

Behavior, brain, and morphology in a complex insect society: trait integration and social evolution in the exceptionally polymorphic ant *Pheidole rhea*

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Received: 2 June 2017 / Revised: 12 October 2017 / Accepted: 13 October 2017 / Published online: 26 October 2017
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Abstract Polyphenisms in social insects arise from flexible developmental mechanisms that may produce behaviorally and morphologically specialized workers. The ant genus *Pheidole* is typically characterized by small minor worker and large soldier subcastes, but larger supersoldiers have evolved in several species, including the exceptionally polymorphic *Pheidole rhea*. To examine worker phenotype evolution in this socially complex ant, we tested the hypotheses that behavior, brain structure, and morphology are integrated within worker size classes due to selection for specialized social roles and that traits have been discretized among these groups. Our analyses revealed significant differences in brain structure and body shape between minor workers and the two soldier size classes. Behavioral repertoires of minors, soldiers, and supersoldiers were similar, but minors performed important tasks at higher frequencies than soldiers and supersoldiers. The extensive overlap in behavioral repertoire between soldier and supersoldier groups correlated with shared neuroanatomical and morphological traits. Although head and body size vary

allometrically among *P. rhea* size classes, scaling analyses revealed little allometry in brain substructure. The covariation of behavior and brain organization between soldier size classes suggests that their functionality may be due to differences in task performance rate or efficiency rather than task repertoire. The early branching position of *P. rhea* in the phylogeny of *Pheidole* and patterns of worker phenotypic plasticity suggest this species exhibits an ancestral state of sociobiological and neurobiological organization that served as a ground plan for diversification in this ecologically dominant ant genus. Selection for divergent social roles may impact the degree to which behavioral, neuroanatomical, and morphological phenotypes are integrated within individuals and between specialized groups. Behavioral repertoire, neuroanatomy, and worker body shape in the strongly polymorphic *P. rhea*, a species of the hyperdiverse ant genus *Pheidole* that branched early in the phylogeny of the clade appear to show a relatively low degree of integration with worker head-body size. The putatively ancestral and developmentally plastic condition in which larger-bodied soldier groups are not distinguishable in behavior, brain scaling relationships, and body shape suggests the advantage of worker head-body allometry may derive from variation in quantitative aspects of behavioral performance rather than specialization on qualitatively different tasks. This appears to be significant to social evolution in the genus.

Communicated by W. Hughes

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00265-017-2396-z>) contains supplementary material, which is available to authorized users.

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Keywords Division of labor · Caste development · Social brain evolution · Neuroethology · Developmental plasticity · Trait integration

Introduction

Suites of adaptive behavioral traits are often associated with variation in neuroanatomy and morphology, and correlated

selection for these traits is significant to phenotypic evolution (Powell and Leal 2012; Holekamp et al. 2013; Tsuboi et al. 2014). Developmental plasticity is especially important to trait integration because multiple levels of phenotypic organization can be coordinated through interacting responses at genetic, epigenetic, and neuroendocrine levels (West-Eberhard 2003; Murren 2012; Wund 2012; Wund et al. 2012; Standen et al. 2014; Ellers and Liefing 2015; Gibert 2017). Such effects are illustrated by social insect caste determination (Molet et al. 2012, 2014; Londe et al. 2015), caste proliferation, and their relationships to behavioral repertory (Londe et al. 2016). Interactions between hormonal states and genotypic and genomic plasticity influence adult morphology, brain gene expression, and social role flexibility in various eusocial species (Lucas and Sokolowski 2009; Ament et al. 2010; Schwander et al. 2010; Friedman and Gordon 2016; Simola et al. 2016; Hamilton et al. 2017).

The origin of social insect worker phenotypes is of particular interest in the ecology of social evolution given its prominence in species diversification (Oster and Wilson 1978; Wilson 2003; Planqué et al. 2016). Selection acting on worker phenotypes is hypothesized to impact fitness through morphological caste differentiation and collateral task specialization (Fjerdingstad and Crozier 2006; Powell 2016), adaptive demographic distributions (Yang et al. 2004), social networks (Mersch et al. 2013; Mersch 2016), patterns of task allocation (Gordon 2010), as well as collective action and other emergent colony-level properties (Gautrais et al. 2002; Langridge et al. 2008; Dornhaus et al. 2012; Jandt et al. 2013). Division of labor in socially complex polymorphic species concerns the evolution of behavioral specialization among physically differentiated workers (subcastes) that is hypothesized to increase ergonomic efficiency and thus enhance colony fitness (Oster and Wilson 1978; Wilson 2003; Powell 2016). By examining phenotypic integration in species with polymorphic workers, significant questions concerning the degree of association of task performance, brain evolution, and morphological differentiation can be addressed.

Ants are an exceptionally rich eusocial insect clade that includes a relatively small number of genera and species exhibiting striking and sometimes extreme worker polymorphism (Fjerdingstad and Crozier 2006). Ecologically diverse and evolutionarily successful, they offer excellent social models to gain novel insights into phenotypic integration. Moreover, exceptional polymorphism provides opportunities to explore brain evolution in respect to selection for adaptive morphological and behavioral variation (Gronenberg and Riveros 2009; Riveros et al. 2012; Ilies et al. 2015). Patterns of worker brain scaling may vary according to ecological challenges and be reflected in differences in sensory perception, higher-order processing, and/or motor demands required for task performance (Sulger et al. 2014; Bulova et al. 2016; Kamhi et al. 2016).

