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**Macroevolutionary integration of phenotypes within and across ant worker
castes**

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26 **Abstract**

27

28 Phenotypic traits are often integrated into evolutionary modules: sets of organismal parts that
29 evolve together. In social insect colonies the concepts of integration and modularity apply to sets
30 of traits both within and among functionally and phenotypically differentiated castes. On
31 macroevolutionary timescales, patterns of integration and modularity within and across castes
32 can be clues to the selective and ecological factors shaping their evolution and diversification.
33 We develop a set of hypotheses describing contrasting patterns of worker integration and apply
34 this framework in a broad (246 species) comparative analysis of major and minor worker
35 evolution in the hyperdiverse ant genus *Pheidole*. Using geometric morphometrics in a
36 phylogenetic framework, we inferred fast and tightly integrated evolution of mesosoma shape
37 between major and minor workers, but slower and more independent evolution of head shape
38 between the two worker castes. Thus, *Pheidole* workers are evolving as a mixture of intra- and
39 inter-caste integration and rate heterogeneity. The decoupling of homologous traits across worker
40 castes may represent an important process facilitating the rise of social complexity.

41

42 **Keywords:**

43 Morphological integration, modularity, caste, dimorphism, *Pheidole*, ants, geometric
44 morphometrics

45

46

47 **Introduction**

48

49 The increase of morphological complexity following divergence in cellular function is a
50 repeating theme in the evolution of multicellular organisms (Wagner and Altenberg 1996). Given
51 cues regarding their developmental fate, cells and tissues express their identical genomes in
52 different ways to produce different traits and thus allow functional specialization. Morphological
53 integration can be considered the extent to which these traits vary in concert, either as a
54 continuation of their shared genetic or developmental origin, or as a unification of parts
55 contributing to a shared function and shaped by selection (Olson and Miller 1958; Klingenberg
56 2008). Sets of integrated traits covary as modules, between which covariation is weaker than
57 within (as in the primate cranium; Cheverud 1982).

58

59 Much as a single genome can underlie different cooperating tissues and traits within the same
60 organism, different traits are also produced among individuals using the same genome. Distinct
61 phenotypes are commonly observed in different sexes (Owens and Hartley 1998), or in
62 individuals adopting alternative reproductive tactics (Emlen et al. 2007) as a result of differential
63 selection. Eusocial insects reflect a major evolutionary transition whereby a unit of selection is
64 comprised of different individuals working together as part of an integrated colony-level
65 phenotype (Wheeler 1911; Hölldobler and Wilson 1990; Szathmáry and Smith 1995) and
66 understanding the evolution and function of these “superorganisms” is a major and enduring
67 interest of evolutionary biology (Oster and Wilson 1978; Seeley 1995; Holldobler and Wilson
68 2009). The castes of social insects can exhibit radically different traits from the same genome: a
69 female egg laid by the queen has the potential to develop into either another queen or a worker
70 caste individual. This phenotypic polymorphism allows functional specialization among
71 individuals in a colony and the rise of social complexity, the feature of eusociality that best
72 defines its potential for division of labor (Oster and Wilson 1978; Hölldobler and Wilson 1990).
73 While worker castes are an ancestral trait shared by nearly all extant ants, several lineages have
74 since evolved further division of labor among workers to form worker castes – known also as
75 subcastes (Wilson 1953; Hölldobler and Wilson 1990; Oster and Wilson 1978; Wills et al. 2017).
76 In the colonies of some species (e.g., *Solenopsis invicta*), worker castes exhibit polymorphism
77 mostly along a single allometric function – shape varies with size along a regular continuum

78 (Wilson 1953). However, for species in other genera (e.g., *Pheidole*, *Colobopsis*, *Carebara*,
79 *Cephalotes*, *Eciton*, *Acanthomyrmex*, *Pseudolasius*), variation reaches “complete dimorphism”
80 into distinct major worker and minor worker phenotypes (Wilson 1953). While there is some
81 contention over nomenclature within the myrmecological community (Urbani 2015), we refer to
82 minor workers and major workers (aka soldiers) as “worker castes” throughout following Wills
83 et al. (2017).

84
85 The evolution of complete dimorphism offers the potential for new dimensions of variation in
86 ants (Wilson 1953, Wills et al. 2017). If phenotypes are disintegrated among worker castes, this
87 can allow for greater functional specialization and different combinations of traits available to
88 the colony-level phenotype (Wilson 1953; Powell 2008; Powell 2009; Wills et al. 2017).
89 However, the evolution of specialized morphology in major workers may be biased by
90 developmental pathways that are shared with minors (Wheeler and Nijhout 1983; Wheeler and
91 Nijhout 1984, Wheeler 1991; Rajakumar et al. 2012), thus there could be limits to divergence
92 among homologous body parts across the different worker castes, or a shared pathway could lead
93 selection on one worker caste to result in a neutral change in the other.

94
95 The ecological and behavioral roles of polymorphic worker ants have long been a fascination of
96 social insect research (Wheeler 1911; Goetsch 1937; Wilson 1953, Oster and Wilson 1978;
97 Powell and Franks 2006; Powell 2008; Powell 2009; Powell 2016; Wills et al. 2017). Likewise,
98 the genomic and biochemical mechanisms underpinning caste differentiation is a central avenue
99 for understanding the evolution of social complexity (Wheeler 1991; Hughes et al. 2003,
100 Anderson et al. 2008; Molet et al. 2012; Rajakumar et al. 2012; Lillico-Ouachour and Abouheif
101 2017, Gospocic et al. 2017, Chandra et al. 2018). However, the macroevolutionary implications
102 of these processes—the patterns of integration and modularity that emerge across the
103 diversification of hundreds or thousands of lineages—are comparatively less well-studied in the
104 ants, although the topic is receiving increasing interest (Pie and Traniello 2007; Pie and Tschá
105 2013; Holley et al. 2016; Powell 2016). These patterns, revealing the degree to which different
106 traits evolve independently within and among worker castes, may be an important clue to both
107 the selective forces driving evolution like ecological subspecialization among worker castes
108 (Powell and Franks 2006), and the potential constraints on evolution like restrictive

109 developmental limitations (as in Fritz et al. 2014). Furthermore, patterns of evolutionary rate
110 heterogeneity or homogeneity within and across worker castes may reflect particular aspects of
111 the phenotype that are under strong selection because they underlie axes of ecological divergence
112 among species (Schluter 2000; Price et al. 2016).

