting behavior, whereas minims performed most fungal-gardening acts. The diversity of tasks performed by medias, including their capacity for learning and memory of foraging routes and leaf selection (Saverschek et al. 2010; Saverschek and Roces 2011; Thiele et al. 2014; Travaglini et al. 2015; Arenas and Roces 2016, 2017), is likely neurally supported by allometric enlargement of their mushroom bodies (Muratore et al. 2022), centers of higher-order processing that may provide circuitry to perform more tasks with a wider array of behavioral and/or cognitive demands (Riveros et al 2009, 2012; Muscedere and Traniello 2012; Muratore and Traniello 2020). Mid-sized medias also had the largest antennal lobes (Muratore et al. 2022), which likely support olfactory leaf discrimination. The overlap in task performance by adjacent worker groups similarly has a potential neuroanatomical correlate: workers in adjacent classes in the media size range do not significantly differ in the volumes of most brain compartments (Muratore et al. 2022). Mosaic brain scaling patterns may therefore reflect gradual behavioral divergence among adjacent worker size groups more closely than statistically identified morphological differentiation.

Minims showed higher fungal gardening RPTP values than other workers and this task was also performed by medias, albeit to a lesser extent. Both minims and medias were observed nursing, although RPTP values for brood care were greatest for small- (0.56) and mid-sized medias (1.0). Worker groups with the highest hygiene RPTP scores (minims and small medias) also showed lower social contact RPTP scores relative to larger medias (i.e., mid-sized medias; large medias and majors did not frequently perform this task). This may relate to hygiene-specialized workers that socially isolate to lower infection risk (Hart and Ratnieks 2001). RPTP values indicate the smallest two workers groups were both more likely to perform hygiene tasks crucial to maintain the health of gardens (Fernandez-Marin and Wcislo 2003; Hölldobler and Wilson 2010), and less likely to engage in social interactions.

A. cephalotes small- and mid-sized medias had the broadest repertoires, performing many tasks flexibly as well as specialized tasks associated with leaf harvesting and likely colony defense. Large- and mid-sized medias showed the highest rates of leaf-harvesting behavior. These results correspond to the estimated optimum of 2.2–2.4 mm HW for leaf cutting in *A. sexdens* (Wilson 1980b). *Atta* majors primarily defend against army ants in nature (Swartz 1998; Sánchez-Peña and Mueller 2002; Powell and Clark 2004; Powell 2011), but were not observed engaging in defense in our study. However, majors were readily recruited to sites of physical disturbances (vibration or air currents), and all workers larger than ~1 mm HW, including majors, aggressively responded to contact with workers of other ant species.

Our integrated analyses of division of labor in *A. cephalotes* provide novel methods to quantify worker size variation and understand morphological and behavioral phenotypes neuroanatomically (Muratore et al. 2022) and transcriptomically (Muratore et al. 2023). The approaches we used to identify task profiles associated with morphological differentiation can be applied to other social insect systems to test hypotheses regarding the origin and evolution of specialization by physical castes. Our analysis of *A. cephalotes* identified five worker size groups and enabled the degree of integration and modularity of morphological traits to be distinguished among subcastes, thus offering insight into how suites of traits may be influenced by selective pressure related to the biomechanics of behavioral performance. Our statistical differentiation of size classes among polymorphic workers expands the study of caste evolution and accentuates the value of integrating morphological, sociobiological, and neuroanatomical analyses of division of labor.

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Author contributions IBM, JFAT, and II designed the study. IBM drafted the manuscript. IBM, JFAT, and II developed the manuscript. IBM, JFAT, and II edited the manuscript. AKH and IBM collected morphometric data. IBM collated and processed raw measurement data. II performed and interpreted clustering analyses of morphological data and other statistical modeling. FHZ collected behavioral data. JFAT secured funding.

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Data availability All behavior and morphology data are available in supplementary tables.

Code availability Code used for analysis is available at: https://github.com/Imuratore-bio/ant-morphology-behavior.

Declarations

Ethics approval We collected ant colonies in compliance with the conditions of USDA APHIS Permit P526P-12-04067 and in accordance with the laws of countries of origin.

Competing interests The authors declare no competing interests.

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Authors and Affiliations

I. B. Muratore[1] · **I. Ilieş**[2] · **A. K. Huzar**[1] · **F. H. Zaidi**[1] · **J. F. A. Traniello**[1,3]

✉ I. B. Muratore
isabella.b.muratore@gmail.com

[1] Department of Biology, Boston University, 100 Summit Street, Newark, NJ, MA 07103 Boston, USA

[2] Healthcare Systems Engineering Institute, Department of Mechanical and Industrial Engineering, Northeastern University, Boston, MA, USA

[3] Graduate Program in Neuroscience, Boston University, Boston, MA, USA