Trait evolution is reversible, repeatable, and decoupled in the soldier caste of turtle ants

Scott Powell[a,1], Shauna L. Price[a,b], and Daniel J. C. Kronauer[c]

[a]Department of Biological Sciences, George Washington University, Washington, DC 20052; [b]Department of Science and Education, Field Museum of Natural History, Chicago, IL 60605; and [c]Laboratory of Social Evolution and Behavior, The Rockefeller University, New York, NY 10065

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The scope of adaptive phenotypic change within a lineage is shaped by how functional traits evolve. Castes are defining functional traits of adaptive phenotypic change in complex insect societies, and caste evolution is expected to be phylogenetically conserved and developmentally constrained at broad phylogenetic scales. Yet how castes evolve at the species level has remained largely unaddressed. Turtle ant soldiers (genus *Cephalotes*), an iconic example of caste specialization, defend nest entrances by using their elaborately armored heads as living barricades. Across species, soldier morphotype determines entrance specialization and defensive strategy, while head size sets the specific size of defended entrances. Our species-level comparative analyses of morphotype and head size evolution reveal that these key ecomorphological traits are extensively reversible, repeatable, and decoupled within soldiers and between soldier and queen castes. Repeated evolutionary gains and losses of the four morphotypes were reconstructed consistently across multiple analyses. In addition, morphotype did not predict mean head size across the three most common morphotypes, and head size distributions overlapped broadly across all morphotypes. Concordantly, multiple model-fitting approaches suggested that soldier head size evolution is best explained by a process of divergent pulses of change. Finally, while soldier and queen head size were broadly coupled across species, the level of head size disparity between castes was decoupled from both queen head size and soldier morphotype. These findings demonstrate that caste evolution can be highly dynamic at the species level, reshaping our understanding of adaptive morphological change in complex social lineages.

adaptive radiation | niche filling | polyphenism | polymorphism | Cephalotes

The extent of adaptive phenotypic change within a lineage is shaped by how key functional traits evolve. In particular, the degree to which traits evolve reversibly, repeatably, and decoupled from each other is seen as critical for the process of adaptive niche-filling and biodiversity production more generally (1–6). Yet the dynamics of trait evolution remain poorly understood in many diverse and ecologically important taxa, especially at the species level, where the process of adaptive trait evolution is most evident (7). This knowledge gap is particularly glaring in the social insects. The rise of these taxa to global prominence in diversity, abundance, and ecological footprint (8, 9) has been underpinned by a major evolutionary transition to societies that function as integrated adaptive units (10–12). The resulting colony phenotype of these organismal societies (10), often called superorganisms (8), is distinct in many respects from the phenotype of unitary organisms (i.e., individual multicellular organisms). Many aspects of the colony phenotype have been studied intensively (8, 11–14), but how it evolves across species as a lineage fills ecological space has remained largely unaddressed. This knowledge is then necessary to explain the adaptive phenotypic evolution of one of the most prominent forms of animal biodiversity.

The defining and special characteristic of adaptive phenotypic evolution in the most derived insect societies (i.e., eusocial species) is that functional traits can be partitioned among different members of the colony. The partitioning of morphological traits among colony members is the most conspicuous example of this phenomenon, and it is typically generated by differential regulation of the genome during development (15, 16). The resulting polyphenic colony phenotype can be anything from a simple variation in adult size to numerous distinct morphological forms, or "castes" (12). Trait specialization within castes and disparity among castes can become extreme, because each caste is freed from the tradeoffs that would be associated with performing the functions fulfilled by the other castes (14, 17–19). For example, soldier and queen castes can have extreme trait specialization for defense and reproduction, respectively, because neither has to perform the alternative function in the presence of the other caste. Unsurprisingly, the function of castes and their role as adaptive traits of the colony have been studied intensively for decades (12–14, 17, 20).

Despite the long history of work on caste function, knowledge of how castes evolve adaptively across taxa is remarkably incomplete. Nevertheless, two general expectations have become prominent in the literature. First, directional and conserved evolution of more phenotypically distinct and functionally specialized castes is expected over time because of the gains in organizational efficiency castes can provide (12, 13, 20). Second, the phenotype of a particular caste is expected to be coupled developmentally to trait expression in other castes within the polyphenic series of the colony (13, 16, 21–23). At broad macroevolutionary scales, we can be sure that morphologically distinct castes are a derived social state, have evolved multiple times, and are largely conserved once

Significance

Specialized castes are seen as phenotypic innovations necessary for ecological and evolutionary success in social insects. Nevertheless, how castes evolve adaptively as a lineage fills ecological space has remained unaddressed. Recent work with turtle ants has established that head shape and size in the iconic soldier caste, specialized for nest entrance defense, determine two key aspects of nesting ecology. Here species-level comparative analyses reveal that the evolution of head shape and size is extensively reversible, repeatable, and decoupled within the soldier caste and relative to the queen caste, underpinning the lineage's diverse nesting ecology. These findings reshape our understanding of caste evolution, rejecting a stable, directional process in favor of a dynamic process of adaptive fitting between phenotype and environment.

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[1]To whom correspondence may be addressed. Email: scottpowell@gwu.edu.