113
114 Ants have colonized and evolved adaptations to many environments, and are among the most
115 abundant terrestrial organisms on the planet. Ants have also radiated to produce a diverse array
116 of morphologies in nearly every region they have colonized (Hölldobler and Wilson 1990). In
117 this study, we focus on overall body size, the relative body sizes of different parts, and the shapes
118 of the head and mesosoma. Body size has been shown to be a major axis of morphological
119 variation among ants (Pie and Traniello 2007, Price et al. 2016; Powell 2016). Previous studies
120 of functional morphology in ants have also focused on the head (e.g., Holley et al. 2016), which
121 contains the main apparatus for feeding (mouthparts, mandibles), manipulating objects
122 (mandibles), and sensation (eyes, antennae). If head shape is the primary focus of ecological
123 adaptation, this trait should evolve more rapidly than others during radiation. We also examine
124 the mesosoma, the main power center of the ant including muscles for bearing loads and moving
125 the legs. While the functional significance of external mesosoma shape is not well understood,
126 the shapes and relative sizes of different regions likely reflects investment in different muscle
127 groups that have functional implications. The sizes and positions of the sclerites (plates of the
128 cuticle which are partially captured here by our landmarks) are associated with homologous
129 attachment points underneath. For example, Keller et al. (2014) showed that the pronotal region
130 associated with the T1 sclerite houses the muscles that lift the head. Other regions of the
131 mesosoma contain stabilizing muscles, muscles to support the legs, and muscles to flex the
132 petiole (Lubbock 1881), all of which have obvious functional implications.

133 Relatively few studies have compared the tempo of evolution across different ant traits,
134 (but see Pie and Tschá 2013; Blanchard and Moreau 2017; Holley et al. 2016). If the shapes of
135 other traits such as the mesosoma (thorax) evolve more rapidly, this may be an indication that
136 they serve a greater functional role in ecological divergence than previously understood.
137 Likewise, if majors exhibit greater rates of change, that may signal that their functional role has
138 changed often following the evolution of complete dimorphism, or that they are important for
139 achieving and maintaining ecological divergence among species.

140
141 To compare morphological integration and evolutionary rate of different worker castes and traits,
142 we focused on the ant genus *Pheidole*. The ants of this genus have, in the course of their
143 approximately 37 million year history, spread throughout 6 continents to produce more than
144 1000 described (and many more undescribed) species (Moreau 2008; Economo et al. 2015a).
145 Perhaps the most notable characteristic of species in this hyperdiverse genus is the clear
146 dimorphism of their workers: a major worker caste with enlarged heads is easily visible in all
147 species (indeed a third super-major form is also observed in some species) (Wilson 2003).
148 Behavioral studies have described different ecological roles for *Pheidole* worker castes, with
149 major workers performing more defense, food processing, and storage tasks than minor workers
150 (Wilson 1984; Tsuji 1990; Mertl and Traniello 2009; Huang 2010). The relatively consistent
151 body plan and caste structure of this genus make it an ideal clade for comparative studies of
152 morphology (Pie and Traniello 2007; Holley et al. 2016). The developmental basis of worker
153 caste differentiation in *Pheidole* has been well studied over the years (Wheeler and Nijhout 1983;
154 Wheeler and Nijhout 1984; Rajakumar et al. 2012; Lillico-Ouachour and Abouheif 2016;
155 Rajakumar et al. 2018), and recent work on the taxonomy, biogeography, and ecomorphology of
156 this group (Wilson 2003; Mertl and Traniello 2009, Muscedere and Traniello 2012; Sarnat and
157 Moreau 2011, Economo and Sarnat 2012, Economo et al. 2015b; Holley et al. 2016; Sarnat et al.
158 2017) make it an attractive model clade for evolutionary research on social insects.

159
160 Several previous studies on the macroevolution of *Pheidole* morphology are particularly relevant
161 for the current investigation. First, in an analysis before a *Pheidole* phylogeny was available, Pie
162 and Traniello (2006) analyzed morphology with linear measurements and found that size
163 differences explained most of the variation in *Pheidole* morphology across species, but majors
164 and minors showed divergent patterns of character correlation. Later, with the benefit of a
165 *Pheidole* phylogeny (Moreau 2008), Pie and Tschá (2013) showed that size varied more quickly
166 than shape variables based on linear morphometrics, but did not explicitly test for modularity and
167 integration. Holley et al. (2016) found that known ecological specialization of majors (seed
168 milling behavior in granivorous species) was related to divergence in head size between major
169 and minor worker castes (although enigmatically, due to a change in the minors), evidence that
170 independent evolution of the two worker castes in relation to ecology can occur. Finally, Sarnat

171 et al. (2017) tested hypotheses for the evolution of exaggerated thoracic spines, an unusual and
172 geographically restricted phenotype in *Pheidole*.

173
174 Despite the insights of these pioneering studies, a comprehensive picture of the roles of
175 integration, modularity, and rate heterogeneity in morphological evolution within and among
176 *Pheidole* castes has not emerged. Using landmark-based geometric morphometrics, and taking
177 advantage of recent progress on reconstructing the *Pheidole* phylogeny (Economato et al. 2015a;
178 Economato et al. 2019) which allows for a more taxonomically and geographically extensive
179 analysis, we perform the most morphologically and phylogenetically comprehensive analysis to-
180 date to attempt to infer a general picture of integration and modularity in size and shape in the
181 *Pheidole* worker castes.

