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Caste-specific morphological modularity in the ant tribe Camponotini (Hymenoptera, Formicidae)



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Abstract

Background: The morphological structures of organisms form tightly integrated but mutually independent character complexes (modules) linked through common development and function. Even though their abundance, diversity, and complex caste systems make camponotine ants ideal subjects to research developmental modularity and phenotypic integration, no studies investigating these phenomena have been conducted in this taxonomic group. This study attempts to identify and visualize integrated character complexes in 14 taxa from the genera *Camponotus* and *Colobopsis* using statistical analyses of morphometry.

Results: The identified modules differ between castes: Minor workers have small heads and long extremities, while major workers have enlarged heads modified for defence, and short, thick appendages; extremities (legs and antennae) are strongly correlated in both worker castes. Gynes show weaker integration of extremities, but a strong correlation of mesosoma and eyes, and highly variable median ocellus size. Gynes infested by mermithid nematodes exhibit reduction of gyne-specific characters and altered patterns of phenotypic integration.

Conclusion: The integrated character complexes described herein can largely be interpreted as functional, caste-specific modules related to behavioural ecology and task allocation within ant colonies. This modular nature of the body plan is hypothesized to facilitate the evolution of novel phenotypes and thus contributes to the tremendous evolutionary success of ants. The study of these modules can help to further elucidate the evolution and ontogeny of castes in camponotine ants, as well as the effects of parasite infestation on the phenotype.

Keywords: Cluster analysis, Division of labour, Modules, Morphometry, PCA, Social insects, Phenotypic integration

Background

Phenotypic integration and modularity

The concept of phenotypic integration was first popularized in 1958 by Olson & Miller [1]. It states that, when viewing an organism as a whole, certain morphological structures covary more strongly than others, forming spatially and temporally interdependent modules due to commonalities in development and function. It thereby becomes evident that the concept of phenotypic integration is closely linked to the topic of modularity: In most known organisms, certain groups of elements –

morphological, genetic, or otherwise – exhibit strong interconnection and correlation, but are relatively independent of other such groups, thereby dividing organisms into distinct, heterogeneous parts or “modules”. The concept of modularity has gained great interest in the fields of evolutionary, developmental, and systems biology within the last decade [2–4]; for reviews see [5–8].

While most research on the phenomenon has been performed on vertebrates [1, 3, 9–11] and flowering plants [12, 13], insects are rather poorly studied in this regard [14]. Depending on the investigated traits and applied methodology, previous authors have reported either intermediate [15, 16] or very high [14, 17] levels of phenotypic integration in holometabolous insects.

To date, there have been very few studies directly investigating how patterns of integration and modularity differ between castes and species of the family

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Formicidae and none in the diverse tribe Camponotini. Most studies investigating integration and modularity in ants are either conceptual in nature [18, 19] or focus on single phenotypes or species [20–22]. Some noteworthy exceptions are publications describing caste-specific patterns of modularity in *Pheidole* [17] or highlighting trade-offs and correlations between defensive structures in ants [23]. Comparing such phenomena across ant castes and taxa is especially intriguing: Firstly, the workers of a colony are closely related and the diversity of castes has been attributed to high degrees of phenotypic plasticity and modularity [18, 19, 24, 25]. Secondly, phenotypically integrated modules frequently correspond to structures associated with particular functions and behaviours, e.g. locomotion, foraging, or defence [10, 11, 17, 23] and may thus be connected to phenomena of functional specialization and division of labour commonly observed in ants exhibiting morphologically distinct castes. By analysing caste- and species-specific patterns of integration and modularity, we can therefore gain a better understanding of the functional and ecological roles of morphological structures and character-sets.

Caste-specific morphological specializations in Camponotini

The ant tribe Camponotini currently includes 1896 species in eight genera [26–28]. Females of the camponotine species are particularly known for their diversity of morphologies within the worker caste [29–32]. The most prominent cases of worker caste dimorphism are found in the genera *Camponotus*, *Colobopsis* and *Dinomyrmex*: most commonly, the smaller minor workers (Fig. 1) engage in foraging, brood care and nest-maintenance, while the major workers (often termed “soldiers”) have large heads and mandibles specialized for defence [28, 30, 33] (Fig. 2a, b, d, f).

Alate sexuals (gynes and males) in the Camponotini are typically characterized by large eyes and ocelli, and an enlarged mesosoma to accommodate the flight muscles [34, 35]. In contrast to the larger gynes (Fig. 2c), males usually have a small body and head, as well as long and fragile extremities [33]. Their short lifespans [36] and mating flights make them much harder to sample than their conspecific females, leading to their scarcity in museum collections and low number of publications describing male phenotypes. As males of the Camponotini are still insufficiently studied and taxonomic relationships in older descriptions are partially doubtful, this study uses the recently described males of *Colobopsis explodens* (see Fig. 6 in [32]) as an example.

In his seminal work, Wilson [37] used morphometric data to characterize different forms of allometry and polymorphism in various species of ants. For the genus

Camponotus in its current state, his results indicated a gradual transition from monophasic allometry to complete dimorphism of the worker caste, which has arisen independently multiple times. He especially mentioned the pronounced dimorphism within the current genus *Colobopsis*, where the specialized major workers bear plug-shaped heads used to close the small entrance-holes in wooden nests. This phenomenon of animals using parts of their bodies to obstruct their nests is termed “phragmosis”. Several cases of independent evolution of diverse phragmotic structures have also been described in at least five other genera and subgenera of ants [33].

Most traditional theories assume the origin of the major worker or soldier caste from minor workers [37, 38]. Other authors, however, have strongly disputed this, regarding majors as a caste derived from queens. This assumption is based on many morphological similarities and observations of larval development [39, 40]. More recent publications, however, propose the evolution of specialized castes as mosaics of pre-existing or ancestral developmental patterns [18, 24, 41]. Others consider the influences of genetic and epigenetic mechanisms [42], disruptive selection [43], or hormonal regulation [44] on caste evolution, leaving the issue open to further investigation.

In addition to the genus-specific phragmotic major workers, minor workers of the Southeast Asian *Colobopsis cylindrica* (COCY) group (Figs. 1a, b, d; 2a, c, e) have evolved a peculiar specialization as well: In territorial combat with other arthropods, they can rupture their enlarged mandibular gland reservoirs and their gastral integument, thereby covering their enemy in a sticky, toxic defensive secretion and sacrificing their own lives in the process [31, 32, 45–49]. The roughly 15 species included in the group have been preliminarily divided into four subgroups (complexes) based on molecular and morphological data (Zettel et al., in prep.). Members of the *Colobopsis saundersi* (Figs. 1a; 2a, c, e), *C. leonardi* (Fig. 1b) and *C. cylindrica* (s. str.) (Fig. 1d) complexes are treated in the present study.

One especially intriguing aspect of the hitherto investigated COCY colonies is the frequent presence of so-called “interlopers” [31, 48, 50] – presumably mimetic and parabiogenic ants belonging to *Camponotus* (*Myrmamblys*) (Figs. 1e, 2f), whose minor workers are often found inside COCY nests [50]. The inclusion of the example species *Camponotus inquilinus* in this study aims to further assess their morphology and compare it to that of their putative hosts.

Mermitogenic phenotypes

The influences of parasitic nematodes of the family Mermitidae on ant-hosts have long been a subject of scientific study [51], especially as the infestation is often

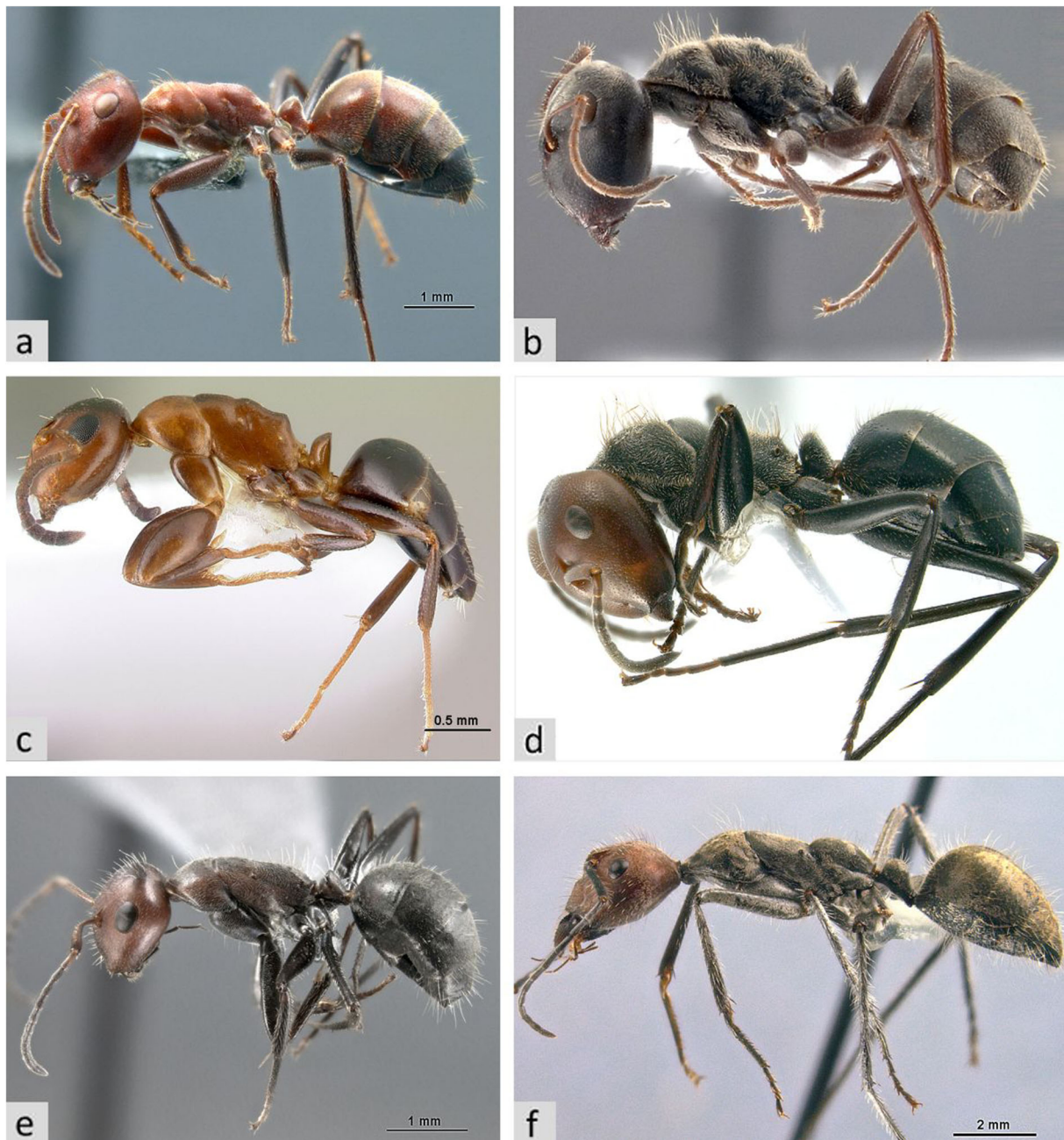


Fig. 1 Example minor worker specimens of investigated taxonomic groups, lateral view; **a**) *Colobopsis explodens*, minor worker (from [32]); **b**) *Colobopsis* sp. "BBQ", minor worker (© A. Laciny); **c**) *Colobopsis truncata*, minor worker (from [62], CASENT 0179881); **d**) *Colobopsis cylindrica*, minor worker (© A. Laciny); **e**) *Camponotus inquilinus*, minor worker (from [50]); **f**) *Camponotus singularis*, minor worker (from [30])

accompanied by aberrant morphologies in the hosts. Such parasitized individuals are known from several subfamilies of ants [51, 52] and have been observed to infest specimens of the genus *Colobopsis* [31, 53]. Studies on *Lasius* [54–56] and *Myrmica* [57] revealed that all known mermithogenic phenotypes develop from larvae destined to be sexuals, i.e., gynes or males, and found no infestation of workers. Parasitized gynes are often morphologically intermediate between castes,

resulting in so-called intercaste phenotypes [51]. Characteristic changes include reduced body size, elongated extremities, enlarged gaster (due to distension by the nematode), reduced size of head, deviations in pilosity and sculpture, as well as reduction of all sexual characters (wings, thoracic sclerites, ovaries, and ocelli, Fig. 2e) [31, 51, 54, 55, 58, 59]. The study of morphological changes brought about by this form of parasitism can provide insight into phenotypic plasticity and