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present (12, 20, 24, 25). Similarly, the phenotype of all castes within a polyphenic series must be coupled to some degree by developing from a single genome and having limited pupal resources to repurpose during metamorphosis, although the mechanisms and constraints governing caste development continue to be debated (15, 16, 26). Yet these insights, intimately connected with the general issues of reversibility, repeatability, and decoupling in trait evolution, may represent only the broad bounds of phenotypic change in social lineages. Notably, the species-level dynamics of adaptive caste evolution have remained largely intractable and therefore unaddressed.

The turtle ants (genus *Cephalotes*) have long been known for their iconic caste system (27) and have recently emerged as an ideal group for studying caste evolution. The caste system is exceptional because in addition to the worker and queen castes common among ants, most species have a soldier caste with elaborate head armor. Moreover, soldier phenotype varies substantially across the 119 extant species (28, 29). Most notably, soldier heads span four distinct morphotypes (*SI Appendix*, Fig. S1) and a four-fold difference in width across species. Turtle ant colonies establish arboreal nests in the abandoned tunnels of wood-boring beetles (18, 28, 30), and soldiers use their heads as living entrance barricades (i.e., phragmotic defense; refs. 31 and 32).

The specialized defensive function of soldiers has been known for more than a century (31), but we now understand both the adaptive importance of soldier defense and the ecological relevance of soldier morphological diversity within the group. First, the fit between soldier heads and beetle-produced entrance holes impacts soldier defensive performance against would-be nest usurpers, with consequences for colony growth into additional cavities and reproductive output (33, 34). Second, members of the four distinct soldier morphotypes differ in nest entrance specialization and defensive strategy, and head width sets the exact size of entrances they defend (18, 30) (Fig. 1, *SI Appendix*, Fig. S2). More specifically, species with square-headed and dome-headed morphotypes have broad, generalized entrance distributions and typically use entrances much larger than the head of one soldier (*SI Appendix*, Fig. S2), while differing in how they cooperatively block these oversized holes (Fig. 1). In contrast, the disc-headed and dish-headed morphotypes both specialize on entrances that fit a single soldier head (*SI Appendix*, Fig. S2), but differ in how the heads mechanically lock into place (Fig. 1). Within this context of entrance specialization and defensive strategy, determined by morphotype, head size then quantitatively determines the specific hole sizes a species selects from those available in the environment. Thus, for a soldier caste of a given morphotype and head size, we can robustly predict the level of entrance specialization, the defensive strategy employed, and the specific entrance sizes utilized. Understanding the ecological function of key traits in this way, spanning the full range of trait values within a lineage, provides the rare opportunity to infer the adaptive significance of trait evolution in comparative analyses (35).

Here we address the species-level dynamics of adaptive caste evolution in the turtle ants, focusing on the degree of trait reversibility, repeatability, and decoupling in the soldier caste. We do

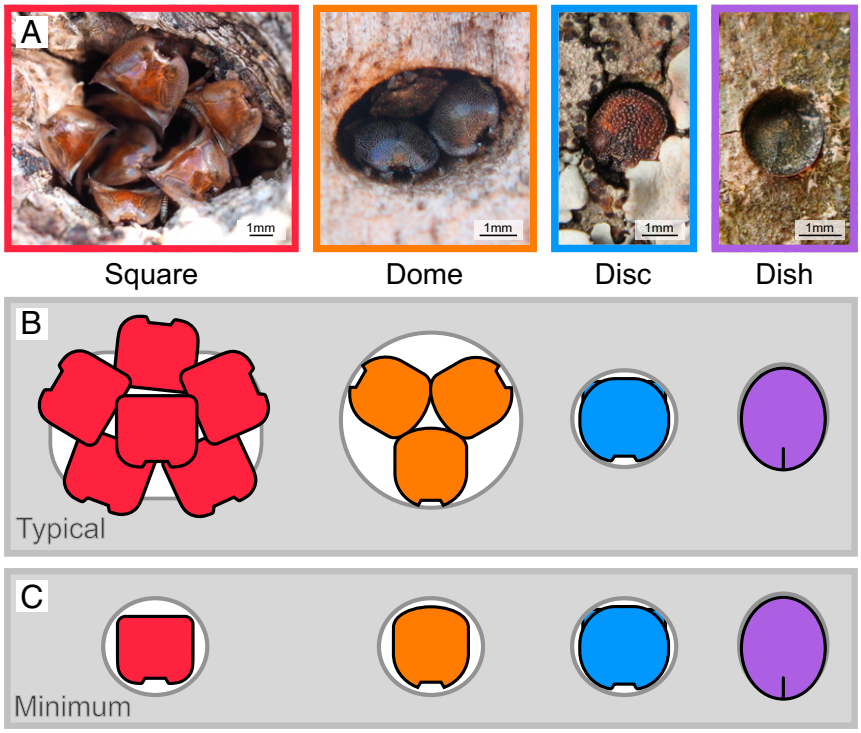

Fig. 1. Usage and defense of preexisting cavity entrances by the four soldier morphotypes in turtle ants. (*A*) Photographs of typical entrance sizes and defensive strategies across representative species of the four soldier morphotypes (photos by S.P.). (*B*) Graphic illustrating typical entrance size and defensive strategy across morphotypes. Square-headed soldiers typically group-block large, often irregularly shaped entrances by haphazardly overlapping their heads like scales; dome-headed soldiers typically group-block moderately large entrances by pushing the domed, posterior region of their heads together and facing the mandibles forward; disc-headed soldiers typically solo-block entrances that only fit a single soldier and mechanically lock the anterior rim of their head disc into the inside surface of the nest entrance; dish-headed soldiers typically solo-block entrances that only fit a single soldier by overlapping the anterior margin of the dish with the entrance hole and pulling back, creating a cap-like seal. For the two group-blocking morphotypes, workers may also participate by wedging their heads into small gaps left around the soldier heads (e.g., smallest individuals in square-headed morphotype photo in *A*) (30). (*C*) Graphic illustrating the minimum entrance size and defensive strategy across morphotypes. The minimum entrance size is limited by the size of the soldier's head in all cases, but only the disc and dish morphotypes typically achieve mechanical locking interactions with the entrance perimeter.