182
183 To frame our study, we propose a set of hypotheses predicting different patterns of
184 morphological integration within and among castes in social insect colonies (see Figure 1). We
185 discuss this in terms of the head and mesosoma (thorax) of *Pheidole* worker castes, but it could
186 equally be applied to any morphological traits shared among castes, or indeed traits shared
187 among other differentiated phenotypes like sexes or reproductive strategies (Simpson et al. 2011).
188 First, different parts of the body *within* a worker caste may be more or less integrated. This
189 integration could reflect developmental biases or biomechanical constraints, for example a
190 specific change in head morphology may necessitate a specific change of the thoracic segments
191 that support or move the head. Second, *across* worker castes the same homologous body parts
192 could be more or less integrated. As different worker castes share not only genomes but
193 developmental pathways, it is plausible that selection on a trait in one worker caste could lead to
194 a change in another worker caste. For example, selection on elongation of the head of a minor
195 worker may lead to similar elongation in the major worker, even if there is no fitness benefit to
196 the change in the major worker. Or, each worker caste could vary independently facilitating
197 different functional roles in the colony.

198
199 We test these hypotheses by assessing the presence and pattern of integration of the head and
200 mesosoma within and among worker castes. First, we assess heterogeneity in rates of evolution
201 across body parts and worker castes; whether evolutionary change tends to follow a pattern in

202 which different parts or worker castes are hot or cold spots of change, or whether there is rate
203 homogeneity within and among worker castes. Second, we look for patterns of modularity in
204 shape and size to test how well an evolutionary change in shape or size of one trait predicts the
205 shape and size of another trait within the same worker caste or in a different one. If there is rate
206 variation, we ask again whether those differences reflect characteristics shared among
207 homologous traits or among worker castes.

208

209

210 **Methods**

211

212 *Photographic Measurements*

213

214 All comparative studies reflect a compromise between depth of individual sampling within
215 species versus breadth across species. In this study we aimed to expand the latter to include as
216 many *Pheidole* species as possible. We acknowledge a drawback of this strategy, which is that
217 we cannot capture the size or shape range of individuals within each species. We measured a
218 total of 1164 specimens from 314 species, measuring an average of 2.18 major worker and 2.20
219 minor worker specimens per species (Appendix S1); to maintain consistency between samples,
220 all measurements were performed by coauthor BL. Myrmecologists use high resolution montage
221 photographs to document ant diversity, following a standardized set of specimen positions that
222 display head and body features from a consistent angle as described by the online resource and
223 repository, AntWeb.org. We made a broad effort to photograph specimens from species used in
224 recent phylogenetic projects (Economo et al. 2015a), supplemented with photographs taken by
225 others and deposited on Antweb.org. We endeavored to collect data on both major and minor
226 workers whenever possible, however photographic data for both worker castes were only
227 available for 214 species or 68% of our total taxonomic sample. To account for potential focal
228 length issues when using 2D photographs taken with different optical systems, we landmarked
229 the same specimen 100 times under six different magnifications. A focal length warping effect
230 was observable but was non-significant, and was within the range of intraspecific variation.

231

232 For each specimen, we placed landmarks using the three standard photographic angles: head
233 view, dorsal view, and profile view. We collected landmarks from features that were consistently
234 in the plane of the camera angle. Specifically, we placed 11 landmarks on the dorsal view of the
235 head (Table S1) and 6 landmarks on the profile view of the body (Table S2; all located on the
236 mesosoma; hereafter head, mesosoma; see Figure 2). To capture information on the posterior
237 head shape, we also included a set of 6 sliding semi-landmarks (7 in major workers) from
238 landmark 3 to 11 (Figure 2). The landmarks on the left side of the head were reflected bilaterally
239 to produce the curve on the right side of the head between landmarks 11 and 1. Fixed landmarks
240 on opposite sides of the head were reflected and averaged to force object symmetry.

241 While these landmarks omit several features that vary among *Pheidole* taxa, and those typically
242 used in myrmecology research and taxonomy (Pie and Traniello 2007), this was unavoidable due
243 to the constraints of choosing homologous landmarks in positions that are not occluded by
244 nearby features (e.g., the anterior pronotum is often occluded by the posterior head lobes).

245

246 *Geometric Morphometrics*

247

248 We performed a generalized Procrustes alignment on each set of landmarks using the R package
249 *geomorph*, employing separate analyses for major and minor workers (Adams and Otárola-
250 Castillo 2013; version 3.0.7). Specimens showing greater than expected distance from the
251 Procrustes mean (i.e., above the upper quartile) were inspected for improper scale entry or
252 landmark order/placement. Photos for which improper specimen positioning was observed were
253 removed from the data set (< 1% of specimens studied). Within each species, we calculated the
254 average Procrustes shape before proceeding with further analyses; we also averaged linear
255 measurements in this manner. To visualize variation in highly dimensional shape characters, we
256 estimated principal component axes and plotted species averages in tangent space (Figure 3c and
257 e). As a proxy for body size, we used the logarithm of the centroid size of mesosoma landmarks
258 as in (Economo et al. 2015a), which behaves similarly to the Weber's Length measurement
259 typically used by myrmecologists (Weber 1938). Only multivariate Procrustes alignment data,
260 and not principle component data, were used in the comparative methods below (Uyeda et al.
261 2015).

262

263 *Phylogenetic Data*

264

265 We used a time-resolved phylogeny reconstructed by Economo et al. (2018) that includes 449
266 ingroup *Pheidole* species, based on a molecular dataset of nine loci. This phylogenetic tree builds
267 upon previous analyses of *Pheidole* (Moreau 2008; Economo et al. 2015a), with the addition of
268 164 taxa and an expanded set of loci sequenced across species. For analyses in this paper, we
269 used the maximum clade credibility tree from a Bayesian posterior set, which was pruned to
270 contain only the taxa present in our morphological data (Figure 3; Figure S1).