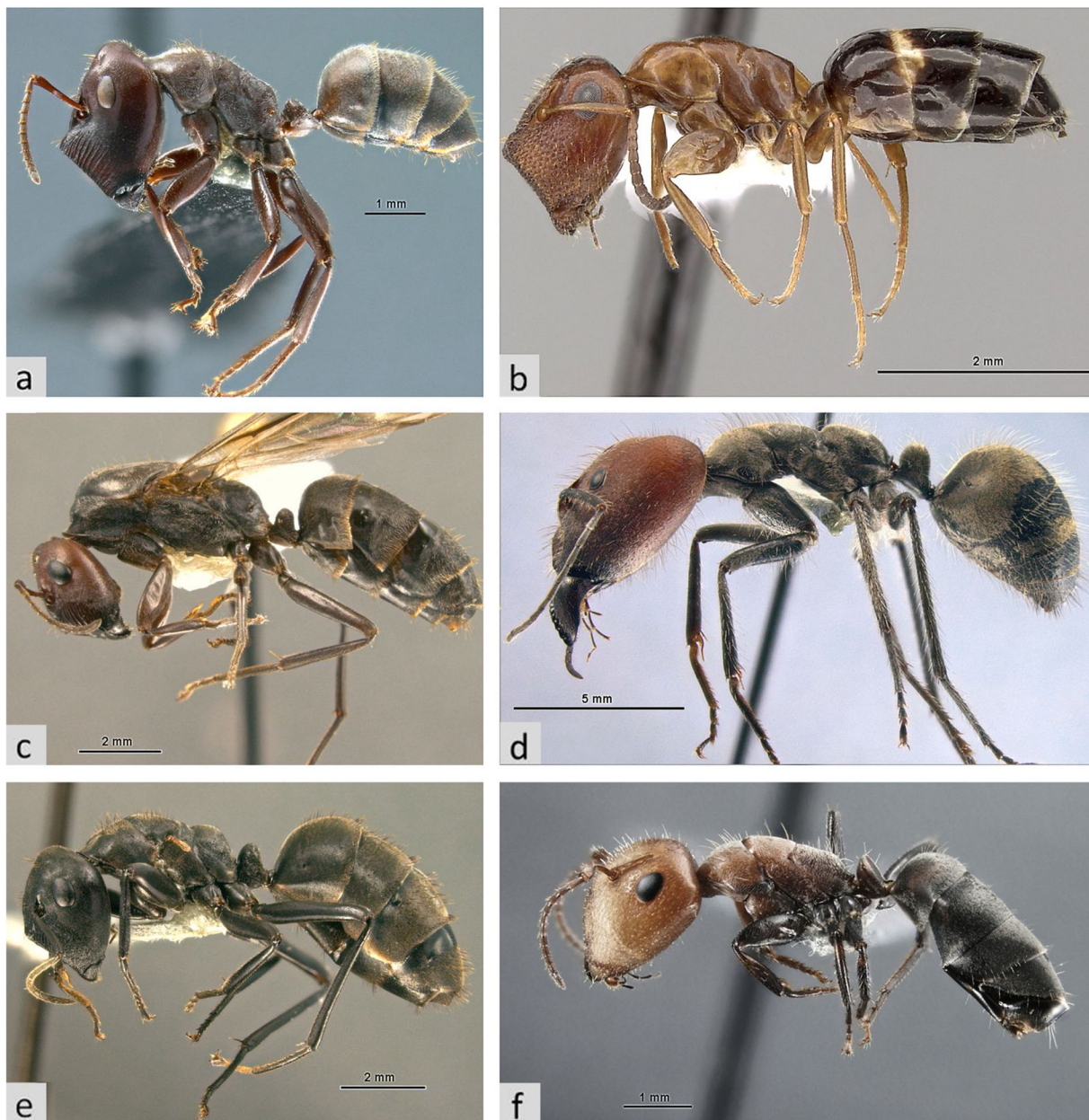


Fig. 2 Example major worker, gyne and MIC specimens of investigated taxonomic groups, lateral view; **a**) *Colobopsis explodens*, major worker (from [32]); **b**) *Colobopsis truncata*, major worker (from [62], CASENT 0249998); **c**) *Colobopsis* sp. "nrSA" alate gyne (from [31]); **d**) *Camponotus singularis*, major worker (from [30]); **e**) *Colobopsis* sp. "nrSA" mermithogyne (MIC) (from [31]); **f**) *Camponotus inquilinus*, major worker (from [50])

developmental modularity in the studied species [18, 19, 24, 31]. Following previous studies in *Colobopsis* and *Lasius* [31, 56], this study hopes to further clarify how developmental perturbation by mermithid parasites influences host-morphology and patterns of integration in gynes of the studied species.

Aims and scope

This study assesses morphometric data of 14 taxa from two camponotine genera with well-known, pronounced

caste dimorphism – *Camponotus* and *Colobopsis*. We thereby aim to address the following research questions: Are the selected morphometric characters suitable to assign the studied specimens to their respective castes? Do patterns of proportions and correlations between these characters differ across castes and taxa? Can we identify caste-specific morphological modules, and are these modules interpretable regarding our current knowledge of behavioural ecology and task-allocation? Answers to these questions will support the interpretation of caste-

characteristic specializations in morphology and behaviour, and lay the groundwork for further studies on caste-development and division of labour in social insects.

Methods

Material examined

In the course of this study, 332 specimens from 14 taxa were measured and examined. The sample was composed of 231 minor workers, six males, 36 major workers, 54 gynes, two intercaste specimens (IC) and three mermithogenic intercaste specimens (MIC). To simplify the analyses, specimens of the COCY group were sorted into species complexes as accurately as current taxonomic knowledge allows; example images for each species or complex are shown in Figs. 1 and 2. The complete list of specimens and depositories is provided in Additional file 1.

Notes on caste definition: While the distinction between minor workers, major workers, gynes and males is based on clear morphological characters (i.e., wings, ocelli, genitals, phragmotic or enlarged heads) and therefore self-evident in most of the examined camponotine species, some classifications had to be performed according to the individual perception of the researchers. Workers of the *leonardi* and *cylindrica* COCY complexes exhibit a continuous transition from minor to major worker; major workers were therefore only classified as such, when a clear phragmotic modification of the head (clypeal shield, cuticular ridges) was evident. Intercaste (IC) specimens were defined as those individuals whose morphology presented as a combination of two or more of the known castes. In three specimens, intercaste morphology was known to be caused by infestation with mermithid nematodes; they were thus denoted as mermithogenic intercaste (MIC) phenotypes.

Measurements

Seven morphological measurements (modified from [31, 32]) were taken from all specimens. In addition, median ocellar width (OcW) was measured in all gynes and MIC specimens possessing ocelli. All examined specimens were card-mounted or pinned, individually numbered, and measured at magnifications from 25.6 × up to 256 × with a Nikon SMZ1500 binocular microscope. All measurements were taken in millimetres and logarithmically transformed for statistical analyses. For a visual depiction of measurements, see also Figs. 15, 17, and 19. Measurement errors (standard deviation, relative error, and standard error) were assessed via 10 repeat measurements of all characters on representative specimens from the minor worker and gyne caste of *C. explodens*. The complete dataset of specimens, measurements, and error statistics is provided as Additional file 2: Table S2.

HW Head width. Maximum width of head in full-face view, including eyes if laterally protruding.

HL Head length. Maximum length of head in full-face view, excluding mandibles, measured parallel to midline from anterior-most point of clypeus to midpoint of occipital margin or to midpoint of an imaginary line connecting the apices of posterior projections (major workers and some gynes of *C. singularis*).

EL Eye length. Maximum diameter of compound eye.

SL Scape length. Maximum length of antennal scape in dorsal view excluding basal neck and condyle.

SW Scape width. Maximum width of antennal scape in dorsal view, usually in distal third.

WL Weber's length. Maximum diagonal length of mesosoma. Measured laterally from anterior surface of pronotum proper (excluding collar) to posterior extension of propodeal lobes.

FeL Femur length. Maximum length of hind femur, measured from base to apex.

OcW Ocellar width. Maximum width of median ocellus (if present).

Imaging of specimens

MicroCT imaging

A selected sample of specimens (*Colobopsis explodens* minor worker, *Colobopsis* sp. "CASA" major worker, *Colobopsis* sp. "BBQ" gyne) was used for X-ray microtomographic imaging to illustrate examples of caste-specific morphology [60, 61] (Figs. 14, 16, 18). All specimens were stored in 70% ethanol prior to imaging and prepared according to the following protocol:

Specimens stored in 70% EtOH, transferred to 1:1 Bouins solution (96–100% EtOH) 3 h – overnight; 70% EtOH (> 30 min.); 96% EtOH (> 30 min.); 100% EtOH (> 30 min.); I2E stain (1% iodine, 96–100% EtOH) overnight; stored in 100% EtOH until scan.

Fixed and stained specimens were then scanned in 100% EtOH or embedded in agarose. To mount specimens for scanning, they were inserted into pipette tips that were glued onto plastic blocks and sealed with Parafilm and / or wax. X-ray microtomographic images were made with a high-resolution microCT system (Xradia, MicroXCT-200, Zeiss X-Ray Microscopy, Pleasanton, CA) with a tungsten microfocus X-ray source and variable secondary optical magnification. Scans were made with an anode voltage setting of 60–80 kV and 50–130 μA with an exposure time of 5–25 s for projection images every 0.25 or 0.20°. Tomographic reconstructions were exported as image stacks with isotropic voxel sizes of 2.2–15.0 μm. Using 4X optical magnification, the minor worker specimen was scanned as a whole, major worker and gyne specimens were scanned in two parts (anterior and posterior half of body) and stitched after reconstruction. Additional scans using 1X magnification were performed for whole body scans of large specimens in some cases.

Images were reconstructed with Xradia software, and processed with Amira 6.3.0 and Adobe Photoshop 7.0.

Stacking photography

To visualize species- and caste-specific morphology, including colouration, pubescence and cuticular micro-sculpture, selected specimens of all investigated species-groups (Figs. 1, 2) were imaged photographically. Images of *C. truncata* minor and major workers were obtained from AntWeb.org [62].

Digital stacked images (Figs. 1a, b, d-f; 2a, c-f) were taken with a Leica DFC camera attached to a Leica MZ16 binocular microscope with Leica Application Suite v3 and stacked with Zerene-Stacker 64-bit. All images were processed with Adobe Photoshop 7.0.