this by combining recent insights into the ecological significance of soldier morphotype and head size with expanded and revised morphological datasets and a recent species-level turtle ant phylogeny (36). Our analyses focus on the following specific questions for our two focal ecomorphological traits of morphotype and head size: (1) to what extent is trait evolution reversible and repeatable within the soldier caste?; (2) to what degree are the two traits evolutionarily decoupled from each other within the soldier caste?; and (3) to what extent is trait evolution in the soldier caste decoupled from the potential upper limits of trait expression set by the queen caste? These questions differentiate trait decoupling within and among castes as two distinct axes of adaptive caste evolution. The extent of within-caste trait decoupling determines how well a lineage can fill the ecomorphological space that is specific to the specialized function of the focal caste. Among-caste trait decoupling addresses the related but separate issue of the degree of functional novelty that can be achieved between the focal caste and the other castes in the polyphenic series. By addressing these questions with turtle ants, we are using an iconic ant lineage to conduct a species-level empirical examination of the dynamics of adaptive caste evolution.

Results & Discussion

Reversibility and Repeatability in Soldier Morphotype Evolution. Not all extant representatives of each morphotype clustered together when mapped to the tips of the phylogeny (Fig. 2), consistent with multiple gains and losses of morphotype within the lineage. Concordantly, a pattern of reversibility and repeatability in soldier morphotype evolution was identified consistently across a number of approaches used to estimate ancestral morphotype states (*Methods*). The best-fitting model under maximum likelihood estimation (equal rates model, Akaike weight 0.95; *SI Appendix*, Table S1) indicated that the ancestral state for modern turtle ants was a square-headed soldier caste and that a total of 11 changes in morphotype state have occurred across the phylogeny (Fig. 2 and *SI Appendix*, Table S1). Eight state changes were associated with transitions to the dome, disc, or dish morphotypes, with each evolving at least twice, while the remaining three changes were soldier losses from ancestors with a square-headed soldier. Notably, the disc morphotype, previously considered the most specialized and derived state (28, 37), appears to have been lost up to five times during state transitions to both the more functionally specialized solo-blocking dish morphotype and the less functionally specialized group-blocking dome morphotype. The same qualitative pattern of reversibility and repeatability in morphotype evolution was estimated for the other two models examined, even though they were substantially poorer fits and the worst-fitting model estimated a different root state (*SI Appendix*, Table S1). Moreover, this pattern of state transitions in all models was highly consistent across a sampling of phylogenetic trees that represented the uncertainty in our phylogeny (*SI Appendix*, Table S2).

The same qualitative results for morphotype evolution were again recovered across models when using stochastic character mapping to estimate state transitions across the phylogeny (*SI Appendix*, Table S3). More specifically, the average number of state changes across models was three to four times the minimum number possible for our five morphotypes, with multiple gains and losses across states. In addition, the simplified binary-state hypotheses of multiple state transitions in group-blocking versus solo-blocking morphotypes and soldier presence versus absence were also supported, with the number of state transitions four times and three times the possible minimums, respectively (*SI Appendix*, Table S4). Considered together, these results underscore the robustness of the biological interpretation of reversibility and repeatability in soldier morphotype evolution.

The consistent support for reversibility and repeatability in soldier morphotype evolution indicates numerous shifts in functional and ecological specialization, with important implications for niche-filling within the lineage. The classic "ergonomic theory" of caste proposes that the evolution of more phenotypically distinct and functionally specialized castes promotes greater collective efficiency and niche invasion (20). This theory is not macroevolutionary in nature and does not preclude reversible or repeated caste evolution, yet the general expectation for directional and conserved evolution of greater caste specialization has become prominent in the literature (8, 12, 13, 20). Indeed, turtle ants were previously considered an example of directional progression in caste specialization, from no soldier as the ancestral state through increasingly more specialized states, using a phylogenetic hypothesis based on morphology (28, 37). Instead, we have seen that major transitions in soldier specialization, represented by morphotype evolution and soldier loss, are more dynamic.