Mosaic brain structure underpinning sociobiological variation in subcaste-related division of labor and behavioral development has been found in the hyperdiverse ant genus *Pheidole* (Muscedere and Traniello 2012; Ilies et al. 2015), which is typically characterized by a completely dimorphic worker caste of task-generalist minor workers and larger workers, often termed majors or “soldiers” (the latter term used herein) that may show different degrees of specialization. The evolution of the soldier subcaste is thought to be key to the remarkable diversification of the genus (Wilson 2003; Lillico-Ouachour and Abouheif 2016), which includes ecological dominance across diverse biomes (Economio et al. 2014). *Pheidole* species differ in specialized worker morphologies (Pie and Traniello 2007; Mertl and Traniello 2009; McGlynn et al. 2012; Holley et al. 2016), subcaste demography (Mertl et al. 2010; Wills et al. 2014), repertoire breadth (Wilson 1984), and behavioral plasticity (Brown and Traniello 1998; Mertl and Traniello 2009; Sempo and Detrain 2010). The development of dimorphic *Pheidole* subcastes is regulated by the reprogramming of critical size and growth parameters through a juvenile hormone (JH)-mediated sensitive period (Wheeler and Nijhout 1981, 1983; Wheeler 1991; Nijhout and Wheeler 1996). Alterations of this developmental system have facilitated the independent and parallel evolution of a third worker size class of *supersoldiers* in at least eight *Pheidole* species (Moreau 2008; Rajakumar et al. 2012). Supersoldiers are larger in size and have disproportionately larger heads than soldiers. These allometric differences between soldiers and supersoldiers are produced through similar developmental mechanisms: JH-mediated increase in larval size as well as the presence of vestigial wing discs (Rajakumar et al. 2012). Finally, supersoldiers are demographically rare in colonies and therefore could have highly specialized social roles (Huang 2010; Huang and Wheeler 2011).

We examined trait integration within worker size classes of *Pheidole rhea* and their discretization of traits in respect to social behavior. In this species, head and body size variation between minor workers and the two larger-bodied worker size classes is discrete, and although the size distribution of soldiers and supersoldiers appears to be at most bimodal, head size scales allometrically to body size, supporting the morphological distinction of supersoldiers (Huang and Wheeler 2011). We tested the hypothesis that selection for distinct social roles has resulted in divergence in brain structure and worker morphology across body size variation. We describe the organization of worker phenotypes in regard to differentiation in behavioral repertoire, brain anatomy, and body shape to better understand phenotypic integration and plasticity in this highly polymorphic insect society.

Methods

Ant collection and culture

Three *P. rhea* lab colonies (each ~ 30,000 workers), initiated by foundresses collected by Dr. M. Huang in the foothills of the Pajarito Mountains and Santa Rita Mountains in Santa Cruz County, AZ, were cultured in the lab at the University of Arizona for at least 3 years before use in the present study. Colonies were maintained at 28 °C in constant darkness inside an artificial nest constructed of test tubes partially filled with water and fitted with tight cotton plugs and were fed cockroaches and sunflower seeds three times a week. One of these colonies was transferred to Boston University for behavioral observations and was maintained in the lab for an additional 2 years at 25 °C, 65% humidity with a 12 h:12 h light/dark regime, and fed 1 M sucrose and live wingless fruit flies or mealworms and sunflower, flax, and chia seeds on alternate days. Colonies were housed in three connected plastic boxes (30 cm × 20 cm coated with Fluon®). A large dental stone nest (23 cm × 18 cm) outfitted with an embedded plastic irrigation tube connected to a reservoir to keep the nest humid formed the center box. This primary nest contained a series of different-size chambers with a red acetate film-covered Plexiglas® top to facilitate observations. On one side, the center box was connected to a foraging arena in which food was provided, and on the other side two smaller dental stone chambers (10 cm × 10 cm each) served as auxiliary nests. For all studies, workers approximating in size three modes of worker head widths (minor = 0.75 mm, soldier = 2.0 mm, supersoldier = 3.0 mm), which we refer to as size classes, were sampled, according to description of size variation in *P. rhea* worker groups (Huang and Wheeler 2011). We categorized *P. rhea* workers conservatively according to their size class rather than subcaste because of unresolved morphological and behavioral implications of the latter term.

Morphological measurements

Consistent with prior research on size variation in *P. rhea* (Huang and Wheeler 2011), we use the term *soldier-polymorphic* to describe the worker caste system of this species, recognizing allometry in body size and acknowledging that the functional ecology of worker size classes and their task arrays are not fully understood. To analyze the distribution of morphological characters associated with worker size variation, 18 measurements (Pie and Traniello 2007) of the head and mesosoma were recorded to the nearest 0.02 mm from individual *P. rhea* workers ($N = 10$ per worker size class) in the ant collection of the Museum of Comparative Zoology at Harvard University. Because this required morphological measurements on workers of a particular size, we could not be blind to the size class categorization of each specimen.

Average measurements for each size class are provided in Online Resource 1. We focused on relative differences in body size and shape across workers in *P. rhea*, herein termed *relative morphological characteristics*, to complement studies that demonstrate allometry in head width between *P. rhea* size classes (Huang and Wheeler 2011). Body shape can be important to phenotypic evolution (Harmon et al. 2010) and has functional consequences for behavioral performance (Losos 1990). We chose to examine a set of size-free relative morphological characteristics to gain multidimensional insight into how potential differences in worker body shape may impact task performance (Tschinkel et al. 2003). Body size differs dramatically across the worker size range in *P. rhea*; we therefore reduced its effect in our analyses by recombining linear measurements (Table 1). Head width was measured at the widest point posterior to the eyes. We used pronotal width (PW) as a body size indicator (Kaspari and Weiser 1999) because of the importance of size in distinguishing features across worker groups.

Worker size and behavioral repertoire

To quantify within-nest and outside-nest behaviors of workers in each size class, data were collected using focal individual sampling. The nest was divided into zones that were randomly selected to record behavior. Ten-minute observations of focal workers ($N = 24$ per worker size class) in a queenright *P. rhea* lab colony (> 10,000 individuals) were recorded by viewing ants with a swing arm microscope at ×7 magnification. The goal of these observations was to establish behavioral profiles for worker groups; therefore, we could not be blind to the size class of focal individuals. Observations were logged on a voice recorder while the colony was in an undisturbed state. A surgical mask was worn during observations because *P. rhea* workers react strongly to air currents and carbon dioxide. The colony used to quantify worker repertoires was generated from a mated foundress collected by Dr. M. Huang. Sampling additional colonies was not possible due to a lack of other colonies originating from founding queens and the inability to collect incipient and/or mature colonies of *P. rhea*, which naturally nest deep within rocky foothills. Nevertheless, we believe that the worker size-related behavioral profiles we present reflect robust sampling and accurately describe the *P. rhea* repertoire, allowing us to relate behavior to worker size and brain structure. Results of complementary studies of behavioral performance by *P. rhea* workers (DGG and JFAT unpublished data) support the repertoire compositions described here.