Statistical analysis

All statistical analyses were conducted in Past3 [63] using log-transformed morphometric data.

A linear discriminant analysis of the entire sample based on a variance-covariance matrix, using the hypothetical caste-assignments (minor, major, male, gyne, intercaste) as groups was conducted. A Jackknife resampling procedure was added to cross-validate the group assignments. The resulting confusion matrix was used to assess the validity of the chosen measurements for caste classification.

To assess the expression of phenotypic integration over the complete character set, and identify the characters and character-combinations most relevant to total variance, a principal component analysis (PCA) based on a correlation matrix with 1000 bootstrap resamplings was

conducted on the entire sample. To enable visual distinction in the resulting scatterplots, the respective castes were assigned a colour, and species (species groups) were allocated a shape, each consistent throughout all featured graphs. Identical PCAs were subsequently conducted for the sample of minor workers and gynes including MIC to identify caste-specific morphological patterns. The sample of major workers proved to be too small for PCA and was only analysed via cluster analysis (see below).

To assess the interdependences of characters and allow an alternative, hierarchical visualization, cluster analyses of the minor worker, major worker, and gyne samples were conducted. To this end, the R-mode clustering function in Past3 was used on the transposed character matrix, applying the UPGMA algorithm and correlation as similarity index, with 1000 bootstrap resamplings.

Separate analyses of the remaining castes or of single species were omitted due to insufficient sample size. Graphical representation of results were created in Past3 [63] and processed with Adobe Photoshop 7.0.

Results

Linear discriminant analysis (LDA)

The linear discriminant analysis (LDA) of all specimens, using seven characters and five caste-assignments as groups, yielded a correct classification of 93.37% after Jackknifing (Fig. 3, Table 1). Axes 1 and 2 shown on Fig. 3 explained 64.59 and 30.91% of total variance, respectively (Table 2). Based on the given morphometric data, all males were correctly classified, as well as all IC/MIC phenotypes except one (classified as a minor worker) and all gynes

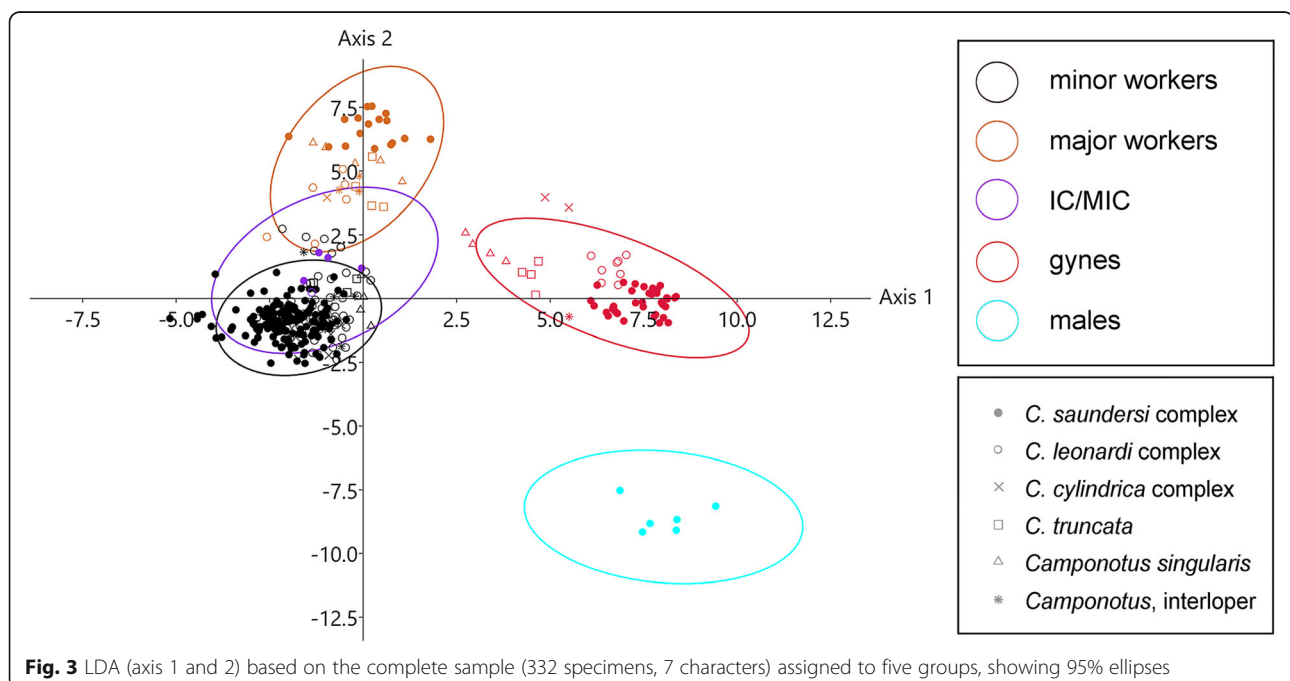


Table 1 Confusion matrix after linear discriminant analysis (LDA)

Caste	Hypothesis	LDA assignment	classification by LDA				
			Minor	male	major	gyne	IC/MIC
minor	231	216	215	0	0	0	16
male	6	6	0	6	0	0	0
major	36	35	0	0	33	0	3
gyne	54	52	0	0	2	52	0
IC/MIC	5	23	1	0	0	0	4

Caste-assignments of 332 specimens before and after LDA with Jackknifing; confusion matrix showing subsequent classifications resulting in 93.37% congruence with the hypothetical assignments

except two of *C. singularis* (classified as major workers). Most interestingly, three major workers and 14 minor workers of the *C. leonardi* complex, as well as two minor workers of *C. singularis* were classified as intercastes.

Principal component analysis (PCA)

Subsequently, a principal component analysis was conducted on the entire sample as well as for the subsamples of gynes and minor workers, to identify which characters or character combinations contributed most meaningfully to the principal component axes and their respective variances.

Complete sample

PC1 contributed 84.53% of the variance and exhibited overall similarly high, positive loadings of all characters. This pattern is often considered a “size axis” [17, 22, 64]. PC2 accounted for 10.16% of the variance (Table 3) and exhibited the highest positive loadings for SL and FeL, and high negative loadings for EL and WL (Fig. 6a, Additional file 3: Table S3). Thus, all specimens included in the sample can be roughly classified in a space ranging from “small body vs. large body” on PC1 – the most extreme cases being *C. truncata* minor workers (Fig. 1c) as the smallest and *C. singularis* gynes and major workers (Fig. 2d) as the largest – and “small eyes and mesosoma with long appendages vs. large eyes and mesosoma with short appendages” on PC2 – the extremes exemplified by *C. singularis* minor workers and *C. truncata* gynes and majors, respectively (Fig. 4). A further noteworthy result of this analysis was the placement of *C. leonardi*-complex major workers: While in all other taxa the major workers were clearly set apart from their conspecific minor workers by a lower placement on PC2

Table 2 Axes 1 to 4 of linear discriminant analysis (LDA)

Axis	Eigenvalue	% of total variance
1	10.641	64.59
2	5.0914	30.91
3	0.639	3.88
4	0.1023	0.62

LDA based on 332 specimens, presented with eigenvalues and the percentage of total variance explained by each axis

(e.g., due to shortened appendages), this was not the case for these major workers, which mainly differed from their conspecific minors in average body size (higher loadings on PC1). A very similar pattern was observed for all the IC/MIC specimens and the single major of the *C. cylindrica* complex, which also mainly differed from conspecific minor workers by higher loadings on PC1. Males were clearly set apart from female castes due to their small size, and enlarged mesosoma and eyes, resulting in low placement on PC1 and PC2 (Fig. 4).

PC3 accounted for 2.76% of the total variance (Table 3) and was characterized by strong negative loadings for HW, HL and SW, as well as strong positive loadings for EL, FeL and WL (Fig. 6b, Additional file 3: Table S3). PC3 is especially suitable to better distinguish between conspecific gynes and major workers of the investigated species: Major workers are consistently placed lower on PC3, due to the combination of large heads, thick scapes, short femora, and a comparatively short mesosoma, while gynes exhibit the contrasting traits (Fig. 5) and IC / MIC specimens occupy intermediate positions. Male specimens were again set apart from their conspecific females by very high placement on PC3, as a result of their small head, enlarged mesosoma and elongated legs (Figs. 5, 6b).

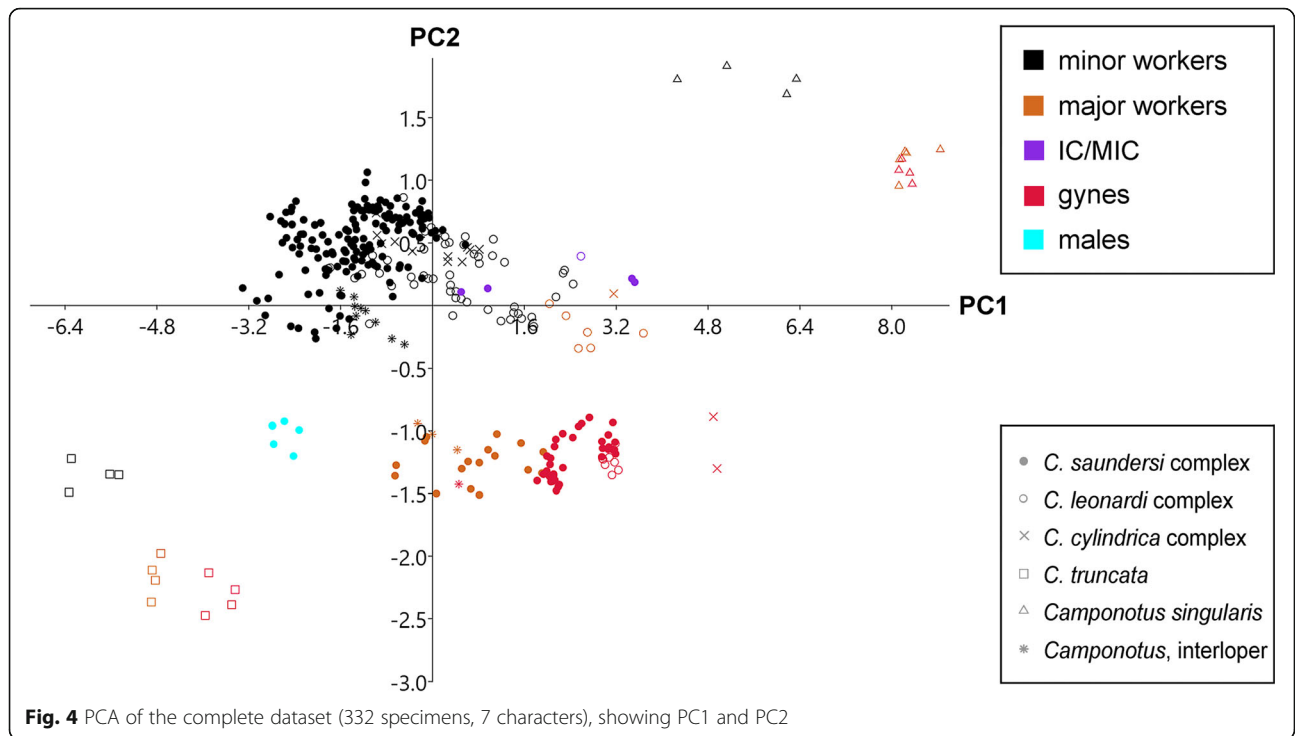
Minor workers

Considering only the 231 specimens of the minor worker caste, the PCA yielded 88.9% variance for PC1, 5.64% for PC2, and 2.29% for PC3 (Table 4). PC1 was again considered mainly an indicator of overall size. PC2 exhibited very strong positive loadings for appendage lengths (SL and