The idea that specialization is a largely irreversible evolutionary trajectory also has been a long-held and oft-cited expectation for a variety of unitary taxa (38, 39). However, phylogenetic comparative analyses have shown that the direction and degree of specialization is often reversible and repeatable within such lineages (40–46), consistent with species traits adaptively responding to fit a diversity of more or less specialized ecological niches. Our analyses show that the same can be true for caste evolution in social insects; the presence and morphological specialization of a soldier caste, along with the associated defensive strategies, are also

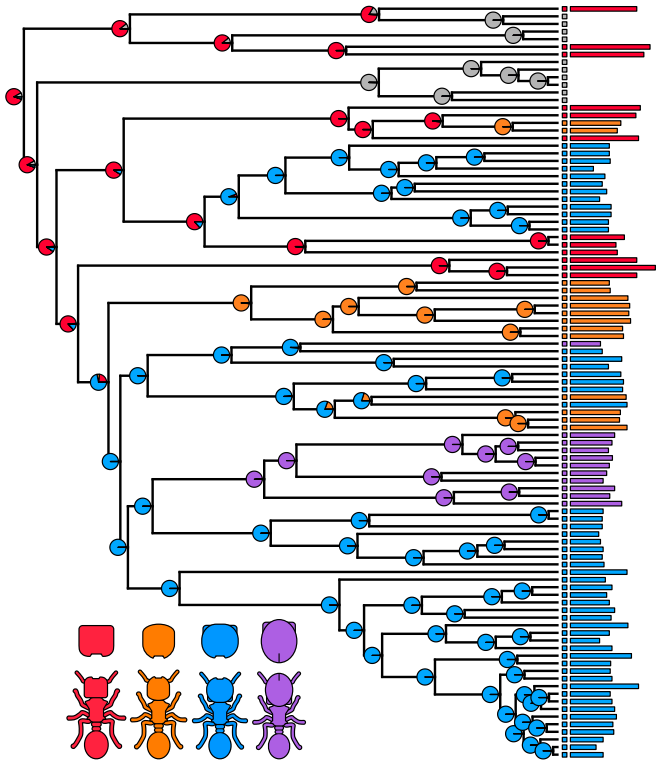

Fig. 2. The evolution of soldier morphotypes in the turtle ants, incorporating head width data for extant species. Soldier morphotype is color-coded as illustrated in the *Inset* (bottom left; red for square-headed morphotype, orange for dome, blue for disc, and purple for dish). Gray denotes the absence of a soldier caste. Pie charts show the maximum likelihood estimates for ancestral morphotype states at the nodes, under the best-fitting equal rates model. Squares at the tips show the morphotypes (colored) or the absence (gray) of a soldier caste in extant species. The variable length bars at the tips are color-coded by morphotype and scaled by head width for those extant species with a soldier. The phylogeny is the maximum clade credibility tree from Price et al. (36) that has been trimmed to include only species for which soldier data are available. Species names and previously defined species groups are given in *SI Appendix*, Fig. S3.

reversible and repeatable traits, likely tracking major shifts in the distribution of entrances that species use. This insight provides the general ecological prediction that any species demonstrating a reversion in morphotype, or soldier loss, should show a concordant shift to more generalized entrance usage and defensive strategy compared with close relatives.

Soldier Head Size Evolution and within-Soldier Decoupling of Ecomorphological Traits. Soldier head width was variable among closely related turtle ant species when mapped to the tips of the phylogeny, without apparent size grouping by morphotype or clade (Fig. 2). Concordantly, the variance in head width was not significantly different among morphotypes (Bartlett test of homogeneity of variances; Bartlett's k-squared = 6.62, df = 3, $P = 0.09$) (Fig. 3). This indicates that the considerable head width variation across the lineage is partitioned within rather than among morphotypes, with no particular morphotype more variable in head width than the others. Accounting for the phylogenetic relationships among taxa, mean head width differed significantly across morphotypes (phylogenetic generalized least squares [PGLS]; estimated $\lambda = 0.56$, morphotype $F_{3,85} = 13.0$, $P < 0.0001$), but pairwise comparisons revealed that this result was driven by a larger mean head width in species with the square-headed morphotype. Mean head width did not differ significantly among the dome, disc, and dish morphotypes (Fig. 3). This result establishes that while the ancestral square-headed morphotype is associated with larger head size, on average, head width evolution is not coupled with morphotype evolution for species with a dome-, disc-, or dish-headed soldier, which constitute the majority of the diversity in the lineage (77 of 89 taxa in the present analysis).

Likelihood models of continuous trait evolution revealed that the broad and variable pattern of head width evolution across species was best explained by the kappa model (Akaike weight 0.996; parameter estimates within 95% CI; *SI Appendix*, Table S5), where the degree of trait divergence is associated with the number of speciation events (47). Notably, the Ornstein–Uhlenbeck (OU) model representing a directional pull in head width evolution toward two trait optima was a poor fit (Akaike weight 0.000003; optima1, 2.41; optima2, 1.58; *SI Appendix*, Table S5). This result rejects the hypothesis that the significant difference in mean head width between square-headed soldiers and the other three morphotypes (Fig. 3) is indicative of two adaptive peaks. The OU model representing a directional pull toward a single adaptive peak was a similarly poor fit (Akaike weight 0.000002; optimum, 2.27; *SI Appendix*, Table S5). Monte Carlo-based pairwise comparisons among models (48) further revealed that the kappa model was a significantly better fit to the data than each of the other models, and that there was considerable power to differentiate between alternative models in all cases (*SI Appendix*, Table S6 and Fig. S4), including the Brownian motion (BM) model as a reference for random incremental change. In addition, the OU models were again identified as a poor fit to the data. The single optimum OU model was rejected in favor of the kappa model in a test with 98.2% power, while the one-optimum and two-optima OU models were both a poor fit to the data, and there was low power to distinguish between them (*SI Appendix*, Table S6). The far better fit of the kappa model across these analyses therefore suggests that the broad and overlapping ranges of head width across all morphotypes is the product of divergent change within the turtle ant lineage.

