

# Sexual dimorphism as a facilitator of worker caste evolution in ants

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## Abstract

Ant societies are primarily composed of females, whereby labor is divided into reproductive and non-reproductive, worker, castes. Workers and reproductive queens can differ greatly in behavior, longevity, physiology, and morphology, but their differences are usually modest relative to the differences relative to males. Males are short-lived, typically do not provide the colony with labor, often look like a different species, and only occur seasonally. It is these differences that have historically led to their neglect in social insect research, but also why they may facilitate novel phenotypic variation – by increasing the phenotypic variability that is available for selection. In this study, worker variation along a size-shape axis corresponded with variation in male-queen size and shape. As worker variation increased within species, so did sexual variation. Across species in two independent genera, sexual size dimorphism correlated with worker polymorphism regardless of whether the ancestral condition was large or small worker/sexual dimorphism. These results, along with mounting molecular data showing that process of queen-worker caste determination has co-opted many genes/pathways from sex determination, lead to the hypothesis that sexual selection and selection on colony-level traits are non-independent and that sexual dimorphism may even have facilitated the evolution of the distinct worker caste.

## Introduction

Male ants are ephemeral in the annual cycle of a colony. They have been likened to ‘sperm missiles’ (a statement attributed to E. O. Wilson) – a transient, but essential function – delivering the male gametes of the superorganism to gynes so that the gynes can become queens and found new colonies, beginning the colonial life cycle anew (Hölldobler et al. 1990). Because of this males are often unseen and unsampled. Males are also haploid, typically developing from unfertilized eggs (males can be fathers, but have no father). Males are thus genetically and behaviorally very very different from the standard (female) ant, and their morphology is also clearly distinct as they typically have small heads with big eyes, a large muscly thorax with wings, and an abdomen tipped with an intromittent organ rather than a stinger. They are very clearly different than females; so different to look a different species (Fig. 1). And so it is clear why males are largely ignored in the empirical and theoretical literature on caste and polyphenism in the social insects – they are different.

Recent studies on gene expression during development have revealed that queen-worker caste determination shares many genes in common with male-female sex determination (Klein et al. 2016, Johnson and Jasper 2016, McAfee et al. 2019, Warner et al. 2019, Roth et al. 2019). In fact, in both an ant and honeybee there was a large suite of shared gene expression patterns between males and workers to the exclusion of gynes (Warner et al. 2019). Furthermore, in a CRISPR gene knockout of early sex determination genes in honeybee it was shown that the *Fem*(feminizer) gene appears to control gonad size regardless of sex (though it also interacts with sex determination)(Roth et al. 2019). The emerging picture is that worker caste differentiation (from queens) arose via the co-option of male genes. That is, workers might be a gene-expression developmental mosaic (Molet et al. 2012) of male and female.

The view that male-ness contributed (and contributes) to worker evolution is logical. Male and female ants,

despite behavioral, genetic, and morphological dissimilarity, arise from the same genome, whether haploid or diploid. Thus the raw material for natural selection to work from is the combination of phenotype expressed by both males and females; the colonial lifestyle of social insects may also buffer novel developmental mosaics from strong selection (Molet et al. 2012). Sexual dimorphism is present in most clades of Hymenoptera at least partly because of specialization by females for parasitism/predation and brood care (in some lineages) (Stubblefield and Seger 1994). Due to sexual dimorphism there was morphological variation for selection to act on – possibly enough to recombine developmental programs and produce a novel phenotype/caste that changed ecological and evolutionary history (Smith and Szathmary 1997), the worker. In this study I examine whether there is evidence for sexual dimorphism explaining variation in worker caste polymorphism. In extant species, does the degree of sexual dimorphism correlate with how variable the worker caste is – in other words, does male-ness have a hand in the evolution and elaboration of the worker caste?

Two species of *Pogonomyrmex* harvester ant, *P. barbatus* and *P. badius*, were used to examine the relationship between ant size and shape across all adult castes. These species were chosen because sufficient samples were available across all castes for detailed measurement and because they differ in regard to both sexual and worker dimorphism. *P. barbatus* has very little sexual dimorphism while in *P. badius*, gynes are more than three times the size of males by dry mass and have an energetic cost to the colony of 4.5X of a male (Smith and Tschinkel 2006). Similarly, workers of *P. barbatus* are characterized as monomorphic while those of *P. badius* are dimorphic, with clearly distinct major and minor worker sub-castes (Tschinkel 1998).