Brain size and structure

Brains of fully sclerotized (mature) workers were sampled from the three mature Arizona laboratory colonies over the

Table 1 Relative morphological characteristics used in this study and their abbreviations

Abbreviation	Description of measure
EML/HW	Maximum length of eye standardized by head width
DEA/HW	Distance between eye and antennal socket standardized by head width
MBR/HW	Maximum width of mandible standardized by head width
HDE/HLE	Head shape (head depth/head length in profile view)
ALH/PW	Pronotal shape (mesosoma height/pronotal width)
TCM/MAL	Distance between first coxa and metapleural gland standardized by maximum mesosoma length
TCP/MAL	Distance between first coxa and insertion of petiole standardized by maximum mesosoma length
TPP/MAL	Distance between insertion of petiole and posterior end of pronotum standardized by maximum mesosoma length
PH1/PL1	Petiole shape (petiole height/petiole length)
PH2/PL2	Post-petiole shape (post-petiole height/post-petiole length)
HW/HL	Head shape (head width/head length in frontal view)
SL/HW	Scape length standardized by head width
PW/MAL	Mesosoma shape (pronotal width/maximum mesosoma length)
HW/PW	Head width standardized by pronotal width
PW	Body size (pronotal width)

course of 3 weeks. *P. rhea* worker brains ($N = 20$ per worker size class) were processed using a modified immunohistochemistry protocol (Ott 2008; Kamhi et al. 2016). Brains were dissected in ice cold HEPES-buffered saline (HBS) and fixed in 1% zinc formaldehyde overnight at 4 °C on a shaker. For temporary storage (<1 month), brains were kept in 0.1 M cacodylate buffer. Brains were then washed in HBS (6×10 min), transferred to Dent's fixative (4:1 methanol/dimethyl sulfoxide) for 1 to 2 h, and stored in 100% methanol until further processing. Brains were rehydrated in 0.1 M Tris buffer before blocking for 1 h in a normal goat serum (NGS) solution (PBSTN) (5% NGS + 0.005% sodium azide in 0.2% Triton-X phosphate buffered saline [PBST]). We used a monoclonal *Drosophila* synapsin I antibody (SYNORF1, AB_2315426) purchased from the Developmental Studies Hybridoma Bank (catalog 3C11) as our primary antibody. After blocking, brains were incubated for four nights in primary antibody diluted 1:30 in PBSTN at 4 °C on a shaker. Subsequently, brains were washed in 0.2% PBST (6×10 min) and incubated for an additional three nights in Alexa Fluor 488 (ThermoFisher) goat anti-mouse secondary antibody (1:100 in PBSTN) wrapped in foil at 4 °C on a shaker. After secondary incubation, brains were washed (6×10 min in 0.2% PBST) and dehydrated in an increasing concentration of ethanol in PBS (5 min each in 30, 50, 70, 95, 100, 100%) before clearing and mounting with methyl salicylate in custom stainless steel well slides. Stained brains were imaged on an Olympus Fluoview BX50 laser scanning confocal microscope with a $\times 20$ objective and optically sectioned in the horizontal plane (3.1 μm steps). Due to the refractive index mismatch between air and methyl salicylate, images were corrected along the z -axis (by a factor of 1.59) to give the true section

thickness of $\sim 5 \mu\text{m}$ (Muscedere and Traniello 2012). Corrected images were manually segmented in Amira (FEI v 6.0) to generate volumes of functionally distinct brain regions for one hemisphere of each brain: optic lobe (OL, visual input), antennal lobe (AL, olfactory input), mushroom body calyces (MBC, sensory integrative input), mushroom body peduncle and lobes (MBP, integrative output and modulation), central body (CB, spatial and locomotor integration), and subesophageal zone (SEZ, head movement and gustation), in addition to the rest of the undifferentiated central brain (ROCB). Regions that spanned both hemispheres (CB and SEZ) were traced in full and their volumes were divided by half to equate to one hemisphere. The relative investment of each of six functional subregions defined earlier was calculated by dividing the volume of the region of interest by the volume of the whole hemisphere. Mean volumes and relative investment for each region per size class are provided in Online Resource 2. Images were coded prior to segmentation to blind the observer during image processing of the identity of each individual.

Statistical analysis of morphological and neuroanatomical traits

All analyses were conducted in R (version 3.1.3). Unsupervised cluster analysis was used to reveal natural groups among *P. rhea* workers based on their relative morphological or neuroanatomical measurements, irrespective of size class assignment. A hierarchical rather than partitional approach was used because such methods do not require specifying the number of clusters in advance and are typically deterministic. Similarly, we had

no a priori expectation on the shapes, sizes, or degree of separateness of clusters; therefore, the average linkage algorithm was used. Among agglomerative hierarchical methods, this algorithm has the advantage of being able to distinguish between weakly separated clusters without being overly sensitive to outliers. Additionally, since the two data sets examined here were of small to moderate dimensionality (6 and 15, respectively) and consisted of similarly scaled variables (relative measurements), the standard Euclidean distance was employed. The package *pvclust* was used to assign approximately unbiased (AU) *P* values with standard error (SE) that are less biased than bootstrap probability, with multiscale bootstrapping ($N = 10,000$ iterations) (Suzuki and Shimodaira 2006). The optimal number of clusters was verified by the consensus of 27 indices as implemented by package *NbClust* (Charrad et al. 2014). Following cluster analysis, a supervised random forest feature selection package, *Boruta* (Kursa and Rudnicki 2010), was used to determine the relevance of the different variables for cluster assignment. This was done in part by generation of shadow attributes (min, mean, and max in Figs. 1b and 3b) used as a reference for the relevance of cluster variables. Any variable

with an importance measure (*Z*-score) below that of the maximum value of the shadow attribute was deemed unimportant to classification (Kursa and Rudnicki 2010).