In further evaluating whether a process of divergent change robustly explains head width evolution, we found that a Lévy process model characterized by periods of stasis and bursts of rapid change (normally distributed jumps process [JN] model) fit the data almost as well as the kappa model (kappa, Akaike weight 0.49188; JN, Akaike weight 0.49097; *SI Appendix*, Table S7). Notably, the JN model is used to represent sudden shifts in trait values between adaptive zones and therefore is broadly concordant with the process of divergent change represented in the kappa model. Thus, the best-fitting models from two different classes of models support the same biological process of divergent pulses in soldier head width evolution within the turtle ants. In contrast, the other pure Lévy process model (normal inverse Gaussian jump process [NIG]), used to represent mostly small but constant phenotypic change within an adaptive zone, was a poor fit to the data and worse than BM (*SI Appendix*, Table S7). Other models that combine Lévy process jumps with BM were also poor fits (*SI Appendix*, Table S7).

The divergent change in soldier head size evolution complements the demonstrated reversibility and repeatability in morphotype evolution. It also suggests that divergent evolution of caste traits may be integral to the adaptive filling of niche space across species. Indeed, a pattern of divergent jumps in soldier morphology, potentially coupled with adaptive speciational events, is consistent with recent theory on caste evolution. From an adaptive ecomorphological perspective, divergent jumps in soldier head size predict step changes in cavity entrance size usage among close relatives. Concordantly, recent theory (49) suggests that disruptive selection in sympatry, driven by the intense competition over nesting cavities (33, 50, 51), can produce new species with divergent head size and a specialized soldier caste. This evolutionary process, explored theoretically, thus produces the same qualitative pattern of divergent jumps in head size that we have recovered in the present empirical comparative analyses.

Considered together, the largely decoupled evolution of soldier morphotype and head size, and the broadly overlapping head size distributions, suggest a complex interplay between traits and resource usage within the turtle ants. Nevertheless, existing knowledge of trait function in soldiers provides an unusual opportunity to infer testable mechanisms of resource partitioning among

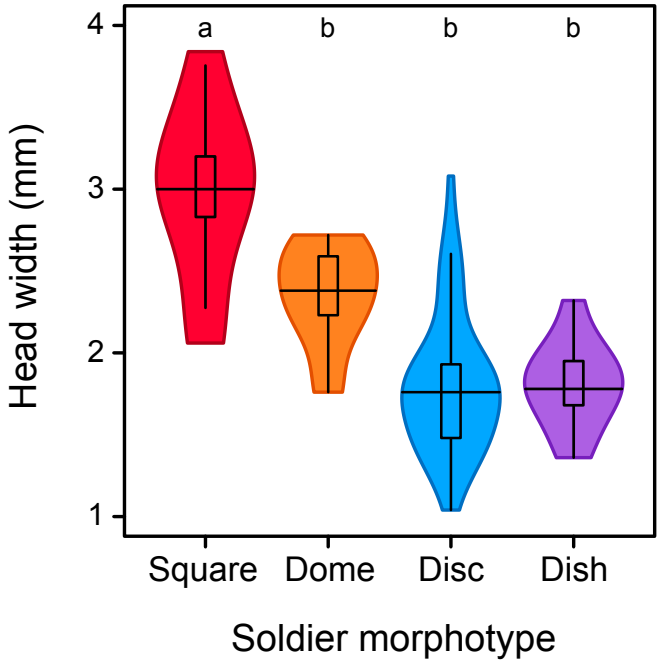

Fig. 3. Violin plots showing soldier head width distributions for the species representing each of the four soldier morphotypes in the turtle ants. Lowercase letters denote significantly different means between morphotypes after accounting for the phylogenetic relationships among species (PGLS; $P \leq 0.05$). Each plot incorporates a kernel density plot of the data and a boxplot with a box encompassing the interquartile range (IQR), a line drawn at the median, and the whiskers extending to the upper and lower quartiles (±1.5 times the IQR). Plots were created with the R package vioplot (67).

species. First, classic tradeoffs between ecological specialization and generalization may explain resource partitioning between species with solo-blocking vs. group-blocking morphotypes but similar head size. The evolution of specialization, and its role in resource partitioning and diversification, remains an important focus of biodiversity research (46, 52–54). Specialization can allow more effective exploitation of a shared resource but at the cost of the capacity to switch to alternative resources (39). While a specialist relies exclusively on more effective usage of a shared resource, the generalist uses it opportunistically and can still switch effectively to other resources (39), thereby reducing resource overlap. In the turtle ants, apparent overlap in minimum entrance usage has evolved between specialist solo-blocking and generalist group-blocking species. Yet this is likely mediated by functionally superior usage of specific entrance sizes by the specialists and a broader distribution of effective entrance usage by the generalists. This hypothesized tradeoff provides the general functional prediction that specialists should block an entrance that fits a single soldier with greater mechanical performance (i.e., resisting pushing and pulling forces) than a generalist, while the generalist should perform better in group-blocking scenarios. Morphotype evolution may also represent innovations that allow access to entrances of a specific size in unfilled ecological contexts. How key innovations facilitate the use of novel niche space remains an active area of biodiversity research (55, 56). In this instance, mechanical superiority of the solo-blocking morphotypes when blocking well-fitting entrances may allow them to survive in more enemy-hostile ecological contexts that would otherwise be inaccessible. This hypothesis also applies to similarly sized representatives of the two solo-blocking morphotypes, because the cap-like seal of the dish morphotype may outperform the disc in the most extreme enemy contexts (Fig. 1).