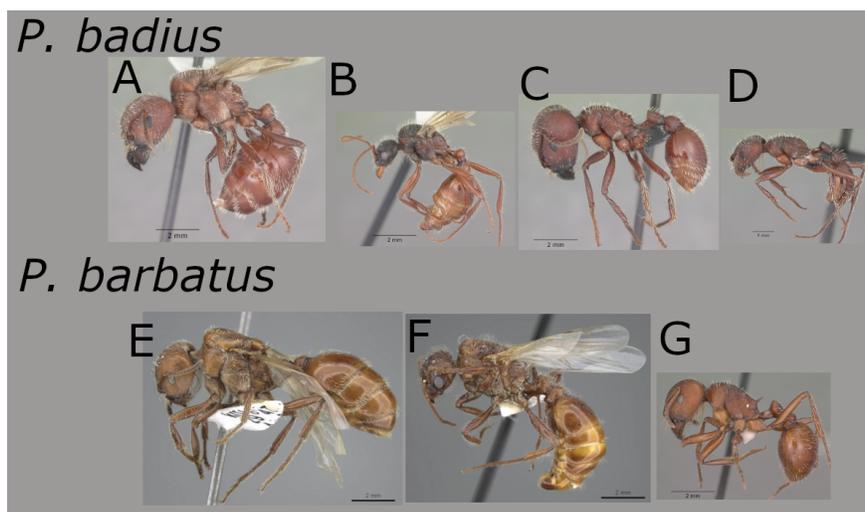


Figure 1. Images of *P. badius* and *P. barbatus* from [www.antweb.org](http://www.antweb.org) size-scaled for comparison (note scales on images). A and E are gynes, B and F are males, C, D, and G are workers. Images A-D photographed by A. Nobile, E-F by M. Esposito, and G by J. Fogarty. In order of letter A to G, specimen codes are casent0104422, casent104421, casent0103057, casent0103056, casent0914095, casent0914094, casent0102894.

I hypothesized that the worker caste of *P. barbatus* would be similar in shape to both males and gynes, as opposed to the traditional notion that females should clearly group to the exclusion of males. In *P. badius*, the gynes and major workers are very similar in basic visual appearance (shape and size) and appear to be co-regulated at the colony-level – that is, their production as a function of colony size is similar. While *P. badius* minors and males are not immediately clearly similar upon visual inspection, they also appear co-regulated at the colony level (Smith and Tschinkel 2006, Smith 2007). And thus for *P. badius*, it was hypothesized that increasing sexual dimorphism would be mirrored by increased worker dimorphism, with males/minors (small castes) and gynes/majors (large castes) grouping together in size-shape space.

In order to generalize the patterns seen across the two *Pogonomyrmex* species, where sexual dimorphism

covaried with worker dimorphism, a generic-level comparison was conducted across two genera that have broad variation in sexual and worker dimorphism, *Pogonomyrmex (sensu stricto)* and *Pheidole*. These two genera were also chosen as they met two other important criteria, 1) a high-quality molecular phylogeny was available, and 2) sufficient samples of all castes were available for measurement on Antweb.org. Based on the prior data on the two *Pogonomyrmex* species it was hypothesized that there would be a positive correlation (controlling for evolutionary history) between sexual dimorphism and worker di/poly-morphism. The use of these two genera have the potential to provide a strong hypothesis test for this correlation as the ancestral state in each case is opposite whereby ancestral *Pogonomyrmex* have monomorphic workers and little sexual dimorphism and ancestral state of *Pheidole* is to high worker and sexual dimorphism.

## Methods

### *Pogonomyrmex* body measurements

*P. barbatus* and *P. badius* were used for measurements as a large quantity of individuals were available from previous field and lab sampling. Samples used for measurement were either stored at  $-80^{\circ}\text{C}$  (most *P. barbatus*) or dried (*P. badius*); for the former, individuals were from years of field and lab sampling, some of the same colonies as were part of previous studies (Smith et al. 2012, 2015, 2018), and similarly for the latter (Smith and Tschinkel 2006, Smith 2007). For both species the objective was to maximize variation in body size for each caste rather than maintain sufficient sampling to examine colony-level effects. *P. barbatus* lab colonies produced microgynes and micromales as well as minim/nanitic-like workers, sometimes after years of lab rearing. These micro individuals were included to help disentangle the effects of size and caste. Because size variation was the primary variable being maximized in the sampling design, some colonies are only represented by single individuals while others with many. A total of 31 colonies of *P. barbatus* were included spread across 25 gynes, 19 males, and 38 minor workers (82 total). A total of 11 *P. badius* colonies were sampled with 17 gynes, 23 males, 21 major workers and 23 minor workers.