Table 3 First three principal components (PC) for the complete dataset

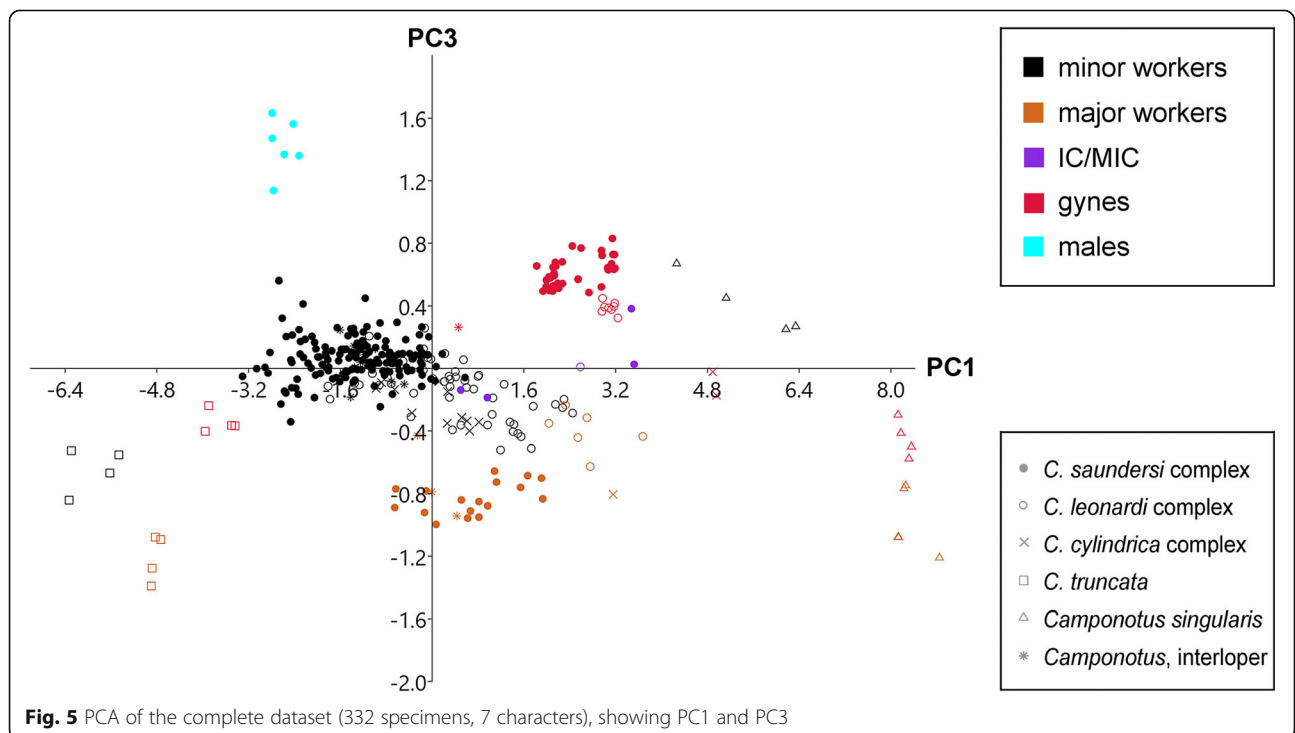
PC	Eigenvalue	% of total variance	Lower 2.5% conf. Limit	Upper 2.5% conf. Limit
1	5.916900	84.5270	81.7020	86.9210
2	0.711179	10.1600	8.5819	12.1580
3	0.193333	2.7619	2.1806	3.4525

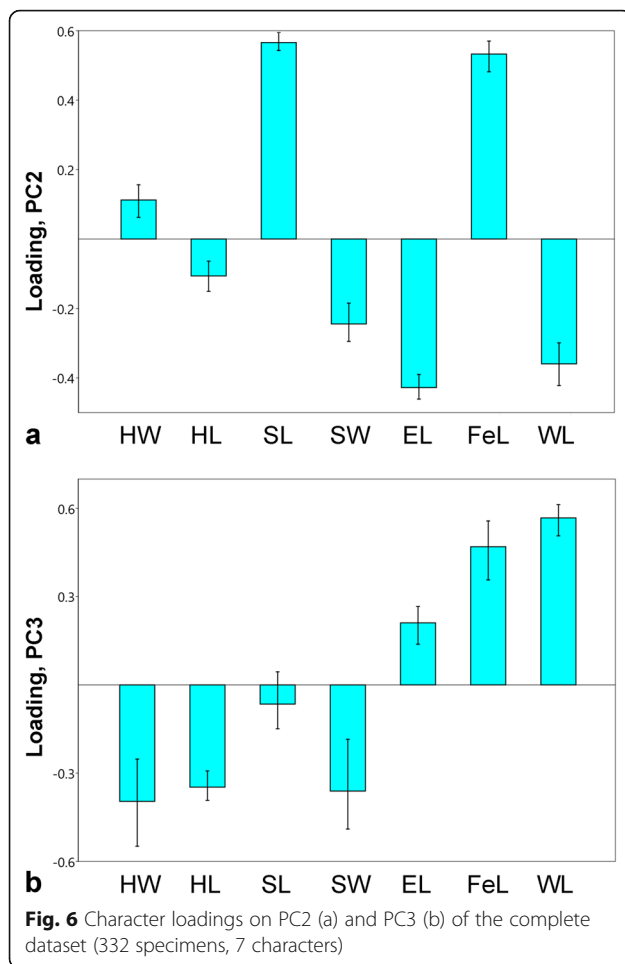
PC 1–3 are presented with their eigenvalues, percentages of total variance, and 95% confidence intervals following 1000 bootstrap resamplings for the complete dataset (332 specimens, 7 characters)



FeL) and strong negative loadings for SW and EL (Fig. 9a, Additional file 3: Table S3). A two-dimensional space created by PC1 and PC2 (Fig. 7) for characterization of the minor workers of different camponotine taxa could thus be approximated as “small body, short and thick appendages, large eyes” (e.g., *C. truncata*) vs. “large body, long

and thin appendages, small eyes” (e.g., *C. singularis*), with the majority of COCY ants occupying an intermediate space. An interesting outlier from this trend are the minor workers of the *C. leonardi* and *C. cylindrica* complexes: Especially the larger individuals exhibit short and thick appendages, more often seen in major workers of other taxa.





A second noteworthy observation is the position of so-called “interlopers” of the species *Camponotus inquilinus* (Fig. 1e): Their very large eyes and comparatively wide scapes place them very low on PC2, but they are not entirely distinguishable from their known hosts (COCY species of the *saunderi* complex, Fig. 1a) within the conducted PCA (Fig. 7). However, they are clearly distinct from the sample of all COCY minor workers when PC3 is also taken into consideration, due to their higher loadings on this axis. PC3 differed from PC2 mainly by the strong positive loading of SW and the weakly negative loading of FeL (Fig. 9b, Additional file 3: Table S3). Minor workers of the *C. cylindrica* clade show a similar pattern, due to their conspicuously wide scapes (Fig. 8).

Table 4 First three principal components (PC) for the minor worker sample

PC	Eigenvalue	% of total variance	Lower 2.5% conf. Limit	Upper 2.5% conf. Limit
1	6.223370	88.9050	85.7630	91.2110
2	0.394777	5.6397	4.3348	7.3820
3	0.160217	2.2888	1.6316	2.8592

PC 1–3 presented with their eigenvalues, percentages of total variance, and 95% confidence intervals following 1000 bootstrap resamplings for the minor worker sample (231 specimens, 7 characters)

Gynes

For the PCA of all gynes (54 specimens) and two *Colobopsis* sp. “nrSA” MIC specimens with visible ocelli, the set of characters was expanded to include the width of the median ocellus (OcW). PC1 held 84.16% of the total variance (Table 5) and was again considered a size-axis, albeit with a slightly weaker loading for OcW (Additional file 3: Table S3). PC2 accounted for 10.91% (Table 5) and had the strongest positive loadings for OcW and WL, and strong negative loadings for SL and HW (Fig. 12a, Additional file 3: Table S3). PC3 accounted for 2.28% of total variance (Table 5) and exhibited strong positive loadings for SW and strong negative loadings for EL (Fig. 12b, Additional file 3: Table S3). Ocellar width is known to be a highly variable character in all examined taxa [30–32]. Eye length and Weber’s length (a measure of mesosoma size) are typically increased and correlated in gynes to accommodate flight. The extraordinarily long and thick scapes of *C. cylindrica* gynes are indicated by their position on PC2, which contrasts with the morphology of all other examined COCY gynes (Fig. 10).

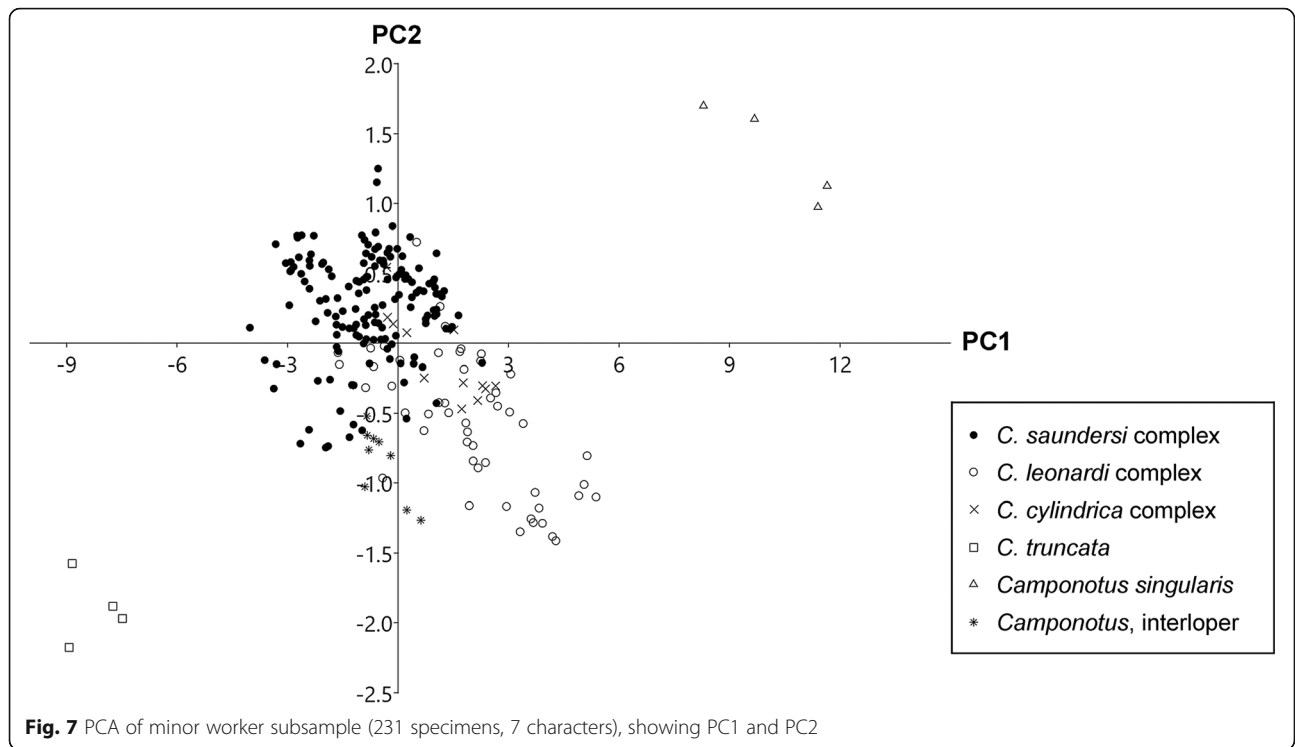
Although similar in overall body size, the MIC specimens were clearly set apart from morphologically typical gynes by their placement on both PC2 and PC3 (Figs. 10, 11), due to reduction of their gyne-specific characters (ocelli, mesosoma).