Among-Caste Decoupling of Ecomorphological Traits. Queen head width was a significant positive predictor of soldier head width across species after accounting for the phylogenetic relationships among taxa (PGLS; estimated $\lambda = 0.55$, $F_{1,72} = 440.1$, $P < 0.0001$) (Fig. 4*A*, solid line). This pattern is consistent with the general expectation that trait expression among castes from the same polyphenic series must be coupled to some degree, because they develop from a single genome and have limited pupal resources to repurpose during metamorphosis (15, 16, 26). Nevertheless, there was considerable variation among species in whether soldier heads are smaller, equal to, or larger than the head of the queen (Fig. 4*A*, position below, on, or above the equivalence line, respectively) as well as the degree of head size disparity among castes (Fig. 4*A*, deviation into white or gray regions). Combined, these patterns indicate meaningful decoupling of soldier and queen head size evolution across the different members of the lineage.

The calculated disparity in head width between soldier and queen castes (soldier head width minus queen head width) provides a metric for directly assessing the extent to which soldier head size is decoupled from the queen and morphotype. Mean head width disparity among castes was not predicted by either queen head width (PGLS; estimated $\lambda = 0.50$, queen head width $F_{1,69} = 0.3$, $P = 0.6$) or morphotype (PGLS; estimated $\lambda = 0.50$, morphotype $F_{3,69} = 0.7$, $P = 0.6$). This pattern further supports a meaningful decoupling of soldier head width evolution from that of the queen. Mean head width disparity was significantly greater than 0 (PGLS; estimated $\lambda = 0.50$, intercept $F_{1,69} = 4.3$, $P = 0.04$), demonstrating that soldier head width is often larger than that of the queen across species. Similarly, neither queen head width (PGLS; estimated $\lambda = 0.39$, queen head width $F_{1,69} = 0.8$, $P = 0.4$) nor morphotype (PGLS; estimated $\lambda = 0.4$, morphotype $F_{3,69} = 0.7$, $P = 0.6$) were significant predictors of mean head width disparity expressed as a proportion of queen head width, to correct for substantial size differences among species. Yet as with absolute head width disparity, the means for proportional head width disparity were significantly greater than 0 across morphotypes (PGLS; estimated $\lambda = 0.4$, intercept $F_{3,69} = 5.6$, $P = 0.02$).