All ants measured were dried and separated into body and appendage segments using a stereo microscope, and glued to card stock (on their right side) to help standardize focal plane and angle among samples. All measurements were done using a stereomicroscope (SZX7, Olympus, USA) with an attached camera (Retiga 2000R, Q-Imagine, Canada) and measured after calibration using iSolutions-Lite software (iMT Technology, USA); magnification of images differed for body parts and castes, but ranged from 25 to 56X.

Body parts measured were chosen for their repeatability and frequency of use in ant studies in order to facilitate cross-study comparisons. Pictures and descriptions of these standard measures are available at [https://www.antwiki.org/wiki/Morphological\\_Measurements](https://www.antwiki.org/wiki/Morphological_Measurements) (edited by S. Shattuck). A total of 16 measurements were taken for each ant. Length: Head length (full-face view, edge of clypeus to head vertex), mesosoma/Weber’s length (profile view, head attachment to petiole attachment), gaster length (dorsal view, post-petiole attachment to end of first gastral tergite along post-petiole to sting axis), the length of the tibia and femur of each leg (fore, mid, hind) was measured; Width: head width 1 (full-face view, head width across the eyes), head width 2 (full-face view, width at clypeus), head width 3 (full-face view, width at mid-point between vertex of head and eyes), gaster width (dorsal view, widest point of first gaster segment perpendicular to length axis); Height: thorax height at three points (profile view, measured at the anterior top of each leg, fore, mid, and hind). Ant length (sum of head, mesosoma, and gaster length) was used as a proxy of ant size when comparing to multivariate statistics (below).

All analyses were done using R 4.1.2 (R Core Team 2021) and the RStudio interface (RStudio Team 2022). Plots were constructed using ggplot2 (Wickham 2016) and where relevant combined using ggpubr (Kassambara 2020).

Non-metric multidimensional scaling (NMDS) was used to compare body size and shape across the measured characters in both *P. barbatus* and *P. badius*. Bray-Curtis distances were used and only two dimensions were extracted for this analysis – the metaMDS function from the package vegan 2.5 (Oksanen et al. 2020) was used, and then points were extracted and plotted. Differences among castes was assessed statistically using PERMANOVA in size-shape space using the function ‘adonis’ from vegan (Oksanen et al. 2020) with pairwise

comparisons made using `adonis.pair` from the `EcolUtils` package (Salazar 2022). Furthermore, differences among caste in shape was tested using ANOVA on the second scaling axis (orthogonal to size).

#### *Measurements from Antweb.org*

Measurements of worker and sexual size were done on specimens from Antweb.org; images were downloaded and measured using ImageJ2 v2.3.0 (Rueden et al. 2017) relative to the scale printed on the image. Head width was defined as the width of the head as measured across the eyes.

To ascertain whether the data available on Antweb.org were representative of natural variation, *Pogonomyrmex* harvester ant samples from Antweb.org were compared to head width measurements that were available from other sources. The overlap of worker size variation as measured from Antweb.org was compared to that reported in a taxonomic overview of the genus in North America (Cole, Arthur 1968). A t-test was used to test whether the proportion of overlap between worker size ranges from Antweb.org and Cole (1984) differed from 1. Note that *P. wheeleri* measurements from Antweb.org ( $N = 2$ ) did not overlap Cole’s range and this 0% overlap was excluded from analysis.

Additional head width measurements were available from four species ( $N = 382$  *P. badius*,  $N = 38$  *P. barbatus*,  $N = 433$  *P. coarctatus*, and  $N = 431$  *P. rugosus*). These additional samples were measured optically using an SZX7 Stereomicroscope by Olympus with a Retiga 2000R camera by Q-Imagine, and iSolutions Lite software by iMT Technology; samples were from various colonies and populations sampled as parts of other studies. Overlap of Antweb.org measurements with these more exhaustively sampled species was qualitatively compared.