271

272 *Comparative Methods*

273

274 To examine the degree of correlated evolution between body regions (i.e., morphological
275 integration), we used the R package *geomorph* (Adams and Otárola-Castillo 2013). We ran a
276 series of pairwise integration tests between body regions and worker castes (Adams and Felice
277 2014). In each test, we estimated partial least squares (PLS) correlations between two sets of
278 landmarks while correcting for phylogeny. The coefficient of correlation (r-PLS) for this
279 regression describes the degree of integration. To calculate a p-value and significance test, we
280 generated 1000 permutations of species' phylogenetically-transformed values for each
281 comparison. To compare integration of body size among worker castes and between the head and
282 mesosoma, we used the coefficient of correlation for the linear regression of phylogenetically
283 independent contrasts, hereafter r-PIC (Felsenstein 1985). To compare the relationship between
284 trait shape and body size, we used a phylogenetic regression implemented for Procrustes shape
285 variables (Adams and Collyer 2018). These and other methods described below were also run for
286 the New World, Old World, and Australasian clades individually (Figure 3A; see online
287 supplement). It is important to note that integration may exceed the values estimated here using
288 PLS, as integration may span multiple PLS axis dimensions beyond the first axis, which is what
289 we compared.

290

291 We used *geomorph* to estimate evolutionary rates for landmarked specimens (Denton and Adams
292 2015). As a significance test for differences in rates between traits, we performed 1000
293 simulations of trait evolution under a joint Brownian motion model, and compared the ratio of
294 independently estimated rates to this simulated null. Given that differences in the number of
295 landmarks can bias the amount of variation and thus rate described by each trait (Denton and
296 Adams 2015), we report rate ratios for each pair of traits (e.g., major head vs. major mesosoma)
297 as a proportion of the simulated null ratio.

298

299 We tested for evidence of evolutionary modularity within each body region (i.e., in addition to
300 the head and mesosoma) again using *geomorph* (Adams and Otárola-Castillo 2013). We split
301 each body region into sets of a priori evolutionary modules (*sensu* Klingenberg 2008) roughly
302 aligned with anatomical axes. Head landmarks were assigned to two potential module

303 configurations, one along the anterior/posterior axis (hereafter: A/P), and one along the
304 sagittal/lateral axis (hereafter: S/L; see Figure S2). The A/P grouping separates the anterior
305 (clypeus) area which is related to the feeding apparatus from the posterior of the head which
306 houses the brain and mandible muscles. The D/V axis separates structures more toward the
307 midline of the head (central clypeus, antennae) from the sides (eyes, occipital lobes). Mesosoma
308 landmarks were also assigned to three potential groupings, one along the anterior/posterior axis
309 with bias towards the anterior (hereafter: A/p), one along a similar axis with bias towards the
310 posterior (a/P), and one along the dorsal/ventral axis (D/V; see Figure S2). These
311 anterior/posterior groupings correspond to landmarks associated with different body segments,
312 while the D/V grouping associates landmarks in the region closer the legs or dorsal part of the
313 body, respectively. In this framework, we compared the covariance ratio (CR; Adams 2016) of
314 each hypothesized set of landmarks to those of simulated sets of landmarks (averaged between
315 orientations rotated up to 90° in 0.05° increments), while accounting for phylogenetic
316 relationships. Each simulation test was run for 1000 iterations (see online supplement).

317

318

319

320

321 **Results**

322

323 *Evolutionary Rate*

324

325 In comparisons of different body regions of the same worker caste, mesosoma shape evolved
326 more rapidly than head shape in both major workers (rate ratio $rr = 6.02$, $p < 0.01$) and minor
327 workers ($rr = 6.14$, $p < 0.01$; Figure 4). In comparisons of similar traits between worker castes,
328 we observed no significant differences in evolutionary rate for head shape ($rr = 1.07$, $p = 0.59$) or
329 mesosoma shape ($rr = 1.10$, $p = 0.59$). In contrast to the rate variation among shape traits,
330 evolutionary rates estimated for size traits showed few differences between worker castes or
331 between the head and mesosoma (Figure S4), with the exception of the major worker's head
332 which evolved relatively slowly.

333

334 Our tests of modularity within body regions suggested the presence of two evolutionary modules
335 in the *Pheidole* head, in an anterior-posterior arrangement, though the use of semi-landmarks
336 may bias this result (see online supplement). We compared evolutionary rate between the
337 inferred modules of head shape (Figure S2). In these analyses, the anterior landmarks exhibited a
338 higher rate of evolution than the posterior landmarks in both major workers (Figure 4; $rr = 1.42$,
339 $p < 0.05$) and minor workers ($rr = 1.42$, $p < 0.001$). We also tested for variation in evolutionary
340 rate among lineages. This analysis showed significant support for differences in evolutionary rate
341 of minor workers' mesosoma shape between biomes, with the most rapid evolution seen in the
342 tropics (see online supplement).

343

344 *Morphological Integration*

345

346 Morphological integration is described here as correlated evolution between morphological
347 shape characters. The strength of this correlation is described using the PLS correlation
348 coefficient (r-PLS), and its significance is assessed by comparison to a simulated null
349 distribution (Adams and Felice 2014; Adams and Collyer 2016). For estimates of body size
350 rather than shape it is measured as the correlation coefficient of independent contrasts (r-PIC).

351

352 We found strong indications of morphological integration between both worker castes and body
353 regions in *Pheidole*, however the strength of these correlations varied depending on the
354 comparison (Figure 5a). Head shape was correlated with mesosoma shape in both major workers
355 ($r\text{-PLS} = 0.53$, $p < 0.001$) and minor workers ($r\text{-PLS} = 0.51$, $p < 0.001$). In examinations of
356 morphological integration between worker castes, mesosoma shape was strongly correlated
357 between castes ($r\text{-PLS} = 0.76$, $p < 0.001$), whereas head shape showed a weaker albeit still
358 significant correlation ($r\text{-PLS} = 0.48$, $p < 0.001$). This difference in worker caste integration
359 effect among body regions was highly significant (two-sample z test; $p < 0.001$). Similar results
360 were observed for analyses performed with semi-landmarks from the head's posterior lateral
361 lobes included. Morphological integration varied somewhat between clades (see online
362 supplement), with the Asian-African clade exhibiting a lower degree of integration for all shape
363 traits.