To further describe scaling relationships (slope, shift, elevation) of brain regions with the rest of the hemisphere (RH = OL + AL + MBC + MBP + CB + SEZ + ROCB – region of interest), standard major axis regression analysis (SMA) was used to compare cluster assignments using the package *smatr* (Warton et al. 2006). If groups based on cluster assignment had similar slopes (shared β) as indicated by a chi-squared test (χ^2), 95% confidence intervals (CI) were calculated. Furthermore, differences among shifts along the *x*-axis (axis shift), indicating differences in mean size, and elevation (grade shifts), which indicate differences in relative region size at a similar RH size, were examined using a Wald statistic (W^2).

Statistical analysis of worker behavioral repertoires

Estimates of behavioral repertoire sizes and degree of overlap were made using *iNEXT* and *SpadeR* in R (Chao et al. 2016; Hsieh et al. 2016), adapting species diversity and assemblage similarity for our behavioral data analysis. Behaviors were thus treated as “species,” observed acts per behavior as counts of “individuals,” and size classes as “sites” in generating sample-based abundance data. In addition to asymptotic richness estimators (Chao et al. 2014), the Simpson diversity index was used to assess behavioral diversity within each size class (Simpson 1949; Magurran 2004), and the Horn size-weighted measure was used to describe repertoire similarity across size classes by weighing behavioral observations by their abundances to mitigate the influence of frequently performed behaviors (Chao et al. 2014; Chiu et al. 2014). Additionally, we combined observed acts into the following socially and ergonomically significant behavioral categories: hygiene and prophylaxis (self-grooming and removal of dead); social contact and interaction (allogrooming, antennation, and trophallaxis); brood care (all brood-directed behavior, encompassing nursing and related alloparental acts); foraging, food transport, and processing (all food collection behavior); movement (walking, climbing, lifting, and carrying nestmates); defensive posturing (adopting a threat posture with flared mandibles and gaster positioned beneath the mesosoma); and inactivity (absence of recordable behavior). Chi-squared tests of equality were used to test the null hypothesis that the proportion of acts in each category was equal across worker size classes. These proportions are provided in Online Resource 3. If the null hypothesis was rejected, pairwise Pearson’s chi-square tests of proportions were calculated with adjusted *P* values (Holm 1979) to detect size-related differences.

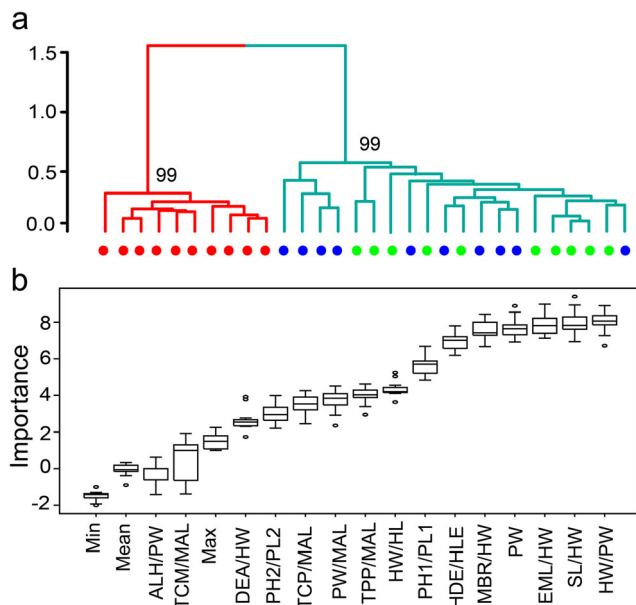


Fig. 1 **a** Dendrogram with AU *P* values (at nodes) indicating two significant clusters among *P. rhea* workers according to morphology. Worker size class is color coded (minor: red; soldier: green; supersoldier: blue). **b** Variable relevance illustrating importance of relative morphological characteristics beyond the shadow attribute threshold. Importance measures from the 18 Boruta iterations as boxplots (bar: median; hinges: quartiles; lower whisker: the larger of either the minimum importance measure or the interquartile range $\times 1.5$; upper whisker: the smaller of either the maximum importance measure or the interquartile range $\times 1.5$; outliers: importance scores that fell beyond whisker definition). Refer to the text for meaning of morphological abbreviations

Results

Worker morphology

A consensus of 14 of 27 indices determined that the optimal number of trait clusters (range 1–15) in worker size distribution data was two. Hierarchical clustering with multiscale bootstrap resampling indicated the two largest clusters were supported in more than 95% of replicates (cluster 1: AU P value = 0.99, SE = 0.001, cluster 2: AU P value = 0.99, SE = 0.001). The first cluster consisted of all minor workers and the second cluster contained soldiers and supersoldiers (Fig. 1a). Variable relevance rejected only TCM/MAL and ALH/PW as unimportant in defining the two cluster assignments (Fig. 1b).