Cluster analysis

To further assess which of the examined morphological characters could be considered as parts of phenotypically integrated modules in development and evolution, R-mode cluster analyses based on a UPGMA algorithm and using correlation as a similarity index were conducted. Bootstrap values resulting from 1000 resamplings are shown in character tree diagrams (Figs. 13, 15, 17, 19). In addition, morphological measurements are illustrated on microCT images for an example specimen of each female caste (Figs. 14, 16, 18); colours of the measurements correspond to colours of integrated modules in the character trees.

Minor workers

In the cluster analysis conducted on the minor workers, the appendages (SL, FeL) formed one strongly correlated module, distinct from all remaining characters. The



characters of head-size (HW, HL) were strongly linked but now clustered with mesosoma length (WL), which can all be considered aspects of “body size”. SW and EL were highly variable and set apart as distinct modules (Figs. 13, 14).

Major workers

The cluster analysis of the major worker subsample (36 specimens, 7 characters) yielded a character tree differing from the topology of the minor workers (Figs. 15, 16). The characters SW and EL were strongly variable and rather

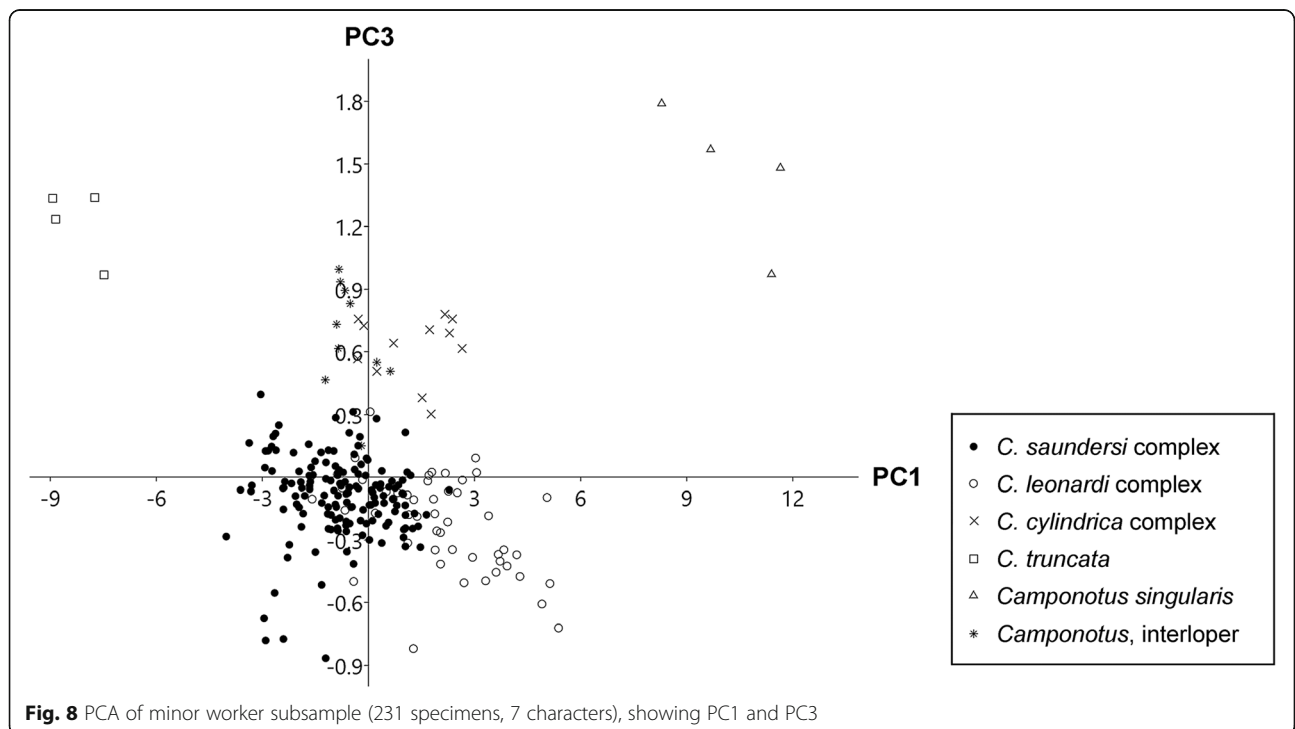


Table 5 First three principal components (PC) for the gyne and MIC sample

PC	Eigenvalue	% of total variance	Lower 2.5% conf. Limit	Upper 2.5% conf. Limit
1	6.732670	84.1580	77.1360	91.507
2	0.873139	10.9140	5.8896	15.845
3	0.182741	2.2843	0.5510	4.117

PC 1–3 presented with their eigenvalues, percentages of total variance, and 95% confidence intervals following 1000 bootstrap resamplings for the gyne and MIC sample (56 specimens, 8 characters)

independent from other characters. The characters defining extremity length, FeL and SL, were here clustered with HW, while the second character of head size (HL) correlated strongly with mesosoma length (WL).

Gynes

For the subsample of gynes, two different cluster analyses were conducted: 1) One analysis used eight characters (including OcW), based on a sample of all gynes including the two MIC specimens with ocelli (56 specimens in total). 2) For better comparison with the worker samples, only the original character set (seven characters, without OcW) was used and MIC specimens were omitted.

The first analysis (56 specimens including MIC, 8 characters, Figs. 17, 18) yielded results differing from those of the complete and minor worker samples: The highly variable OcW was set apart from all other characters. In contrast to all other analyses, the gynes did not present such strong correlation of appendages. Instead, the appendage characters were each strongly correlated to a measurement of head-size: SL was linked to HW, FeL to HL; SW was again set apart from these modules. The influence of the characteristic gyne-specific morphology with both eyes and mesosoma enlarged for flight was also apparent, thus linking EL and WL.

When removing the two mermithogenic specimens and the character of median ocellar width from the sample, the resulting character tree (54 specimens, 7 characters, Fig. 19) was only marginally different from the previously examined sample: The strongly correlated HL and FeL were now more closely associated to the cluster of EL and WL than to the one composed of SL and HW; SW was again set apart as a distinct character.

Discussion

The merits of studying phenotypic integration

First proposed in 1958 by Olson & Miller [1], the concept of studying integrated groups of characters in organisms is not a scientific novelty. However, modern methods and insights from such diverse fields as molecular genetics, developmental biology, evolutionary ecology, palaeontology, and philosophy of science [5] now make interdisciplinary studies on the origin and

evolution of complex patterns of covarying traits more interesting and promising than ever before.

An important question in terms of evolutionary biology is whether phenotypic integration acts as a facilitator for natural selection and adaptation or whether it should rather be regarded as an evolutionary constraint [5, 8]. While strong interdependence among traits certainly channels and limits the evolutionary trajectory of the species in question [5, 65], authors of previous studies have alternatively considered the possibility that functional integration is in itself an adaptation. Since functionally related parts show coordinated variability [10], integration may serve to increase evolvability by making the population more capable of evolving in likely directions of selection [8, 66].

It thereby becomes evident that a better understanding of how a complex phenotype is composed out of tightly integrated, but mutually relatively independent modules can be an important factor in shedding light on the ontogenetic development and evolution of all organisms, as well as the adaptive value and functionality of specific morphological structures.

With the exception of honeybees [15, 16] and a select few species of myrmicine ants [20–22], social insects have been largely overlooked in previous assessments of integration. A noteworthy result of a recent comparative study [14] is the high degree of integration in adult holometabolous insects in comparison to plants, vertebrates and hemimetabolous insects. The authors attribute this to the relative lack of spatial and temporal variation during the development of the adult phenotype in the pupal phase. In vertebrates, this same study also reported the strongest mean correlations within the group of limb characters. In addition, investigations on birds (Carduelidae, Columbigiformes) [3, 10] showed strongly integrated modules corresponding to structures necessary for optimal flight and foraging. Interestingly, this corresponds to the results of the study at hand: Particularly the limb characters (scape length and femur length) tend to be closely integrated in workers, whereas gynes show higher levels of correlation between eyes and mesosoma. This can be interpreted regarding functional aspects of structures necessary for foraging and flight, as well as the common genetic underpinnings by homologous developmental control genes [67, 68]. In light of these findings, it seems

worthwhile to further investigate patterns of phenotypic integration in the family Formicidae: Not only are their caste systems excellent subjects for the study of phenotypic plasticity and modularity [18, 19, 25], they also exhibit patterns of phenotypic integration similar to those found in previously studied vertebrates, pointing to possible parallels in functional demands or selective pressures.

Linking specialized caste morphology and division of labour

Ants and other eusocial insects are known to function as superorganisms, in which each animal is considered less as an individual and more like a cell within a larger body [33]. In this context, the evolution of functionally and morphologically specialized female castes is the foundation for division of labour within the colony and thus the enormous evolutionary success of ants and other eusocial Hymenoptera [69]. In the context of phenotypic integration and modularity, members of distinct castes themselves have been compared to other “functionally specialized modules” such as different cells within one organism or the different limbs of arthropods [70].

The number of different tasks that need to be performed in order to maintain a functioning colony ranges from 20 to 40, yet only 20% of ant genera possess more than one worker caste – among them extremely speciose genera such as *Camponotus* and *Pheidole* [28, 72]. However, no known ant genus contains more than three distinct physical worker castes [71]. Therefore, the behavioural repertoire resulting from functional specialization must be sufficient to perform the necessary tasks efficiently, but may not be too narrow to allow for multiple different tasks to be executed by each colony member [70].

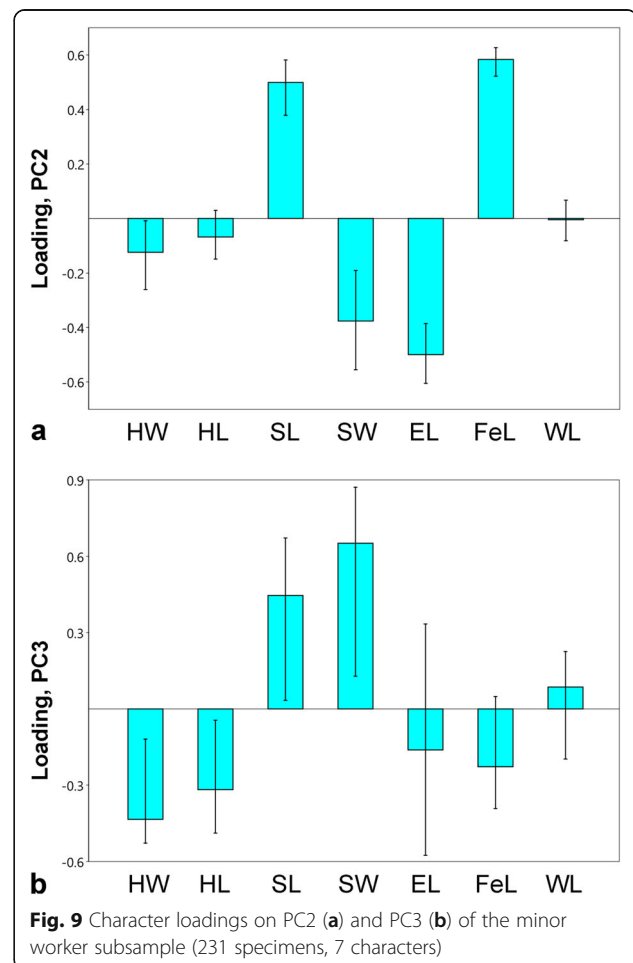
In the study at hand, we have attempted to identify morphological modules characteristic for each female caste and interpret these patterns in light of current knowledge about the investigated species’ caste-specific behaviours. To facilitate statistical analyses despite the scarcity of material for certain castes and species, we elected to pool the individuals of all taxa for each caste. While this approach provides an overview of caste-specific modules, it does not allow a conclusion about the evolutionary origin of these patterns in each of the investigated taxa [6–9]. Thus, a distinction between trait covariation within a caste due to functional constraints, common selective pressures, or genetically governed developmental processes [65, 67, 68] is not feasible at this point. This should be the subject of further studies on the separate species, when larger samples of all castes become available.