364

365 We performed hierarchical clustering on correlation coefficient matrices for shape integration
366 and size integration (Figure 5b, Figure 5c). Overall, *Pheidole* showed much greater
367 morphological integration in size than in shape. Morphological integration was greater for size
368 traits (r-PIC 0.8 – 0.95) than for any shape traits (maximum r-PLS = 0.76). This integration in
369 size was greater within worker castes than between them (Figure 5c). Morphological integration
370 of shape traits was greatest between the mesosoma of major and minor workers, which evolved
371 as though it were a single module. Head shape was weakly integrated with other traits for minor
372 workers, and least integrated for major workers (Figure 5b).

373
374 The scaling relationship between the sizes of different parts is a common theme in evolution and
375 development. As expected, we found a tight relationship between mesosoma size (Weber's
376 length; Weber 1938) and head length; this was evident in both majors and minors (Figure S3).
377 Relationships between the shape of the head and mesosoma and body size were observable,
378 however they were very weak and poorly predictive (all R-squared values < 0.03; Table S3).

379

380

381 **Discussion**

382

383 Our results showed varying evolutionary rates and degrees of evolutionary integration within and
384 among worker castes. Overall, evolutionary rate and integration this followed the predictions of
385 different hypotheses (Figure 1). In particular, the mesosoma exhibited integration among
386 homologous traits in different worker castes, while the head exhibited a weaker degree of
387 integration. We found that the mesosoma evolved faster than the head and with a greater degree
388 of morphological integration between castes (Figure 4), but in general evolutionary rate was
389 similar for homologous traits in different worker castes. We found a complex pattern whereby
390 the evolution the head shape of major workers was largely decoupled from that of other traits,
391 but was not necessarily evolving faster.

392

393 The evolutionary rate of carapace shape was highly divergent across the different parts of the ant
394 (head vs. mesosoma). This observation was most evident with regards to mesosoma shape, which
395 evolved roughly 1.5x faster than head shape (when corrected for variance differences). Moreover,

396 we found that the anterior portion of the head near the mandibles and mouthparts is evolving
397 more quickly than the posterior half. However, there were no significant differences in
398 evolutionary rate among homologous traits between majors and minors. Thus, homologous traits,
399 and not traits within a caste, tended to evolve at similar rates (Figure 1).

400

401 The inferred rate similarity among traits does not alone imply the traits themselves are correlated
402 in their evolution (i.e. they could be evolving at similar rates but on different trajectories), thus
403 we also investigated which sets of traits were correlated during evolution. Here, we found a
404 different pattern, whereby the evolution of mesosoma shape was tightly linked across major and
405 minor workers, but head shape was more decoupled between the two castes. In this way, the
406 head of the major worker was the least integrated with other traits, and the mesosoma of the
407 worker was the most integrated. Previous research in *Pheidole* found that integration among
408 linear measurements was weaker for minor workers than major workers (Pie and Traniello 2007).
409 In contrast, our analyses found weaker integration between head and mesosoma shape for majors
410 than minors. Thus, no one integration hypothesis was supported – either between homologous
411 traits, or between traits within a caste – but rather a mixture of the two.

412

413 The fact that mesosoma shape evolved more rapidly than head shape is somewhat surprising, as
414 the head would presumably be the most related to feeding ecology, a key trait that varies across
415 ant species. One potential explanation is that head shape is under stronger stabilizing selection.
416 However, another potential conclusion is that fast mesosoma evolution reflects relative size and
417 arrangement variation in the underlying muscles that control load-carrying and locomotion,
418 which could reflect functional differences in how the ant carries, moves, and performs different
419 tasks. The primary axis of mesosoma variation runs from a stocky shape to a more gracile and
420 elongate one, and most changes are happening repeatedly within limited bounds. There is reason
421 to expect that stocky shapes are common in belowground-foraging species, and that more gracile
422 characteristics are associated with aboveground-foraging and associated defensive traits like
423 spines (Weiser and Kaspari 2006; Sarnat et al. 2017). *Pheidole* are known to vary in the extent to
424 which they live and forage in the leaf litter or on vegetation (Mertl et al. 2010), and there could
425 be tradeoffs inherent the designs adapted for moving and foraging on horizontal vs. vertical
426 surfaces. This would also explain why major and minor mesosomas are tightly integrated in

427 shape, because they face similar biomechanical challenges due to living and moving in similar
428 environments. Thus, these phenotypes may represent ecomorphs that are repeatedly evolved in
429 each newly colonized region, as in *Anolis* lizards (Mahler et al. 2013). However, given the
430 paucity of behavioral observations for most ant species around the world, further study is
431 required to understand this trait's functional and biomechanical significance. Furthermore, the
432 linking of external geometry with variation in underlying function and performance remains an
433 important avenue for future work on comparative anatomy and biomechanics in ants.

434
435 We find support for the hypothesis that the shape of minor and major worker castes can evolve
436 independently (Holley et al. 2016), promoting the evolution of ecological specialization. We
437 emphasize that this is not simply a statement that head shapes are different between majors and
438 minors, which is obvious, but that they can evolve on diverging trajectories (i.e. the major is not
439 just a consistent transformation of the minor). This allows for increased evolutionary “degrees of
440 freedom” in the functional specialization among castes. However, this finding was specific to the
441 head region, as mesosoma shape was tightly integrated across castes. The fact that rates of shape
442 evolution were 1.5 times greater for the highly integrated mesosoma than for the head (Figure 4)
443 suggests that integration in this case does not constrain, but may rather accelerate rates of
444 evolutionary divergence in shape among species (Cheverud 1995; but see Márquez and Knowles
445 2007).

446
447 Allometry is a common theme and pattern in development and evolution, and strong
448 relationships between the sizes of different body parts are expected during evolution. Matching
449 this expectation, we found that head and mesosoma sizes were tightly linked both within and
450 among castes (Figure 5). In contrast to the pattern for cranial evolution in birds (Klingenberg and
451 Marugán-Lobón 2013), relationships between shape traits and body size were significant, but
452 poorly predictive (Figure S3; Table S1). While we were not able to account for allometric
453 relationships within species due to our study design, we did find that cross-species relationships
454 between body size and shape traits were not strong enough to potentially drive other patterns
455 reported in this study. Our estimates of evolutionary rate for size traits showed that the size of
456 each trait evolved faster than its shape (Figure S4), confirming a similar observation by Pie and
457 Tscha (2013). Interestingly, major worker heads evolved at the slowest rate for size and among

458 the slowest for shape despite being the least integrated with other body parts (which should thus
459 release it from constraint by pleiotropic effects; but see Cheverud 1995). This suggests that this
460 trait is more evolutionarily conserved; future studies investigating the evolutionary consistency
461 of major worker tasks (as in Mertl et al. 2010) and their biomechanical needs would be valuable
462 in explaining this pattern.