Worker size and behavioral repertoire

Estimated repertoire richness and diversity was calculated for each worker size class based on the number of acts observed for each group ($N \approx 500$). Coverage was estimated above 99% for all three worker groups (Chao and Jost 2012; Fig. 2). Estimates of repertoire richness (\pm SE) varied across size classes (minors, 27.99 ± 5.28 ; soldiers, 18.10 ± 0.38 ; supersoldiers, 17.0 ± 3.74). We found low Simpson's diversity indices ($1/D \pm$ SE) for all workers (minors, 6.09 ± 0.50 ; soldiers, 6.51 ± 0.27 ; supersoldiers, 6.58 ± 0.22), which could be explained by a few overrepresented tasks, such as grooming, across all groups. Substantial overlap in behavioral repertoire via Horn size-weighted similarity indices (\pm SE) was found in pairwise comparisons across size classes (minor–soldier, 0.72 ± 0.03 ; minor–supersoldier, 0.70 ± 0.04 ; soldier–

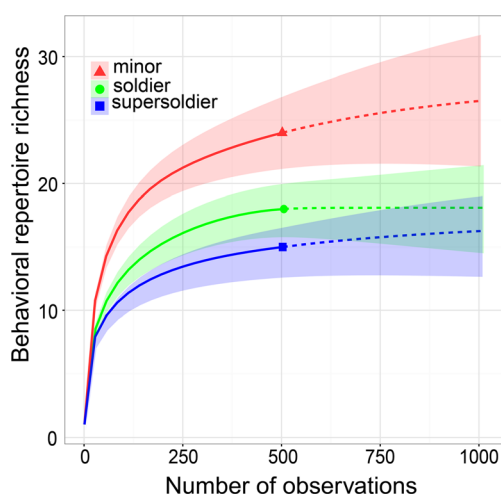


Fig. 2 Sample sized-based rarefaction and extrapolation sampling curves estimating behavioral repertoire richness of worker size classes (solid lines: interpolation, dashed lines: extrapolation, shaded area: 95% confidence intervals)

supersoldier, 0.93 ± 0.01). This overlap, especially between soldiers and supersoldiers, was also evident in analyses of proportions of acts in behavioral categories (Table 2). Soldiers and supersoldiers did not significantly differ from each other in hygiene and prophylaxis or social contact and interaction and engaged significantly more in these behaviors compared to minor workers. There were no significant differences across size class in foraging, food transport, and processing behaviors. Minor workers had a significantly greater proportion of activity in brood care and movement and adopted defensive posturing significantly more often than soldiers and supersoldiers. Soldiers were in a defensive posture significantly more often than supersoldiers and soldiers and supersoldiers had a significantly greater proportion of inactivity compared to minor workers.

Brain size and structure

A consensus of nine of 27 indices surveyed for optimal cluster number (range 1–15) indicated the presence of two groups according to brain phenotypes across *P. rhea* workers of all sizes. Supporting this suggestion of two groups, AU P values based on multiscale bootstrap resampling were significant beyond 95% for the two largest clusters (cluster 1: AU P value = 1.00, SE < 0.001, cluster 2: AU P value = 1.00, SE < 0.001). The first cluster was comprised of all supersoldiers and 17 soldiers, whereas the second cluster contained all minor workers and three soldiers (Fig. 3a). An examination of variable relevance indicated all six brain compartment proportions were important in cluster assignments (Fig. 3b).

SMA revealed all six brain subregions shared similar slopes ($P > 0.05$) across clustered groups and could therefore be examined for axis shifts to analyze differences in overall mean size of a region, or grade shifts, indicating different proportional investment in a region (Fig. 4). Due to pronounced differences in overall brain size across groups, each subregion indicated a significant axis shift (Table 3 and Fig. 5). Grade shifts were apparent in all regions but the OL (Fig. 5). All subregions with the exception of the positively allometric OL and CB displayed isometry with the RH (Table 3 and Fig. 5).

Discussion

Our analyses suggest differentiation in behavior and brain anatomy between minor workers and soldier groups but not between soldiers and supersoldiers in *P. rhea*. Although three worker groups can be distinguished by body size and head allometry (Huang and Wheeler 2011), the lack of further distinction between soldier and supersoldier groups in behavior and neuroanatomy, as well as body shape, does not provide

Table 2 Comparisons of proportions of observed acts in behavioral categories across worker size classes

	Three-sample test of equality of proportions		Pairwise comparisons, Holm corrected <i>P</i> values		
	χ^2 (<i>df</i> = 2)	<i>P</i> value	Minor–soldier	Minor–supersoldier	Soldier–supersoldier
Hygiene and prophylaxis	11.50	0.003	0.048	0.015	0.64
Social contact and interaction	22.92	< 0.001	0.014	0.002	0.48
Brood care	124.72	< 0.001	< 0.0001	< 0.0001	1
Foraging, food transport, and processing	1.90	0.39	N/A	N/A	N/A
Movement	54.92	< 0.001	< 0.0001	< 0.0001	0.22
Defensive posturing	52.99	< 0.001	< 0.0001	< 0.0001	0.043
Inactivity	108.59	< 0.001	< 0.0001	< 0.001	0.81

evidence of selection for discrete, size-related social roles and correlated divergence in neural phenotypes in this species. The extensive behavioral repertoire overlap between soldiers and supersoldiers suggests that workers in these size classes are not performing qualitatively different tasks but may instead vary in the frequency, efficacy, or efficiency with which acts are performed. If soldier and supersoldier workers perform qualitatively similar tasks, we expect their sensory, motor, and cognitive demands to be similar, resulting in conserved brain phenotypes. This was supported by the results of cluster analyses. However, supersoldiers may be quantitatively specialized on performing certain tasks in ways that improve fitness outcomes through repetition of behavior and/or biomechanical advantages associated with head capsule structure and cephalic musculature. For example, all workers in some capacity may defend the nest from intruders, but because of the size, structure, and power of their mandibles, supersoldiers and soldiers may be more effective at neutralizing threats than smaller workers. Similarly, supersoldiers may be more effective at processing large or exceptionally hard seeds. Such differences could correspond to variation in threshold responsiveness to task cues (Beshers and Fewell 2001) and other factors that might affect the induction of behavior. Our behavioral measurements do not provide enough detail to fully resolve these differences. We also acknowledge that rare but critical behaviors, such as enemy-specific defenses, were not observed in the laboratory and may be dependent on subcaste (Wilson 1976; Feener 1987; Huang 2010).