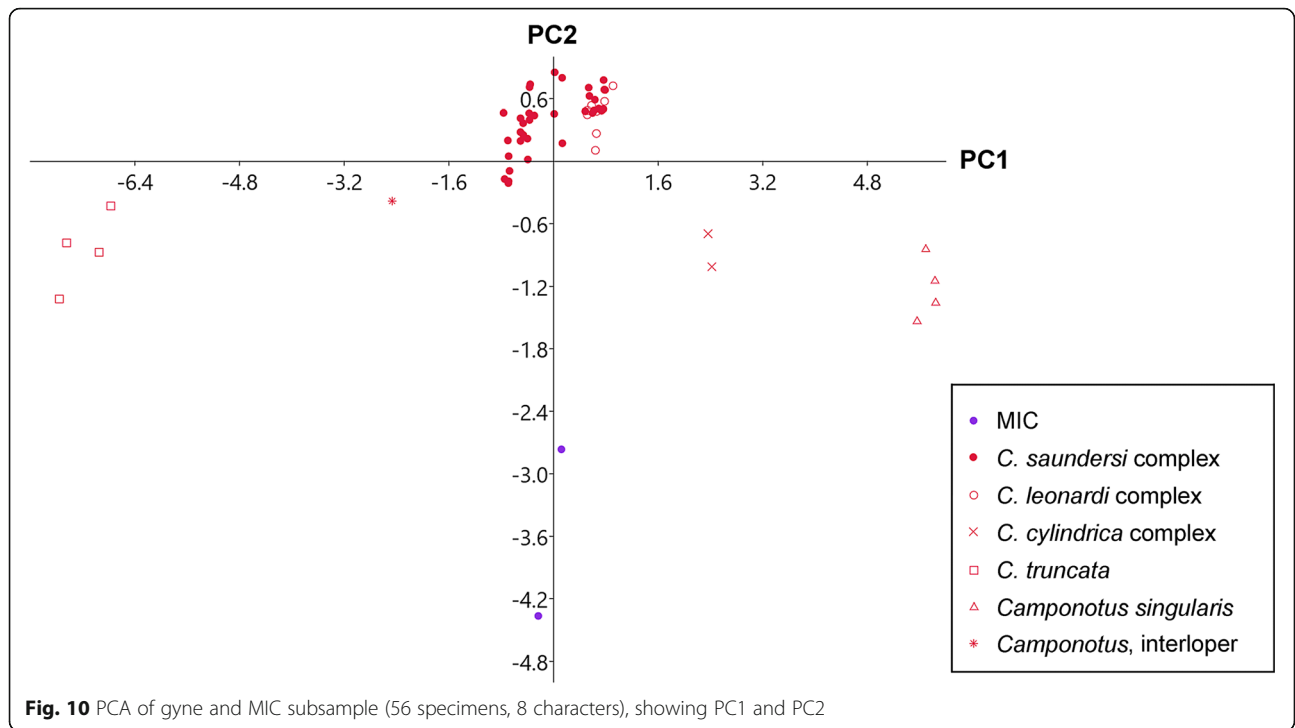
Minor workers

The subsample of minor workers was by far the largest at 231 specimens. Across all examined taxa, minor workers

were the most abundant and most readily available caste. This is also corroborated by previously published data, which place optimal caste ratios for camponotine species at 80% minor workers vs. 20% major workers [73, 74]. These high numbers, together with the apparent intraspecific variability in size [30–32], suggest a possible division of labour not only between minor and major workers, but even within the minor worker caste itself. Although behavioural data on the examined taxa are still sparse, similar results have been described for *Camponotus japonicus* var. *aterrimus* [75] and *Camponotus sericeiventris* [76].

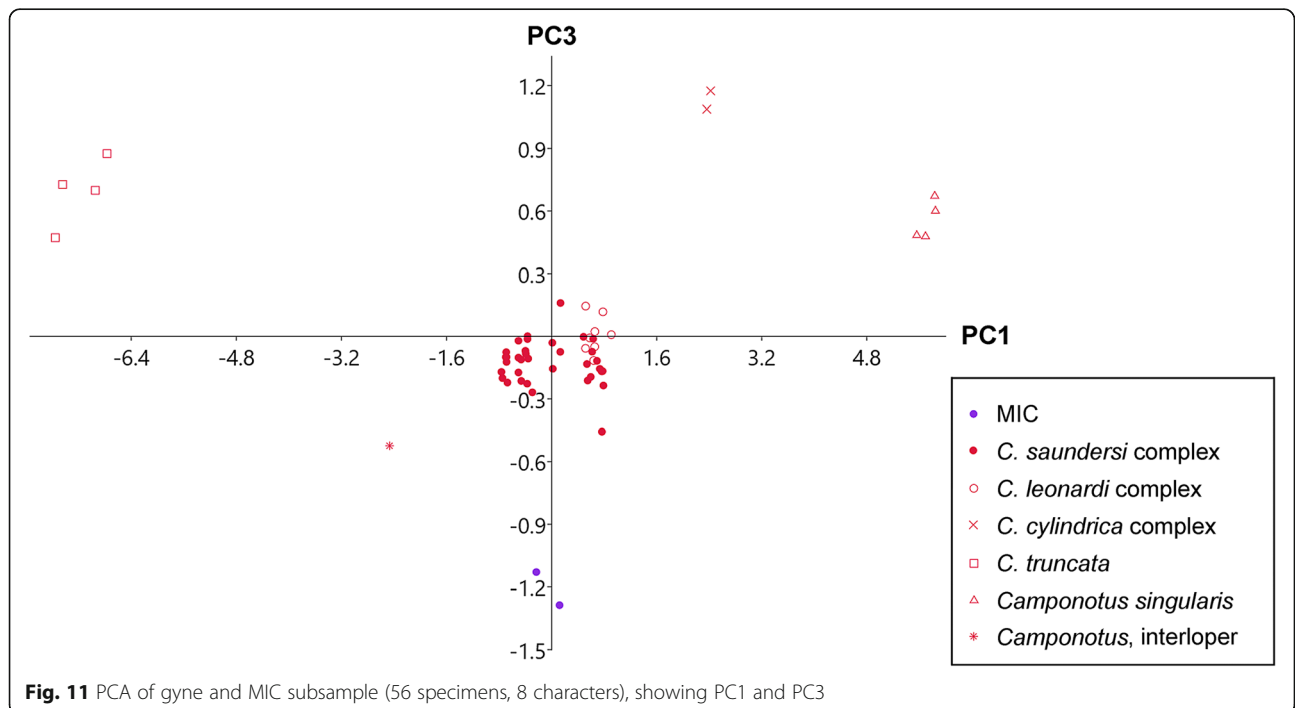
Considering the results of the PCAs and cluster analyses, the most strongly integrated modules for the minor worker caste are the complexes of head size (HL, HW) and the extremities exemplified by SL and FeL (Figs. 9, 13, 14, Additional file 3: Table S3). As shown in previous studies on three of the individual taxa [30–32], long legs and scapes tend to be correlated with well-developed, elongated mouthparts (maxillary and labial palpi) as well. This module thus contains structures for locomotion (legs) as well as sensory function (palpi, antennae). A PCA-based analysis conducted on the myrmicine genus





Pheidole generated similar results: The minor worker caste was characterized by overall high loadings on PC1, reflecting their variability in body size, while PC2 was strongly influenced by scape length [17]. These parallels even in distantly related genera are likely linked to minor

workers' vast array of tasks within the colony (e.g., foraging, brood care), which rely on mobility as well as well-developed sensory abilities [17, 70, 71, 77]. As shown for two species of *Camponotus* [77], and the genus *Eciton* [78], gynes and major workers possess shortened antennae



and maxillary palpi, as well as smaller corresponding brain structures than foraging minor workers of the same species. All appendages are further linked in evolution and development through a common genetic underpinning by developmental control genes, such as homothorax (*hth*) and extradenticle (*exd*) [69], which may explain the modular patterns observable in many of the examined taxa.

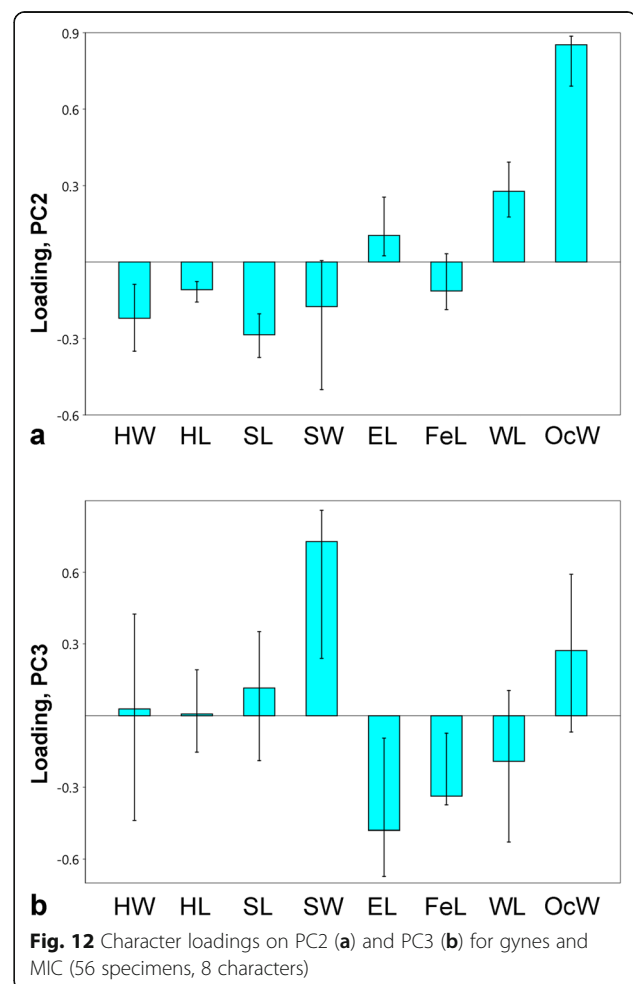
The minor worker caste is also the protagonist in two fascinating phenomena observed in connection with the group of COCY ants – suicidal explosion in the COCY group itself [32, 45, 48] and inquiline behaviour of *Camponotus (Myrmamblys) inquilinus* minor workers (“interlopers”) in nests occupied by COCY species [31, 48, 50]. Interestingly, this study has found no specialized adaptations of outer morphology or proportions in either group. Considering only the “interlopers” and their hosts of the *C. saundersi* complex, both occupy intermediate positions within the scatterplots and are only distinguishable by the proportions of SW and EL. When examined under a microscope, they also differ in structural details of the integument, hair length and head shape (domed in *C. inquilinus*), but are strikingly similar in size and colouration [50]. Although the biology of the *C. inquilinus* group is largely unstudied, their known association and even mimetic resemblance to ants of the COCY group may well enable them to benefit from the ecological dominance and suicidal defence of the “exploding ants” [31, 32, 46, 48, 50]. How they avoid detection by their host colony via putative alterations to their cuticular hydrocarbon profile will be the subject of future studies (M. Hoenigsberger et al., in prep.).