463
464 In principle, correlations in size and shape among traits/castes could be caused by either
465 selection or developmental constraint. This kind of comparative analysis does not by itself allow
466 for inference of the underlying selective or developmental mechanisms responsible for the
467 patterns of integration that we identify. However, there is a strong body of work on the
468 developmental basis of caste differentiation in *Pheidole*, and especially the role of JH as a
469 developmental switch cues, that can inform the likelihood of some potential explanations.
470 Notably, classic (Wheeler and Nijout 1983, 1984; Wheeler 1991) and more recent (Rajakumar et
471 al. 2012; Lillico-Ouachour and Abouheif 2016) work shows that experimental manipulation of
472 pheromone exposure can alter the relative sizes of *Pheidole* majors and minors, and manipulation
473 of rudimentary wing discs can alter the relative sizes of the head and body (Rajakumar et al.
474 2018). Moreover, in other insects, it has been shown that relative sizes of different body parts
475 can be experimentally selected for (Frankino et al. 2005; Stillwell et al. 2016). If researchers can
476 manipulate relative size with apparent ease using chemical cues or artificial selection, this
477 implies that evolution may not be constrained from doing the same. We expect that general
478 diversification of body size is likely to due to selection on loci that control body size overall,
479 rather than independent selection on the size of each part. However, the fact that relative sizes of
480 different parts have been maintained in evolutionary time implies selective advantages of the
481 relative sizes of body parts within and among castes (Gould 1966).

482
483 To our knowledge, less is known about the developmental basis of the shape characters we are
484 capturing in our landmark system, so developmental constraints or biases may explain some of
485 the evolutionary correlation in shape we observe. However, the evolutionary modules in the head
486 inferred by our analysis (Figure S2) do not correspond to the head developmental modules
487 inferred by Yang and Abouheif (2011) in their examination of *Pheidole* gynandromorphs. If both
488 studies are correct, this would imply that developmental modularity does not underlie the

489 macroevolutionary modularity we infer, leaving selection and non-genetic influences, as well as
490 methodological issues with comparing fixed landmarks and semi-landmarks, as the most likely
491 explanations for why different regions of the head appear to evolve separately or independently.
492 An interesting future direction would be to attempt to experimentally investigate the
493 developmental bases of the axes of shape variation we identify in our study.

494

495 One noticeable feature of the genus *Pheidole*'s global diversification has been the re-evolution of
496 similar environmental and behavioral niches in different geographic regions, each radiation
497 following from a single colonization event (Moreau 2008; Economo et al. 2015a). While
498 morphological evolution in this clade has been largely conserved throughout its history (Pie and
499 Traniello 2007), similar body size phenotypes have consistently re-evolved following each
500 clade's colonization of a new biogeographic realm (Economo et al. 2015a). In this study we
501 observed that New World and Old World radiations of *Pheidole* occupied mostly overlapping
502 portions of morphospace (Figure 3), whereas the Australasian clade occupied a smaller, but still
503 overlapping portion of this same trait space. We found this pattern for size and shape of both
504 head and mesosoma. It remains unclear why some portions of morphospace, and large body size
505 in particular, have not evolved in Australasian taxa. One potential explanation is that niche filling
506 in this most recent radiation is ongoing – indeed the Australian clade is the youngest of the
507 continental radiations and is still in a more elevated phase of its diversification (Economo et al.
508 2019).

509

510 *Conclusion*

511 The morphological and functional differentiation of castes is thought to be a key evolutionary
512 innovation underlying the success of ants and other social insects. Patterns of macroevolutionary
513 integration and modularity within and among castes may provide clues to the selective forces
514 shaping diversification in ants, and the developmental biases and constraints involved in trait
515 divergence (West-Eberhard 1979). We find that size evolution is tightly integrated and evolving
516 with homogeneous rates both among parts in a single caste, and across the worker castes. In
517 contrast, our results using geometric morphometric estimates of body shape indicate that while
518 mesosoma shape shows homology integration, head shape has become largely disintegrated
519 between major and minor workers (Figure 3c). Head morphology and its associated musculature

520 is associated with ecological specialization in many taxa, often but not exclusively due to feeding
521 functionality, thus the differences in head shape between major and minor workers probably
522 represent divergence in their tasks in the colony (Smith 1987; Futuyma and Moreno 1988; Mertl
523 and Traniello 2009). In this case, evolution of developmental pathways facilitating independent
524 evolution of major and minor worker phenotypes could represent key innovations enabling
525 lineages with this trait to occupy multiple specialized strategies at once, or to discover new team
526 strategies emergent from their polymorphism (Wheeler & Nijhout 1981, 1984; Wheeler 1990;
527 Anderson and McShea 2001). Interestingly, the independent evolution of the head does not lead
528 to faster rates of evolution, and in fact mesosoma shape evolves 1.5x faster than head shape in
529 *Pheidole*. We hypothesize that this rapid evolution of the mesosoma reflects a pattern of frequent
530 adaptation to different biomechanical needs in different microhabitats, but future work is needed
531 to test this hypothesis.

532

533 While body-size polymorphism is a common trait in ants, “complete” polymorphism (i.e., in
534 shape) is rarer but noticeably present in some of the most diverse ant clades (Wills et al. 2017),
535 an observation that hints at a role for polymorphism in adaptability (Wilson 2003). We propose
536 that, beyond the benefits of body-size polymorphism, the reduction of morphological integration
537 between distinct behavioral strategies, inclusive of sexes, castes, and alternative reproductive
538 tactics (West-Eberhard 1979), could be a recurring key innovation that enables the evolution of
539 adaptive polymorphism and promotes rapid diversification. Further comparative studies on the
540 evolution integration and modularity across radiations of ants with worker polymorphisms, and
541 any concurrent changes in diversification rates and patterns, would be useful for testing this
542 hypothesis.