Differences in behavioral performance and brain structure separating the two soldier groups from minor workers appear to be substantial. Although there is considerable repertoire similarity between soldier size classes, minor workers perform on an absolute basis more types of behaviors and are proportionally more active in brood care, movement, and defensive posturing. It may be that such qualitative and quantitative differences in behavior require different neuroarchitectures. Minors have distinctive neuroanatomical phenotypes consistent with increased behavioral demands, characterized by disproportionate allocation of neuropil to olfactory and

integrative brain centers, as indicated by grade shifts. This supports previous research on three dimorphic *Pheidole* species that identified consistent intraspecific and interspecific differences in neural support for expansive behavioral repertoires. For example, mature minor workers, which have the largest task repertoires (Seid and Traniello 2006) across *Pheidole dentata*, *Pheidole morrissi*, and *Pheidole pilifera*, had larger mushroom bodies (Muscedere and Traniello 2012; Ilieş et al. 2015). Interestingly, minors had lower integration in neuroanatomy (Ilieş et al. 2015) and morphology (Pie and Traniello 2007), suggesting differences in modular

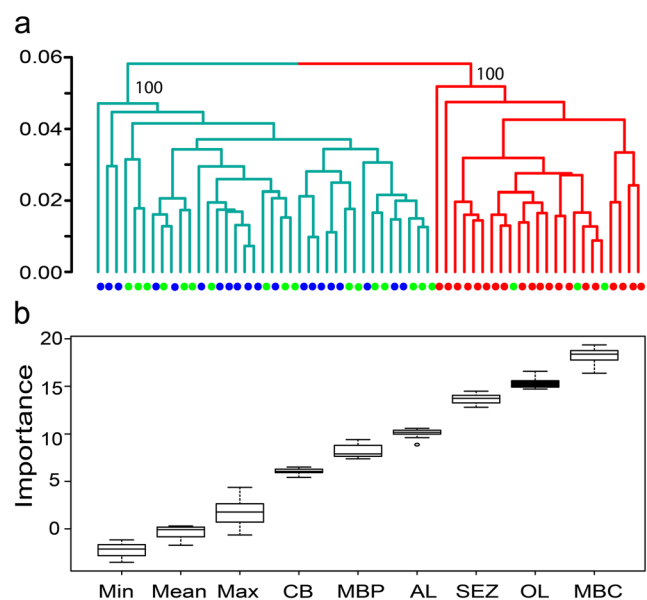
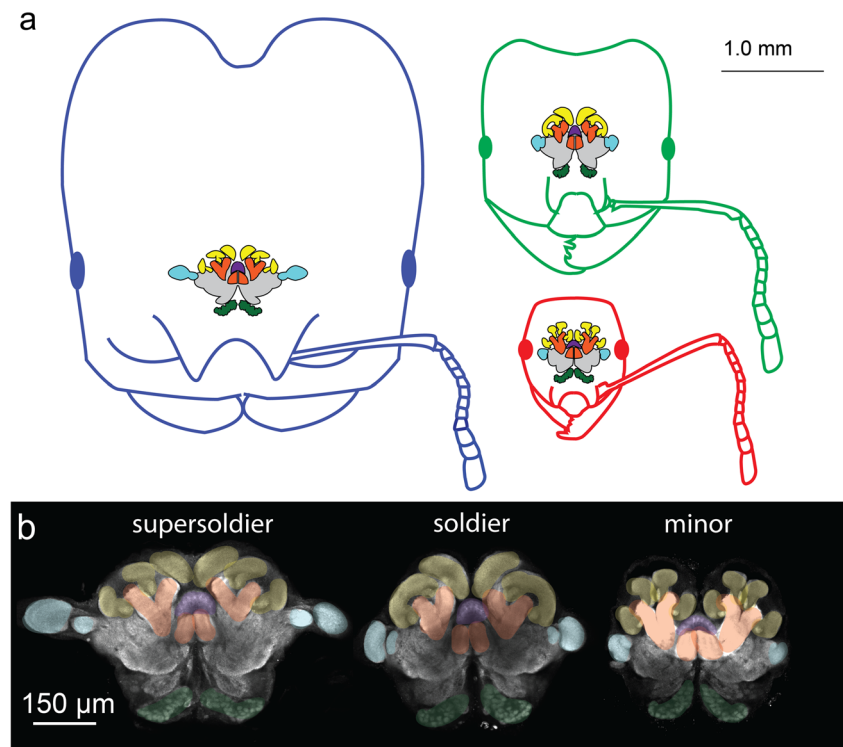


Fig. 3 **a** Dendrogram with overlaid AU *P* values (at nodes), indicating two significant clusters among *P. rhea* workers according to brain composition (minor: red, soldier: green, supersoldier: blue). **b** Variable relevance of brain compartments all beyond the shadow attribute threshold. Importance measures from the 10 Boruta iterations as boxplots (bar: median; hinges: quartiles; lower whisker: the larger of either the minimum importance measure or the interquartile range \times 1.5; upper whisker: the smaller of either the maximum importance measure or the interquartile range \times 1.5; outliers: importance scores that fell beyond whisker definition). Refer to the text for the meaning of neuroanatomical abbreviations

Fig. 4 **a** Diagram illustrating brain scaling relationships across three worker size classes (minor: red, soldier: green, supersoldier: blue) to scale. **b** False-colored representative micrographs from each size class (OL: blue, AL: green, MBC: yellow, MBP: orange, CB: purple, ROCB: gray); the SEZ is not shown



coordination of traits across phenotypic levels of organization during *Pheidole* subcaste evolution and development. Although interspecific comparisons led us to hypothesize that morphological, behavioral, and neuroanatomical evolution would be linked in *P. rhea*, our present results suggest that in *P. rhea* body size and head allometry may not be well-coupled to shape, brain structure, and behavior. We acknowledge the limitations of correlative studies of brain volume and behavior (Healy and Rowe 2007) and note that there may be other differences in neuroarchitecture across worker size classes not detected in analyses of macroscopic neuroanatomy.

However, cellular studies (DGG and JFAT unpublished data) do not suggest functional differences in synaptic organization in the mushroom bodies, brain regions that serve important functions in higher-order processing and the genesis of behavior, across worker size class in *P. rhea*.