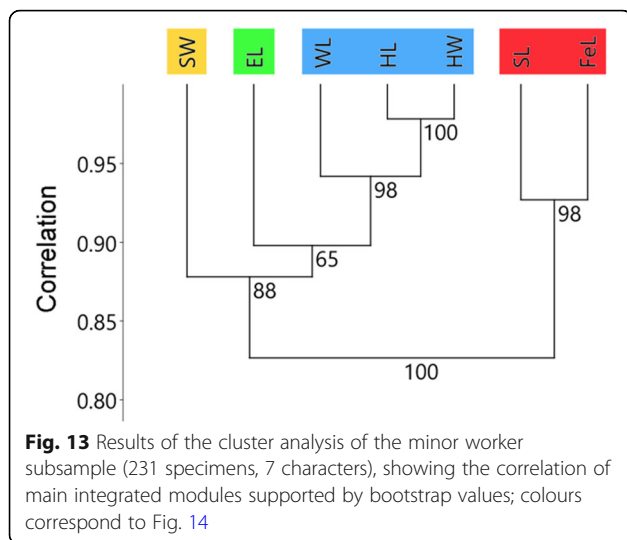
Major workers

Although the limited number of major workers available for this study limited their suitability for statistical analysis, their placement in the PCA based on the entire sample of specimens permits a number of meaningful conclusions. Major workers of all examined species were consistently placed lower on PC2 and PC3 than their conspecific minor workers and gynes (Figs. 4, 5). Taking into account the character loadings on the respective axes, it becomes clear that this is the result of a caste-specific set of traits: A large head (HW, HL) and thick scapes (SW) result in a low placement on PC3, short extremities (SL, FeL) contribute to strong negative loads on PC2. Exceptions to this pattern are only found in the basal COCY groups (Zettel et al., in prep.) represented by the *C. leonardi* and *C. cylindrica* complexes: Here, major and minor workers are not as clearly set apart from each other, as these groups exhibit intermediate morphologies not clearly definable as major or minor workers. This is also reflected in the classification yielded by the LDA (Fig. 3, Table 1), which labelled three major workers and 14 minor workers of the *C. leonardi*

complex as intercastes. As disruptive selection is thought to be at least partially responsible for the evolution of distinct castes [43], it is possible the distinct minor and major worker phenotypes observable in, e.g., the *C. saundersi* complex arose later in the evolution of COCY ants, thus leading to the varying degrees of caste dimorphism observable today (Zettel et al. in prep.). As phragmiosis in the major worker caste is also known from the *C. truncata* group, this trait may have evolved multiple times within the genus *Colobopsis* [31, 79].

Overall, the major worker caste in Camponotini is morphologically adapted to its two main functions within the colony: nest defence and storage of nutritious liquids, leading to enlargement of the head and mandibles, as well as the gaster [30, 80, 81]. Similar patterns of major workers with a comparatively stout body, enlarged heads and short appendages are known from members of other ant subfamilies as well, e.g. *Pheidole* [17], *Cephalotes*, *Oligomyrmex* [39] or *Eciton* [82]. Within the genus *Colobopsis*, the defensive function is shifted from active, aggressive “soldiers” with enlarged mandibles





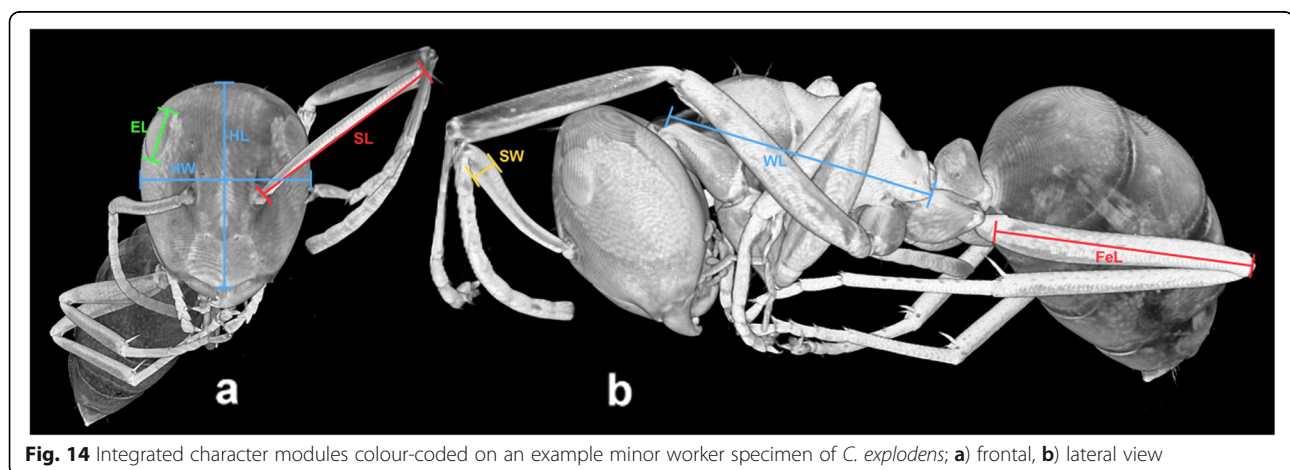
towards passive phragmotic plugging of nest entrances with specialized shield-shaped heads [31, 32, 37, 79]. Both, active combat as well as phragmotic behaviour can possibly benefit from a heavy-set body with short, thick appendages to reduce the probability of injury [30, 39].

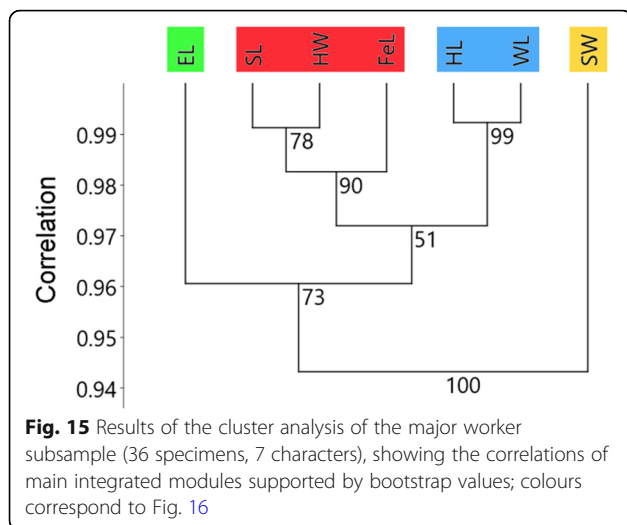
Gynes and MIC

Considering the morphology of the gynes examined within this study, several noteworthy parallels can be drawn to the results of previously conducted investigations: Both, the high loadings on PC2 and the modules apparent in the cluster analysis point to a high level of integration between EL and WL in gynes (Figs. 10, 11, 12, 17, 19, Additional file 3: Table S3). This is most probably linked to aspects of life history and behavioural ecology relevant for alate camponotine gynes: Known flight-specific morphological adaptations in sexual castes of the Formicidae include the presence

of ocelli, enlarged eyes, and a large mesosoma to accommodate the well-developed flight musculature [34, 35]. Thus, the characters of EL and WL may form a functional module in gynes, explained by their crucial role in complex behaviours, such as mating flights, dispersal, and mate-choice. In contrast, gynes showed lower variability of femur length and less pronounced integration of the extremities (SL, FeL) than minor workers (compare Figs. 9a, 12a). This previously unreported finding may also be connected to the life history of alate gynes, which lacks the selective pressures for ambulatory locomotion and sensing of chemical cues relevant for minor workers. In the case of the antennae, this is known to be reflected in the corresponding neurological structures of the brain in two investigated species of *Camponotus* [77]. The results of the PCA and cluster analysis point to an extremely high variability of median ocellar width (OcW) (Figs. 12, 17, Additional file 3: Table S3). This phenomenon was also observed in studies focusing on three of the individual camponotine species examined in the present study: *C. singularis* [30], *Colobopsis* sp. nrSA [31], and *C. explodens* [32]. Current literature yields no evolutionary or developmental explanation for this fact. The authors presume a high level of plasticity for this trait, possibly linked to size-dependent developmental thresholds or a lack of selective pressure towards a particular ocellar size. This is also suggested by the fact that median ocelli or vestiges thereof have been observed in particularly large major workers and intercaste specimens [30, 31, 40].

In the case of MIC specimens (Fig. 2e), vestiges of ocelli, wings and gyne-like mesosomal architecture are due to their origin from queen-presumptive larvae [57]. The infective larval stages of parasitic nematodes of the family Mermithidae infect ant larvae via oral uptake of a paratenic host (e.g. oligochaetes) and develop