543

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552

553 **Figure Titles**

554

555 Figure 1: Hypothesized scenarios for the evolution of differentiated phenotypes. Worker castes
556 or body parts united in the same box represent a pair of integrated traits. The scenarios we
557 propose can be arranged in order of their extent of integration among homologous traits in
558 different castes and among different traits within a caste.

559

560 Figure 2: Example photographs of *fervens* minor worker (A) and major worker (B) assembled by
561 photo-montage according to AntWeb specifications. Landmarks, in white, were placed on
562 homologous features on the head (C) and mesosoma (D). Semi-landmarks, in blue, were spaced
563 equally on the left side of the head between landmarks 3 and 11, and between landmarks 11 and
564 1.

565

566 Figure 3: A phylogeny of the ant genus *Pheidole*, with clades colored by their geographic region,
567 is shown in (A). Note that each clade represents a single colonization event (see Economo et al.
568 2015a). Comparisons of values for like traits in different castes are shown for head size (B), head
569 shape (C), mesosoma size (D), and mesosoma shape (E). Ellipses reflect 95% confidence
570 intervals, and are colored according to clade as in (A). For the shape data displayed in (C) and
571 (E), the first principle component is shown for display purposes (and is not used in subsequent
572 comparative methods), along with the percentage of variance it explains and deformation grids
573 describing extreme values along the axis (produced using *geomorph*; Adams et al. 2018).

574

575 Figure 4: Evolutionary rates are displayed here as a morphogram heat map (Martin &
576 Wainwright 2011). Comparison ratios between traits digitized using different numbers of
577 landmarks (e.g., head and mesosoma) are given as ratios compared to a simulated null ratio.
578 Arrows and brackets indicate statistical tests of rate differences compared to a simulated null,
579 with accompanying numbers describing the estimated rate ratio for the two traits. * $p < 0.05$,
580 ** $p < 0.01$, *** $p < 0.001$

581
582 **Figure 5:** Morphological integration between among body parts within and among worker castes
583 is shown by arrow width in (A). Hierarchical clustering of integration relationships for trait
584 shape is shown in (B) and for trait size in (C), with the strength of relationships indicated by the
585 heatmap and displayed value – r-PLS for trait shape and r-PIC for trait size. *p < 0.05, **p<0.01,
586 ***p<0.001

587
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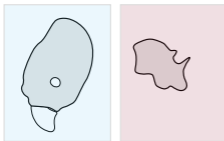
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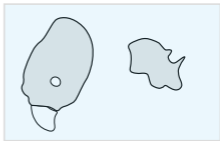
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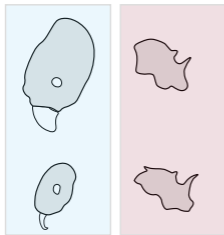
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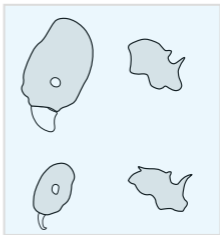
Integration within castes

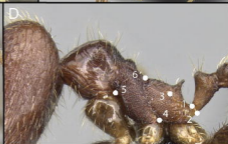
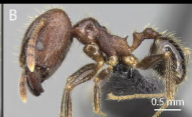


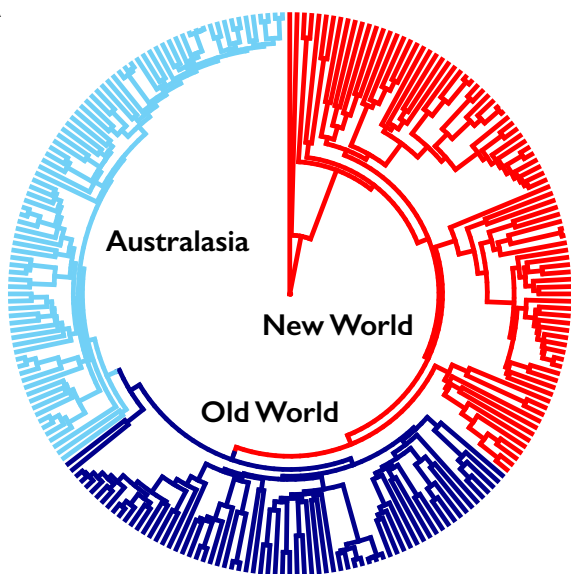
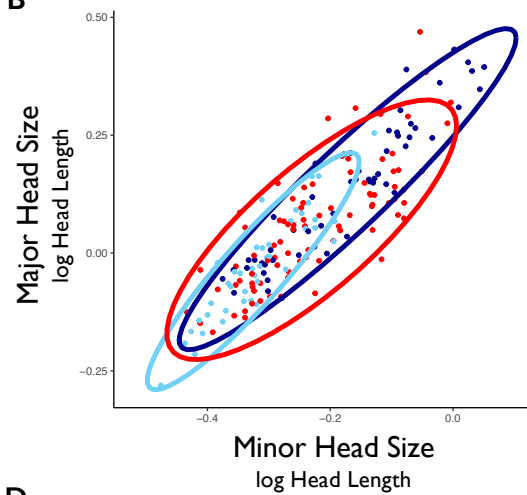
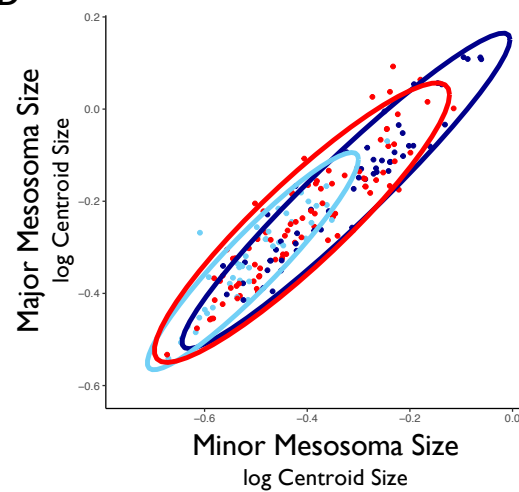
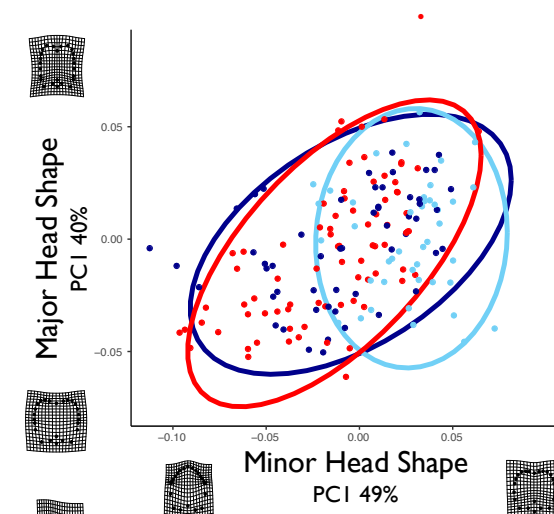
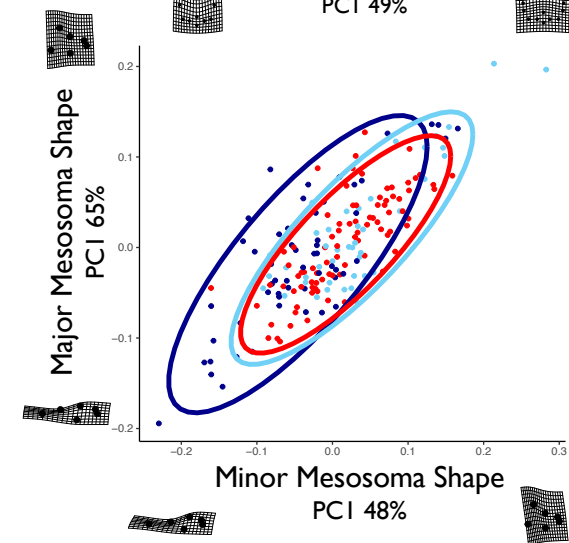
Integration among homologous traits