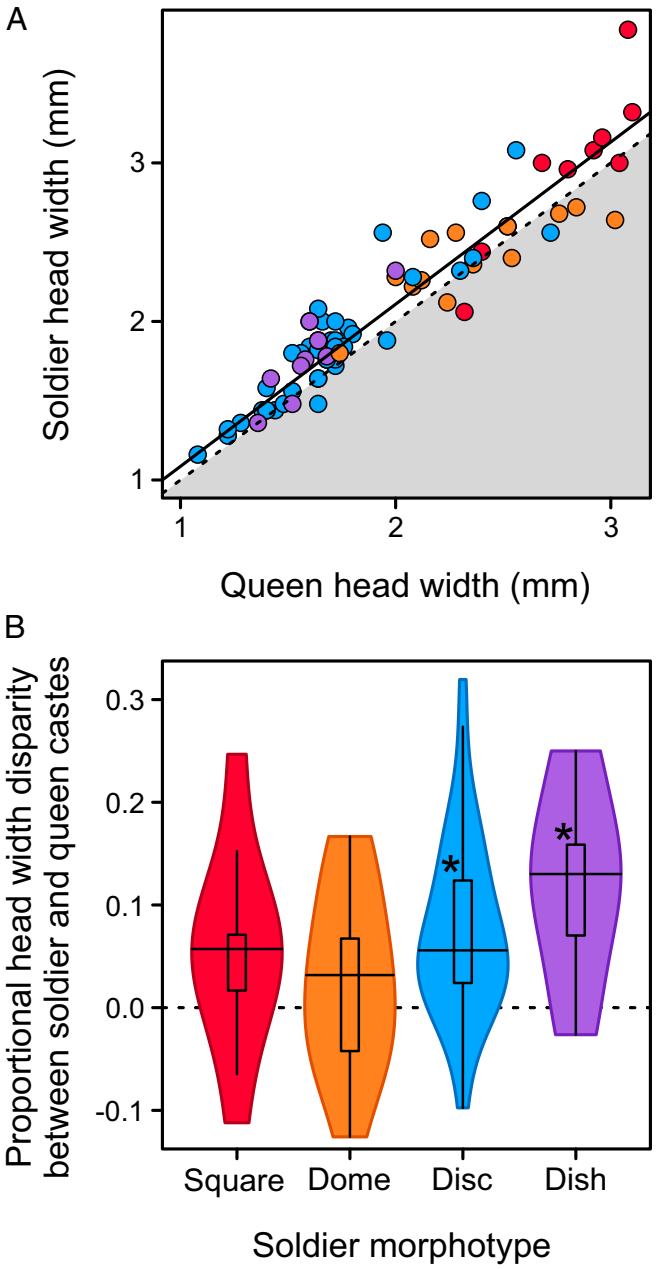

Fig. 4. The disparity in head width between soldier and queen castes in the turtle ants. (*A*) Soldier head width on queen head width. The solid line shows the best PGLS fit to the data (PGLS; estimated $\lambda = 0.55$, $F_{1,\ 72} = 440.1$, $P < 0.0001$). The dashed line shows equivalency (1:1) in head width between castes. White and gray regions identify species in which the soldier head is larger or smaller than the queen head, respectively. (*B*) Violin plots showing the proportional disparity in head width between soldier and queen castes across the four soldier morphotypes. The horizontal dashed line shows equivalency (1:1) in head width between castes. Asterisks denote morphotypes in which mean proportional disparity in head width is significantly greater than 0, indicating that the soldier head is larger than the queen head, after accounting for the phylogenetic relationships among species (PGLS; $P \leq 0.05$). Each plot incorporates a kernel density plot of the data and a boxplot with a box encompassing the interquartile range (IQR), a line drawn at the median, and the whiskers extending to the upper and lower quartiles (±1.5 times the IQR). Violin plots were created with the R package vioplot (67).

This overall pattern of positive proportional caste disparity was driven by the disc and dish morphotypes (Fig. 4*B*). Indeed, soldier head width is up to 32% and 25% bigger than queen head width in these morphotypes, respectively.

Taken together, these results indicate that within the observed range of disparity among castes, the evolution of soldier head size is decoupled from both the absolute and relative queen expression of this trait and soldier morphotype. In addition, while the evolution of head size disparity between castes can be negative or positive in all morphotypes, soldier heads are larger than queen heads on average, with positive caste disparity greatest in the disc and dish morphotypes.

The extent of decoupled head size evolution between soldier and queen castes reveals a substantial capacity for the evolution of novel adaptive function within a polyphenic caste series. How the phenotype of a particular caste is developmentally constrained by the other phenotypes within the polyphenic series has been studied intensively and is still being debated (15, 16, 21, 23, 57). Soldier head size must ultimately be constrained by what development can achieve with available pupal resources and the single genome shared by the polyphenic series. Yet the key insight from our species-level analyses is that the evolution of a critically important functional trait in the soldier caste is extensively decoupled from queen trait expression within the broad bounds set by developmental constraints. Again, existing knowledge of trait function in the turtle ants allows us to infer that this newly identified pattern of caste evolution likely has important consequences for adaptive niche-filling. Specifically, decoupled trait evolution among castes means that species with similar queen phenotypes can evolve substantially different soldier head sizes, and thus fill differentiated nesting niches. The species with soldier heads larger than queen heads are of particular interest in this respect. These are species that have evolved a novel range of functional capabilities in the soldier caste relative to the queen; with larger heads, they can effectively defend larger nest entrances than would otherwise be possible. The evolution of soldier head size larger than that of the queen, a common and variable pattern of the decoupled caste disparity in our analyses (Fig. 4), may then be especially important in the adaptive diversification of the turtle ants.

Conclusions

Our species-level analyses of an iconic ant lineage indicate that the evolution of ecomorphological traits within a lineage can be extensively reversible, repeatable, and decoupled within and among castes. These findings indicate that at the species level, castes are a more dynamic product of adaptive evolution than previously expected. In particular, decoupled trait evolution within and among castes may be especially important in facilitating the adaptive diversification of derived social lineages. Importantly, these comparative insights have also yielded a set of clear predictions for further experimental interrogation of the adaptive relationship between soldier traits and ecological function. The extent to which our findings will be mirrored in other social taxa with both convergent and alternative caste phenotypes remains to be examined. Yet equivalent species-level analyses of traits with known ecological function will be critical for advancing our understanding of adaptive caste evolution. Similarly, as analyses of the dynamics of trait evolution advance for both social and unitary lineages, there will be opportunities to examine how adaptive trait evolution differs across levels of organismal complexity. While patterns of trait evolution at one level of organismal complexity might not be generalizable to organisms at other levels, the contrast in evolutionary outcomes from common underlying processes is likely to be highly informative for explaining global patterns of biodiversity. Therefore, much may be learned from a theoretically and methodologically unified comparative approach to the study of trait evolution across levels of organismal complexity.

Methods

Ecomorphological Traits and Datasets. The morphological datasets for soldier morphotype and soldier and queen head width were compiled from all available data and images in de Andrade and Baroni Urbani (28), data and specimens from the collections of S.P., and from examining type specimen images on AntWeb.org (*SI Appendix*, Table S8). Trait values were standardized on the largest known caste specimens for all species. This approach thus provided robust trait maximums that capture ecologically meaningful functional limits of each species (further details in *SI Appendix*). The soldier morphotype dataset was compiled by cross-referencing all available information to apply our newly revised morphotype categories (Fig. 1 and *SI Appendix*, Fig. S1). For the soldier and queen head width datasets, de Andrade and Baroni Urbani (28) served as the primary data source, with values updated or added from the collections of S.P. for castes that were poorly collected or unknown when the primary data source was published (*SI Appendix*, Table S8).

Phylogeny and Trait Evolution Analyses. All analyses were run in a standard installation of R version 3.5.1, with functions from additional packages as specified below. Analyses in the main text that incorporated phylogenetic information used the maximum clade credibility chronogram from Price et al. (36). This phylogenetic tree combines molecular and morphological datasets for extant and fossil taxa to recover the tip-dated relationships among 115 *Cephalotes* species. This taxon sampling represents 97.5% of the described species plus additional undescribed species. The backbone of this tree, defining clade relationships and positions of previously defined species groups (*SI Appendix*, Fig. S3), is identical to the earlier *Cephalotes* phylogeny recovered using only molecular data for ∼50% of known species (58). The total evidence complete species phylogeny (36) used in the present analyses thus effectively places additional species in the well-supported clades of the previous molecular tree. In addition, the morphological character matrix used in the total evidence phylogeny did not contain the newly defined morphotype categories or head width data analyzed in the present study. This approach ensures that the phylogeny is independent of the morphological traits analyzed here. For each analysis, the phylogeny was trimmed to include only the relevant taxa using R packages picante v1.6-2 (59) and geiger v2.0.6 (60).