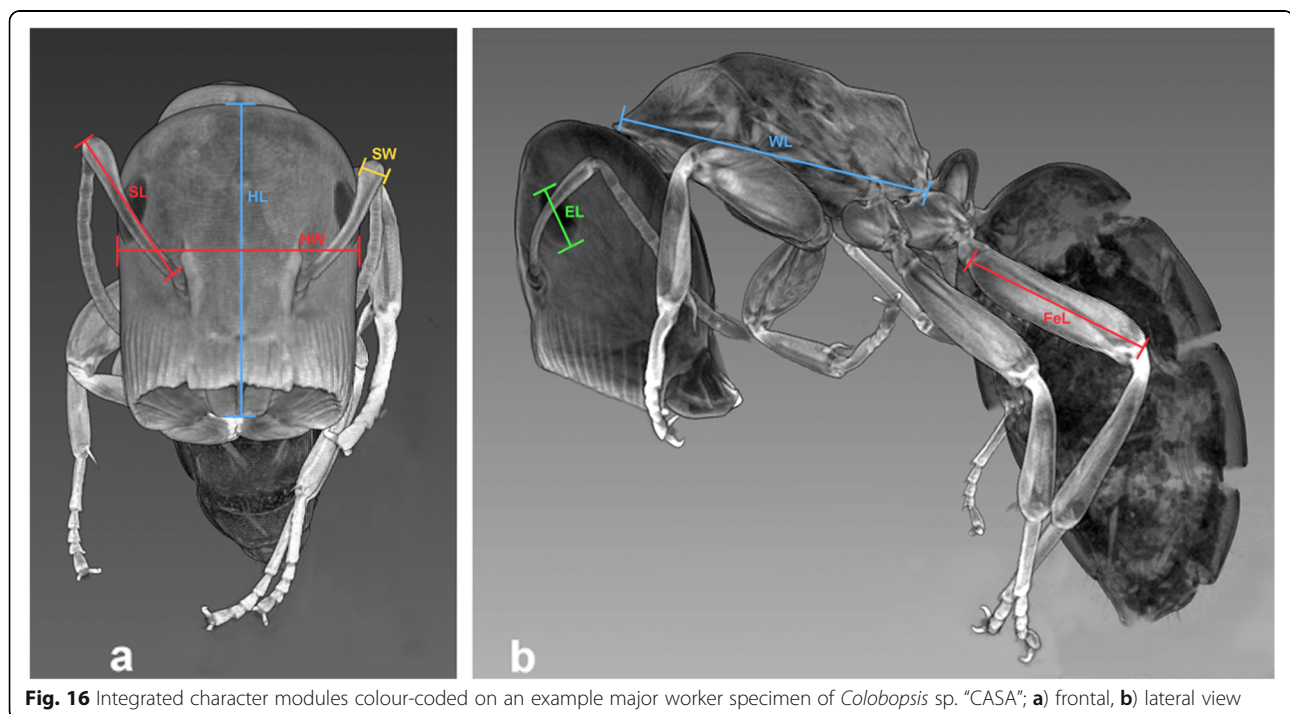


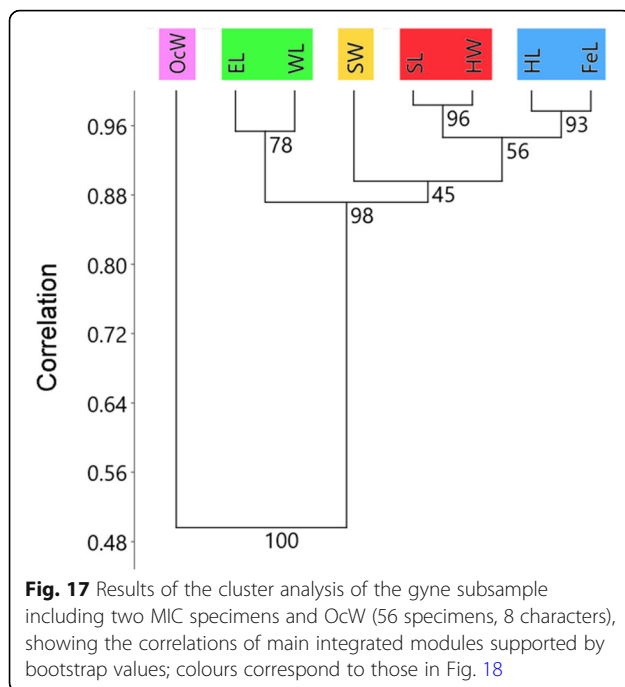


in synchronicity with them [54]. Whether mermithogynes of the studied species have specific tasks within their native colonies is hitherto unknown. As they are usually flightless and have reduced ovaries [51, 55, 83], mermithogynes cannot take part in reproduction. However, there are no reports of antagonistic behaviour by nestmates against infested individuals. On the contrary, they have been observed to stay with their native colony, aid in brood care and beg workers for food [83]. More recent theories suggest that the

survival of intercaste phenotypes may be facilitated by mechanisms of colonial buffering [17]. Eventually, the mature parasite will compel its host to seek a moist habitat for its release and engage in suicidal behaviour (drowning) [53, 54, 83].

Gynes infested by mermithids exhibit apparent, drastic disruptions of the morphological patterns known from healthy gynes: Mermithogenic phenotypes can lie anywhere on a range from almost normal worker morphology (“mermithergates”) to gyne-like specimens with only slightly reduced features (“mermithogynes”) [57]. This is also apparent in the studied sample: Although their reduced ocelli and mesosoma placed them consistently lower on PC2 and PC3 compared to normally developed gynes, the two conspecific mermithogynes differed considerably from each other (Figs. 10, 11). Within the LDA and PCA of the complete sample (Figs. 3, 4), IC and MIC specimens were consistently plotted as intermediate between minor and major workers, further corroborating their status as “intercaste” specimens. The observed morphological aberrations included phenomena previously described from mermithogynes of other host taxa, such as reduction of ocelli and mesosomal architecture [51, 54, 55, 57, 58], as well as an apparent developmental trade-off between elongated appendages and reduced wings [54]. How exactly mermithid nematodes affect the morphology of their hosts – whether by hormonal disruption [17, 57] or through other influences – and why they can induce such a wide



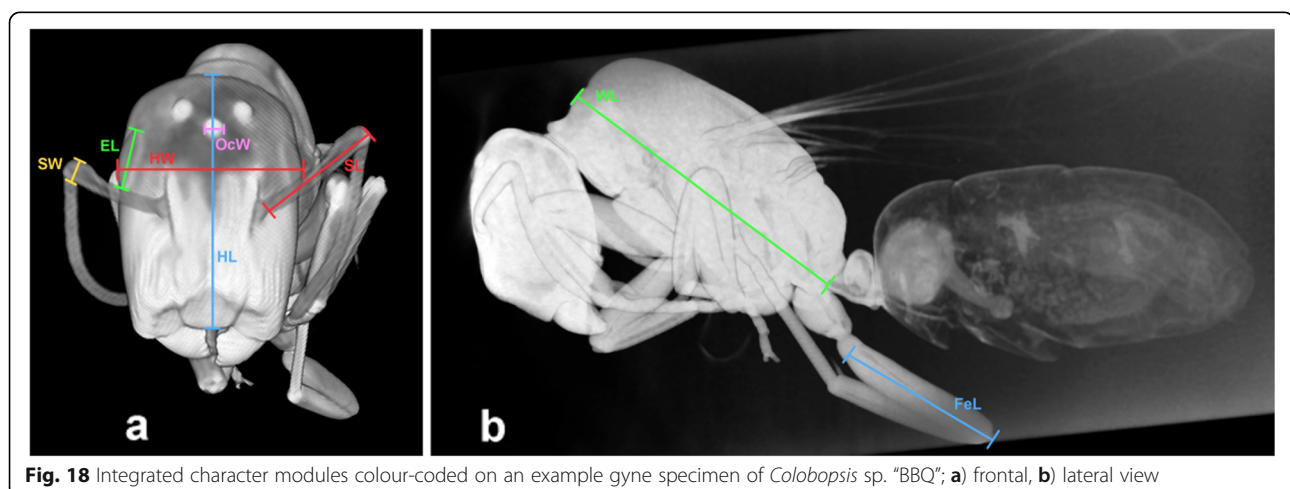


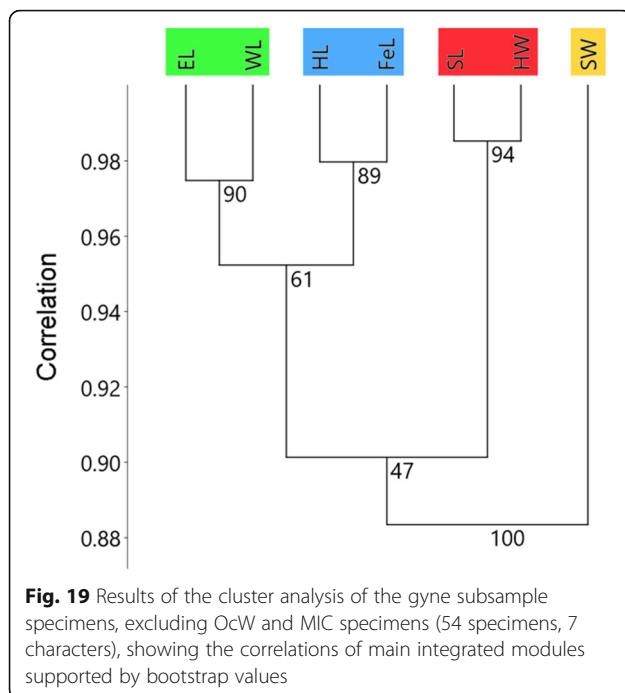
range of phenotypes, will be investigated in future studies (A. Laciny, in prep.). Uncovering the developmental mechanisms behind mermithogenic phenotypes may even lead to a better understanding of developmental plasticity, robustness, and of the evolution of specialized castes: Previous studies have proposed a link between the developmental programs facilitating this plastic response to environmental perturbations and the evolution of novel phenotypes [17, 18].

Conclusions

While the investigation of phenotypic integration and modularity of the body plan has a long tradition in the

fields of vertebrate biology and botany [1, 12], studies on insects are rare [14] and social insects have been largely overlooked. In this study, we present the first detailed investigation of phenotypic integration in the ant tribe Camponotini, based on morphometric data of two camponotine genera – *Camponotus* and *Colobopsis*. As these ants possess complex caste systems with distinct phenotypes (minor worker, major worker, gyne, and male), special focus was placed on caste-specific morphology and character complexes (modules) presumably related to behaviour and division of labour within the colony. The selected characters were shown to be suitable for caste characterization, resulting in over 93% correct classification by LDA. Using multivariate statistical methods (PCA, cluster analysis), we were able to detect species- and caste-specific proportions and visualize multiple tightly integrated modules composed of covarying characters specific to each female caste: For minor workers, strong integration of extremities (legs, antennae) was a distinctive feature. In major workers, extremities also clustered with head width. Gynes differed from workers by a strong correlation of eye length with mesosomal length. In mermithogynes (gynes infested with parasitic nematodes), gyne-specific characters were reduced and overall proportions consequently altered. The caste-specific character modules are interpreted in light of their putative functional relevance to task-allocation for each caste: Locomotion and sensory perception are crucial for foraging activity in minor workers [77], while major workers are morphologically equipped for phragmosis (*Colobopsis*) or active defence (*Camponotus*) [37], and gynes rely on well-developed visual abilities and flight musculature for dispersal and mating [34, 35]. Similar character modules linked through common development and function have previously been described in vertebrates [10, 14]. Furthermore, modularity of the body plan and plastic responses to environmental stress





(e.g., parasites) are thought to have contributed to the diversity and ecological dominance of the family Formicidae [18, 19]. Further studies on the subject could therefore lead to valuable insights into the factors facilitating the evolution of novel phenotypes in ants and even uncover ontogenetic and evolutionary principles common to a wider range of organisms.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s40850-019-0048-7>.

Additional file 1: "S1 Depositories and Material.pdf". Complete list of sampling localities, depositories, taxonomic placement, caste assignment, and collection data for the specimens analysed in this study.

Additional file 2: Table S2: "S2 Measurements.xls". Complete list of specimens examined in this study, measurements (in mm), and measurement error statistics.

Additional file 3: Table S3: "S3 Character Loadings.xls". Loadings on principal components (PC) 1–3, for the complete dataset (332 specimens, 7 characters), the minor worker sample (231 specimens, 7 characters), and the sample of gynes and MIC (56 specimens, 8 characters).

Abbreviations

COCY: *Colobopsis cylindrica* group; EL: Eye length; EtOH: Ethanol; FeL: Hind femur length; HL: Head length; HW: Head width; IC: Intercaste; LDA: Linear discriminant analysis; MIC: Mermithogenic intercaste; microCT: X-ray microtomography; OcW: Median ocellus width; PC: Principal component; PCA: Principal component analysis; SL: Scape length; SW: Scape width; UPGMA: Unweighted pair group method with arithmetic mean; WL: Weber's length (diagonal length of mesosoma)

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Authors' contributions

AL and HLN conceptualized the study and performed statistical analysis. AL compiled the set of specimens and conducted morphometric measurements. BDM and AL conducted microCT scans. HZ and AL constructed figures and conducted stacking photography. ISD organized funding, sampling expeditions and availability of materials. AL, HLN and HZ wrote the manuscript text, to which BDM and ISD contributed. All authors have read and approved the final manuscript.

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Availability of data and materials

The dataset supporting the conclusions of this article is included within the article and its additional files. The datasets of measurements and specimens are available as Additional files 1 and 2. The investigated specimens are deposited and available for study or loan at the respective institutions as described in S1.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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