Our understanding of the conserved genetic toolkits and developmental plasticity that generate soldiers and supersoldiers has recently been advanced (Rajakumar et al. 2012; Lillico-Ouachour and Abouheif 2016). Extensive experimental work has illustrated how alterations of *Pheidole* worker phenotype and caste evolution in the genus may occur

Table 3 SMA analyses on log-transformed volumes of regions of interest against log-transformed RH comparing individuals grouped by cluster analysis

	OL	AL	MBC	MBP	CB	SEZ
Shared β	1.68	1.13	1.10	1.13	1.33	1.13
95% CI	1.40, 2.04	0.94, 1.35	0.95, 1.28	0.96, 1.33	1.09, 1.63	0.97, 1.31
$\beta \neq 1$?	Yes	No	No	No	Yes	No
χ^2	28.95	1.75	2.22	2.57	7.83	3.02
<i>P</i> value	< 0.0001	0.42	0.33	0.28	0.02	0.22
Grade shift	No	Minor > soldiers	Minors > soldiers	Minors > soldiers	Minors > soldiers	Soldiers > minors
W^2	1.67	20.55	31.39	16.13	16.70	9.23
<i>P</i> value	0.20	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.002
Axis shift	Soldiers > minors	Soldiers > minors	Soldiers > minors	Soldiers > minors	Soldiers > minors	Soldiers > minors
W^2	103.10	51.84	59.20	58.07	48.73	105.40
<i>P</i> value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

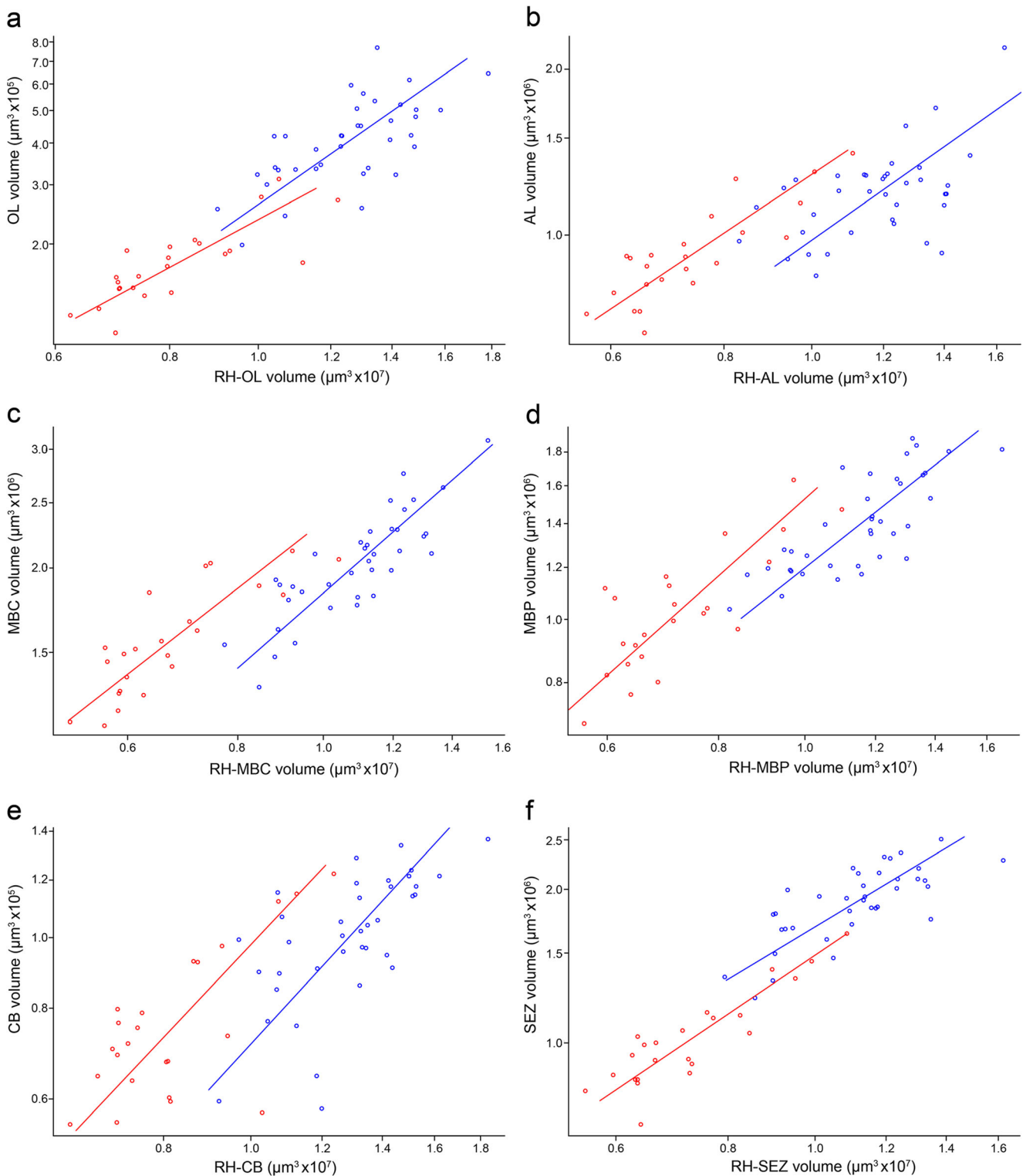


Fig. 5 Scaling relationships between the volumes of **a** OL, **b** AL, **c** MBC, **d** MBP, **e** CB, and **f** SEZ and the rest of the hemisphere volume across the two significant clusters of workers (minors: red; “soldiers:” blue) according to brain composition. Raw volumes plotted on logged axes

by changes in developmental thresholds (Wheeler and Nijhout 1981, 1983, 1984; Rajakumar et al. 2012). *P. rhea* is polyandrous (Huang et al. 2013); patriline-related genetic bias may

therefore contribute to worker phenotype through differentiation mechanisms involving JH cascades and worker provisioning of larvae. How genomic information is translated

through development to brain structure and behavior requires additional research.