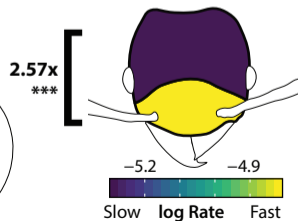
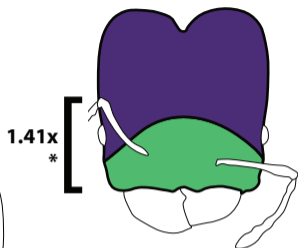
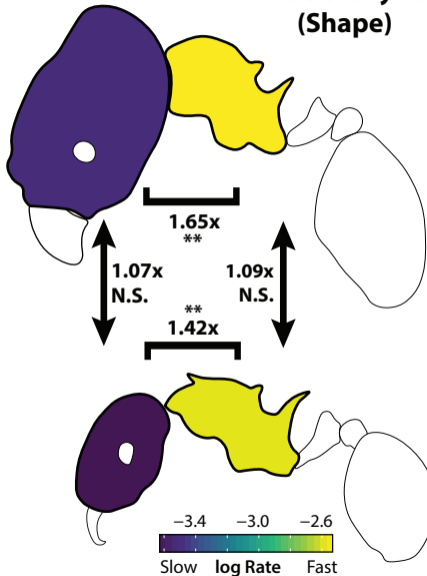
Complete Integration



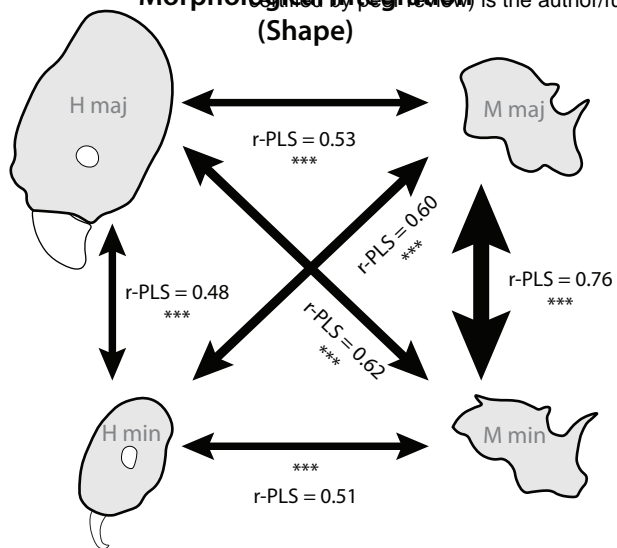


A**B****D****C****E**

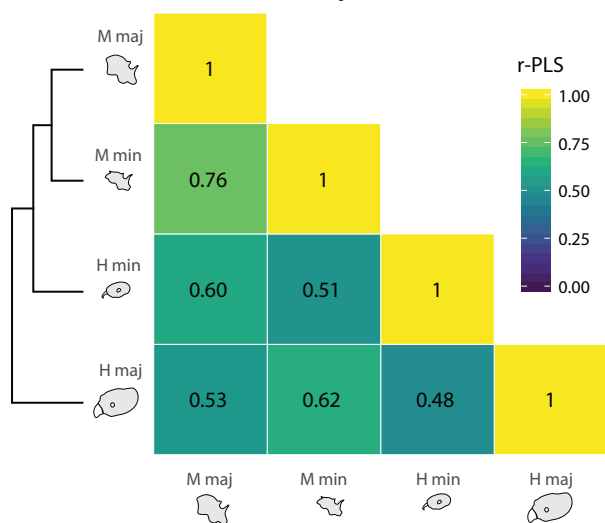
Evolutionary Rate (Shape)



Morphological Integration (Shape)



Morphological Integration (Shape)



Morphological Integration (Size)