Analyses of soldier morphotype evolution, including species known to lack a soldier, used a dataset of 99 species after excluding the 16 species for which the soldier state, and therefore head width, are not known. This dataset was further reduced to 89 species for analyses of soldier head width in combination with morphotype, necessarily excluding the 10 species known to lack a soldier. Finally, analyses that contrasted trait evolution among soldiers and queens included only the 74 species for which both soldier and queen data are known.

To assess the degree of reversibility and repeatability in the evolution of soldier morphotype, we used multiple approaches to estimate ancestral character states. These analyses focused on the broad insight of whether a pattern of reversibility and repeatability was robustly identified across approaches, without prior expectations about specific models of state transition or state ordering. The first approach incorporated five states—no soldier, square soldier, dome soldier, disc soldier, and dish soldier—and estimated ancestral states using maximum likelihood estimation, as implemented in the R package ape (61). The three default models of state transitions—equal rates, symmetric, all rates different—were fitted to the data. Akaike weights were used to determine the best fit to the data among models using phytools (62), and the number and pattern of morphotype transitions was assessed across all models.

To assess the possible influence of phylogenetic uncertainty on these analyses, the same set of models was fitted to a sampling of trees from the posterior distribution of the published phylogenetic analysis (36). The second approach used stochastic character mapping to estimate ancestral character states across the same three models, with analyses implemented in phytools under the default setting for estimating the state transition matrix and the prior distribution on the root node (62). A total of 1,000 stochastic character maps were generated for each model, allowing average state transitions to be calculated and summarized.

The third approach reduced our overarching hypothesis of reversibility and repeatability in morphotype evolution to two simplified binary state hypotheses, to maximize the power for ancestral state estimation on our phylogeny. The first binary state hypothesis addressed reversibility and repeatability in the evolution of group-blocking morphotypes (combining square and dome morphotypes) vs. solo-blocking morphotypes (combining disc and dish morphotypes). The second binary state hypothesis addressed reversibility and repeatability in the presence vs. absence of a soldier caste.

Ancestral states were estimated using maximum likelihood estimation, as in the first approach (61), but the binary states naturally reduced the models to equal rates and forward and backward rates different.

Head width evolution across morphotypes was assessed with the Bartlett test of homogeneity of variances and PGLS models with morphotype as a discrete predictor variable, using a combination of functions in the R packages nlme v3.1-139 (63), ape v5.1 (61), geiger v2.0.6 (60), and phytools v0.6-44 (62). BM, estimated λ, and OU covariance structures were examined in the PGLS analyses, with the estimated λ covariance structure yielding the best-fitting model in all cases, as determined by comparison of Akaike weights. Analyses were run with all possible level-encoding orders to identify significant pairwise differences between level means, as well as level means significantly greater than 0 when appropriate.

The dynamics of head width evolution were examined using the model fitting approaches integrated in the R-package pmc v1.03 (48). This package fits likelihood models for continuous character evolution from the R package geiger v.2.0.6 (60) and OU models for the R package ouch v2.11-1 (64, 65), while also using a Monte Carlo-based approach to calculate parameter confidence intervals and assess significance of fit and statistical power for pairwise model comparisons. We fitted all the models supported by geiger that were appropriate for our dataset (BM; single-optimum OU, early burst, trend, lambda, kappa, delta, white). Our analyses of head width evolution across morphotypes (Fig. 3) further suggested that a two-optima OU model should be examined, which was also fitted to the data. Akaike weights were used to determine the best fit to the data among the models, using phytools (62). The best-fitting model was then compared for significant fit and statistical power in pairwise tests against each of the other models supported by geiger, following the recommended procedure when contrasting a set of models without a clear progression in complexity (48). The single-optimum OU model was also evaluated in a pairwise test against the two-optima OU model to compare different OU variants. (PMC v1.03 did not allow direct comparisons between the two-optima OU model and the other non-OU models that we tested.) Finally, to assess the possible role of pulsed processes in head width evolution, we fitted a set of Lévy process models that incorporate jumps (a compound Poisson with JN, NIG, combined BM and JN processes [BMJH], and combined BM and NIG processes [BMNIG]), following Landis et al. (66) and as implemented in the R package pulsr. Akaike weights were then used to examine model fit across the Lévy process models, the best-fitting kappa model from previous analyses (*Results and Discussion*), and BM and OU models for contrast.

Analyses addressing decoupling of ecomorphological traits among soldier and queen castes used the calculated values of absolute head width disparity (soldier head width minus queen head width) and proportional head width disparity (absolute head width disparity divided by queen head width). As in our within-caste analyses, relationships between traits and calculated disparity metrics were assessed with PGLS models but incorporating both discrete and continuous predictor variables depending on the relationship being addressed.

Data Availability. The morphological datasets for these analyses are provided in *SI Appendix*. The phylogeny used in these analyses is available in a previous publication (36).

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