#### *Phylogenetic contrasts:*

Only species with at least one measure from each caste on Antweb.org were used in analysis; species of the same species complex were substituted if the species in the phylogeny was not fully represented in Antweb.org; species complex information was obtained from species information in Antwiki.org. The worker ratio was the largest divided by the smallest worker head width, while the sexual ratio was the mean gyne head width divided by the mean male head width. The gyne:worker ratio was calculated as the average gyne size divided by the minimum worker size.

Genus-level phylogenies (Pheidole: (Moreau 2008), Pogonomyrmex: (Johnson and Moreau 2016)) were used to address non-independence of species.

Relationships of size variation between castes were tested using a linear model with phylogenetic correction. Phylogenetic correction was done using independent contrasts as calculated using the `crunch` function in the `caper` package in R (Orme et al. 2018).

## Results

#### *Species Comparisons:*

Qualitatively, as hypothesized, in *P. barbatus* with limited dimorphism, females (workers and gynes) did not group by shape to the exclusion of workers, but rather males were intermediate in shape between workers and gynes (MDS2 in Fig. 2C). Contrary to the hypothesis, though, in the dimorphic *P. badius*, workers most differed from sexuals in shape and each workers and sexuals had distinct size-shape axes (Fig. 2D). Interestingly, the workers in *P. barbatus* do have a significant size by shape relationship ( $F_{1,35} = 44.52$ ,  $P < 0.0001$ ,  $R^2 = 0.55$ ) while shape is not predicted by size in males and gynes of *P. barbatus*. In *P. badius*, there is not a significant size-shape relationship in any caste, though visually this appears to possibly be a sample size artifact. That said, size predicts shape for each workers and sexuals, and these slopes are parallel (total model:  $F_{3,78} = 183$ ,  $P \ll 0.0001$ , Size x shape:  $P \ll 0.0001$ , interaction:  $P = 0.14$ ). Also very interestingly, the size-shape slope for *P. barbatus* workers is not different from that of *P. badius* workers, potentially suggesting conservation of the growth mechanisms governing shape change with size in this genus.

Predictable traits helped separate castes in each species; note, below, trait size as referenced is trait size

accounting for body size. The distance from the origin in Fig. 2C-D corresponds to the strength of correlation between each MDS axis and the measured characters. In both species, trunk/mesosomal height helped differentiate sexual castes due to the thoracic enlargement that accommodates wing muscle. Leg length (for all legs and leg segments) and head size tended to help separate workers from sexuals in *P. barbatus*, but helped separate minor workers/males from major workers/gynes in *P. badius*. Gaster size tended to help separate gynes in both species. These qualitative comparisons of castes by the measurements correspond with casual observation and common sense.

Quantitatively, as expected, the first multivariate scaling axis is highly correlated with ant length in both species, thus approximating size (*P. barbatus*:  $F_{1,77} = 757$ ,  $P \ll 0.0001$ ,  $R^2 = 0.91$ , *P. badius*:  $F_{1,80} = 1269$ ,  $P \ll 0.0001$ ,  $R^2 = 0.94$ ) (Fig. 2A-B). In both *P. barbatus* and *P. badius*, all castes were distinct in size-shape space (PERMANOVA: *P. barbatus*:  $F_{2,76} = 14.366$ ,  $P < 0.001$ , *P. badius*:  $F_{3,78} = 89.881$ ,  $P < 0.001$ ; all castes different from each other at  $P < 0.005$  in both species) (Fig. 2C-D) as well as in shape/MDS2 (ANOVA: *P. barbatus*:  $F_{2,76} = 51.57$ ,  $P \ll 0.0001$ , *P. badius*:  $F_{3,78} = 197$ ,  $P \ll 0.0001$ ; all castes different from each other at  $P < 0.005$  in both species).