The early branching position of *P. rhea* in the well-resolved molecular phylogeny of *Pheidole* (Moreau 2008) coupled with the ability to induce supersoldier production in other *Pheidole* species (Rajakumar et al. 2012) indicates that soldier polymorphism and its conserved genetic toolkit evolved very early in the genus. Soldier subcaste polymorphism appears to have occurred in association with granivory: all soldier-polymorphic species collect and/or cache seeds and are geographically limited to the arid southwestern regions of North America (Moreau 2008). It has been suggested that caste proliferation evolved to efficiently utilize larger seed resources; diet shifts may explain polymorphism in other ant genera, including granivorous species (Ferster et al. 2006). We found that soldiers and supersoldiers had disproportionately large subesophageal zone compared to minor workers, possibly reflecting the importance of mandibular control in food processing. Field studies of *P. rhea* support the idea that supersoldiers are recruited to unusually large seeds and other food resources that smaller workers do not appear to be able to efficiently process (DGG, JFAT personal observations). Although we did not find significant differences across worker size classes in foraging, food transport, and food processing in our laboratory study, additional field research is needed to determine the association of morphology and behavior in *P. rhea*.

Pheidole worker morphology is driven primarily by changes in size (Pie and Traniello 2007). Granivory in dimorphic *Pheidole* is associated with differences between soldier and minor worker head size but not soldier head size alone (Holley et al. 2016). In other myrmicine ant genera, shape may also be important in caste evolution associated with diets containing seeds (Ferster et al. 2006). If body size variation allows *P. rhea* to exploit new food resources through biomechanical advantages of size-matching and/or processing capability, then selection for frequency- and/or efficiency-related performance, rather than behavioral discretization, may explain the lack of neuroanatomical divergence between soldiers and supersoldiers. *P. rhea* workers of all sizes engage in food transport and processing; therefore, if supersoldiers specialize on processing larger food items, their sensory processing and integration demands should not be qualitatively different than those of the smaller class of soldiers, with which they show extensive repertoire overlap. It is likely that biophysical requirements of load carriage and/or seed milling or food-source processing are more important in providing support for a quantitative shift in behavior and that sensory and higher-order processing modifications are unnecessary. Moreover, sensory cues provided by seeds that initiate selection, transport, and/or processing are likely shared across seed sizes.

In *P. rhea*, we identified substantial overlap in repertoire size across worker body size variation in spite of head size allometry and identified differences in the proportions of

ergonomically relevant behaviors performed by minor workers and soldier size classes with the exception of food processing, which did not differ across size classes. These differences, when mapped onto divergent neuroanatomical and morphological phenotypes, suggest that the ancestral soldier-polymorphic social organization of *Pheidole* may be characterized by a generally plastic repertoire across worker size variation and more subtle specialization in quantitative aspects of task performance. *P. rhea* polymorphic soldiers are comparable in repertoire size to the most behaviorally expansive soldiers in dimorphic *Pheidole* species, with the exception of *P. morrisi* soldiers whose repertoire most closely resembles that of minor workers of this species (Wilson 1984; Patel 1990). The comparatively wide repertoire of *P. rhea* soldiers may be underpinned by a generalist soldier brain phenotype. Further study of other soldier-polymorphic *Pheidole*, such as *P. obtusospinosa*, in which supersoldiers are specialized to defend against army ants (Huang 2010), would be valuable in determining the extent of behavioral and neuroanatomical plasticity in a later-branching species with more discretized social roles. Patterns of brain region scaling in *P. rhea* are mostly isometric, unlike other *Pheidole* that have positive allometries in regions suggested by covariance analyses to be components of the “social brain” (the AL, MBC, MBP; Ilieș et al. 2015). Ancestral social organization, repertoire overlap with qualitative and quantitative shifts in behavior, and isometric brain scaling support a role for conserved developmental programming in *P. rhea* caste evolution.

Pheidole is characterized by variation in worker and colony phenotypes (Wilson 2003; Mertl and Traniello 2009; Mertl et al. 2010; Lilloco-Ouachour and Abouheif 2016). The plastic ancestral phenotype represented by *P. rhea* may have served as the basis for ecological adaptations that generated hyperdiversity through the estimated 58.4–61.2 million year history of the genus (Moreau 2008). If ancestral states in ants were characterized by greater behavioral plasticity (Powell 2008), then increased subcaste specialization may have been due to disruptive (Planqué et al. 2016) and/or intense directional selection during ecological diversification and subsequent specialization of worker phenotypes. Directional selection followed by stabilizing selection in association with behavioral and/or ecological specialization could refine the wide size range of ancestral soldier groups into the narrower complete dimorphism present in most species in the genus. The great variation in head width ratios between minor and soldiers in dimorphic *Pheidole* suggests that either large (i.e., supersoldiers) or small size classes of soldiers could have been subject to this refinement and the other size class could have been selectively eliminated. This selective reduction in soldier body size variation would not necessarily correspond to a reduction in behavioral competency and its supportive neuroarchitecture in derived *Pheidole* soldiers because these traits are integrated independent of soldier size variation.

Phenotypic evolution thus appears to have been facilitated by the integration of plastic traits ancestral in the genus.

Acknowledgments We thank S. Cover for access to museum specimens and valuable insights from his extensive field experience. We are grateful to Dr. M. Huang for access to colonies used in the neuroanatomical and behavioral studies and to Dr. W. Gronenberg for sharing his expertise in histology and comments on the manuscript. We thank Drs. M.L. Muscedere, S. Arganda, J.F. Kamhi, and Y.M. Giraldo, as well as A.P. Hoadley for their comments and technical support, and Dr. E. Abouheif and one anonymous reviewer for their constructive insights on the manuscript. This research was supported by National Science Foundation grant IOS 1354291 to JFAT and a Grant-in-Aid-of-Research from the Society of Integrative and Comparative Biology to DGG.

Data availability The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standard

Conflict of interest The authors declare that they have no conflict of interest.

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