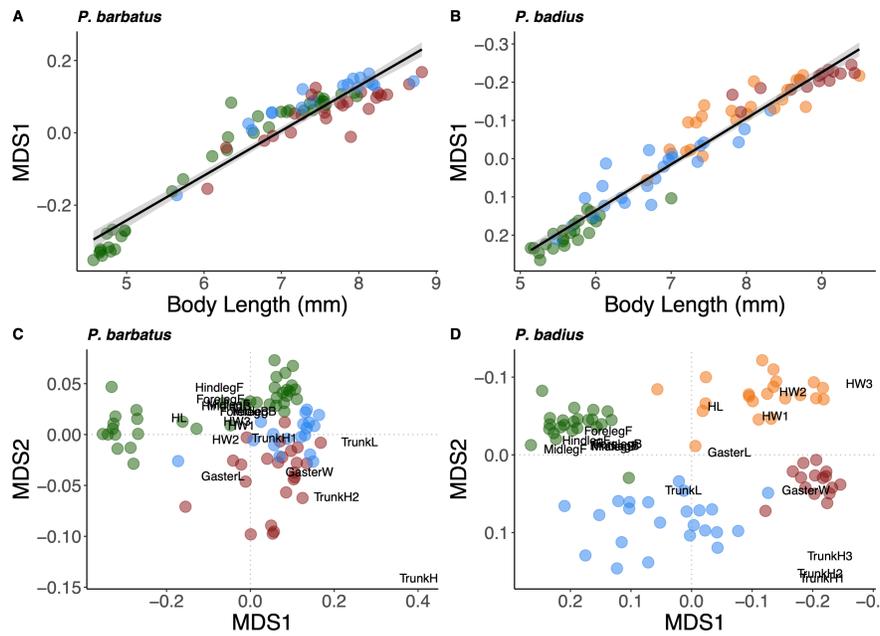


Figure 2. Body size by shape plots using nMDS for both *P. barbatus* (left, A and C) and *P. badius* (right, D and D). In A-B, MDS1 is regressed onto untransformed body length to demonstrate that the first multivariate axis (MDS1) is a proxy for body size. C and D thus show size on the x-axis and the first orthogonal multivariate factor (MDS2) is a dimension of shape. Superimposed on the plots are the correlation between individual measurements and the multivariate factors. In all plots, red = gynes, blue = males, green = (minor) workers, and orange = major workers. Note, MDS1 was rotated in *P. badius* to make the plots easier to compare.

#### Antweb.org Proof of Concept:

Overall, the specimens available on Antweb.org seem to be relatively good estimates of the natural variation present in these species (Fig. 3). The proportion of overlap between Antweb.org and the worker head width ranges reported by Cole (1984) does not differ from 1 ( $t = 1.26$ ,  $df = 16$ ,  $P = 0.23$ ). It should be noted, though, that several species on Antweb.org show a much greater degree of variation (200-600%) compared to Cole (1984) (Fig. 3 inset). When the Antweb.org sample range is compared to four intensively measured

species there is 70-88% overlap; Antweb.org measurements show more variation in one of the four species (*P. rugosus*, Fig. 3).

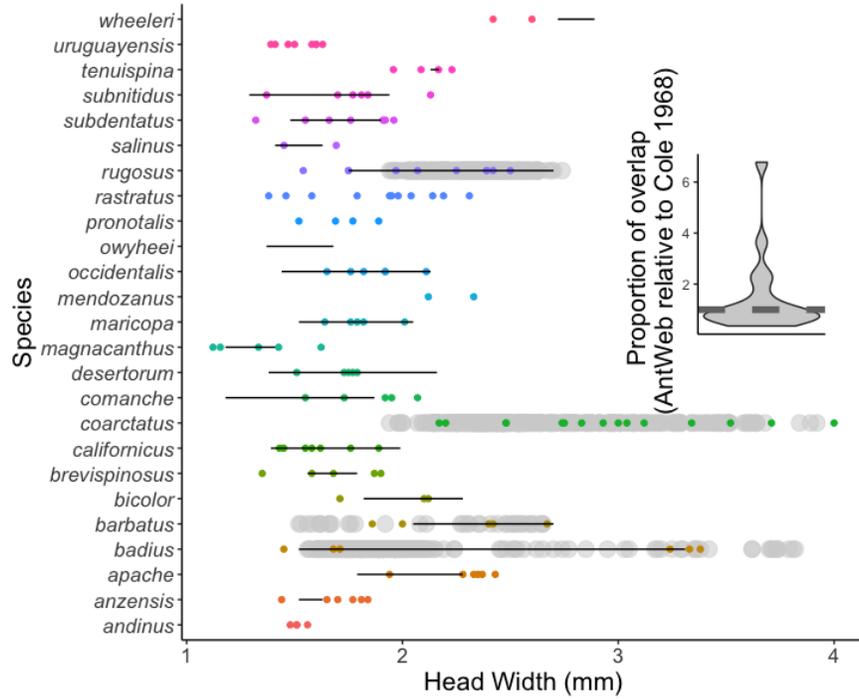


Figure 3. Head width measurements of workers from various sources. Colored points are measurements from Antweb.org, background grey points are measurements by CR Smith, and lines represent the min and max head size as reported by Cole (1984). The inset is a violin plot showing the distribution of worker size range overlap as measured from Antweb.org and by Cole (1984). An overlap of 1 would mean that both sources report the exact same size range for a species; an overlap less than one means that Antweb.org underestimates variation relative to Cole. The proportion overlap between these two sources is not different than 1 ( $t = 1.26$ ,  $df = 16$ ,  $P = 0.23$ ). The measurements present on Antweb.org are largely representative of worker variation in the species examined.

*Generic Comparisons:*

In both *Pogonomyrmex* and *Pheidole*, the sexual size ratio is a significant predictor of the worker size ratio, once controlling for phylogeny (*Pogonomyrmex* :  $F_{1,8} = 5.421$ ,  $P = 0.048$ ,  $R^2 = 0.33$ , *Pheidole* :  $F_{1,15} = 20.77$ ,  $P = 0.0004$ ,  $R^2 = 0.55$ ) (Fig. 4 inset). The gyne to worker size ratio is also a significant predictor of the worker size ratio in both genera – to a greater extent than the sexual size ratio is (*Pogonomyrmex* :  $F_{1,8} = 9.283$ ,  $P = 0.016$ ,  $R^2 = 0.48$ , *Pheidole* :  $F_{1,15} = 70.23$ ,  $P \ll 0.0001$ ,  $R^2 = 0.81$ ).

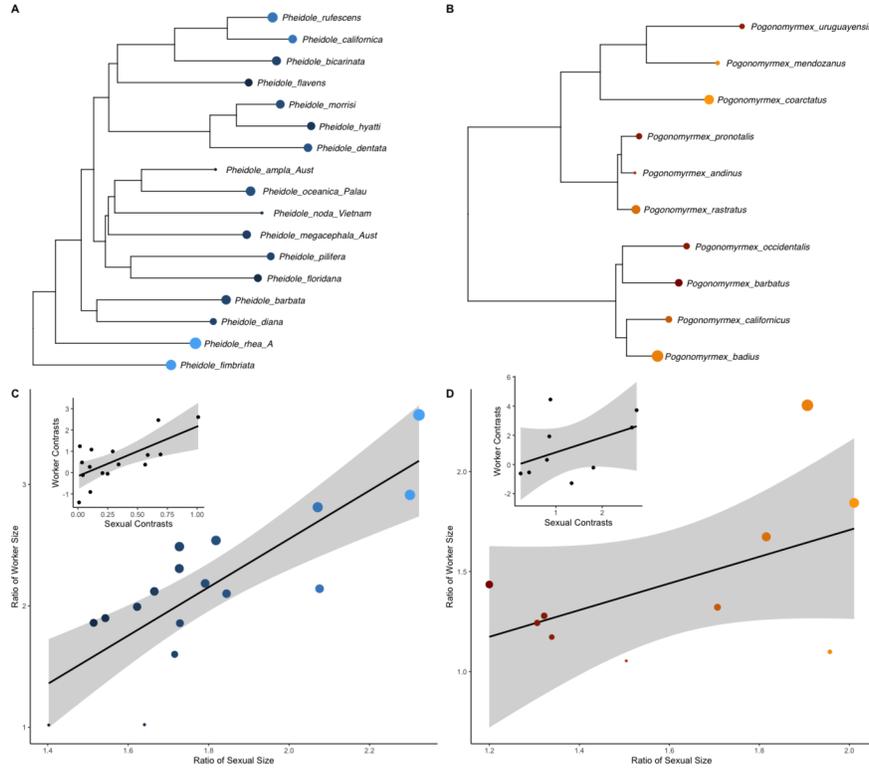


Figure 4. Phylogenies of *Pheidole* (A) and *Pogonomyrmex* (B), along with scatterplots (C and D) showing the relationship between sexual size ratio (gyne/male size) and worker size ratio (largest/smallest size). Graph insets are plots of phylogenetically independent contrasts between sexual size ratio and worker size ratio. Point size is proportionate to worker ratio and color is proportionate to sexual size ratio (lighter color is larger).

## Discussion

As hypothesized, sexual dimorphism covaries with worker polymorphism. This pattern is evident when comparing the size-shape space of individual species as well as at the level of entire genera. While quite logical, that male and female characters do not evolve independently, even in the absence of sexual selection, males have largely been absent from empirical and theoretical studies of the hymenopteran social insect castes.

While this study documents how sexual dimorphism relates to worker polymorphism in two genera of highly social and very derived ants, it is logical to think that sexual dimorphism was important for the origin of the worker caste and all of its subsequent elaboration/modification. Because sexual dimorphism is likely the ancestral state of the Hymenoptera (Stubblefield and Seger 1994), the ancestral size-shape space in the wasp ancestor of the ants was the phenotype space available for selection that ultimately resulted in the worker caste. After all, the differences among castes is primarily the difference in the timing, location, and dosage of a shared set of genes, and so the worker was/is both part male and part female. Variation in mating and social systems among ant species varies the degree of sexual selection and thus sexual dimorphism. Thus it is likely that there is a correlation between mating systems and worker polymorphism. It may even be that the observed correlation between worker caste variation and genetic diversity within nests (Fjerdingstad and Crozier 2006, Anderson et al. 2008, Smith et al. 2008b, Hughes and Boomsma 2008, Smith et al. 2008a), that results from mating systems like polyandry, is mechanistically confounded by the phenotypic results of sexual selection (e.g., sexual, or natural, selection on male body size to increase mating probability).

The causal arrow in the relationship between sexual and worker dimorphism likely points in both directions. As above, sexual dimorphism may have provided the raw material (size-shape) for the evolution of the worker caste, but selection on worker characteristics, and on the population of workers in colonies (colony-level selection)(Oster and Wilson 1979), likely facilitates/inhibits the phenotypic variation available to sexual selection. Strong natural selection on worker size variation, for example, increases the size-shape space and may facilitate the production of novel sexual forms.

Importantly, the causal direction of the relationship is far less important than recognizing the non-independence of the two processes. Selection on worker polymorphism, for example, increases the phenotypic space available to sexual selection, not necessarily whether that selection occurs. Selection for increased variation in the size-shape of any caste increases the raw material for selection – it increases opportunity. As previously theorized, the colony environment buffers its members from individual-level selection and can be an incubator of sorts for novelty, especially via modifications of the social environment (Rajakumar et al. 2012, Molet et al. 2012).

Not surprisingly, queen-worker dimorphism was a better predictor of worker polymorphism than was sexual dimorphism because female castes are developmentally more similar than are the sexes. Previous studies have documented the correlation between queen-worker and worker polymorphism across species (Fjerdingstad and Crozier 2006), though these factors are not always correlated (Lecocq de Pletincx et al. 2021). The development of workers and queens is more similar than that of workers and males (Anderson et al. 2008). For example, queen-worker differentiation takes place in early development while worker caste differentiation occurs later; note, the most upstream mechanisms of female caste determination can occur prior to fertilization or in early embryogenesis (Schwander et al. 2008), but most differentiation (Lillico-Ouachour and Abouheif 2017), and canalization (Qiu et al. 2022), occurs later in larval development. Hymenopteran males and females differ genetically where male-destined eggs are typically unfertilized, and at least in honeybees the mechanism involves heterozygosity at a single locus, CSD, that then feeds into the conserved sex differentiation pathway of insects (Beye et al. 2003, Roth et al. 2019). Many studies are now demonstrating that sexual determination and differentiation and caste determination and differentiation are not mechanistically independent, both pathways utilize many of the same genes (Klein et al. 2016, Johnson and Jasper 2016, Warner et al. 2019, Roth et al. 2019).

One of the most surprising results of this study is the size-shape axis in both *Pogonomyrmex* species. In *P. barbatus* there is a size-shape relationship among workers, and in *P. badius* this relationship is the axis that differentiates both worker castes and both sexes (though workers and sexuals have different intercepts). The major worker caste in *P. badius* is an evolutionary novelty in the sense that it is a unique and derived character for the species, completely absent in all other extant North American *Pogonomyrmex*. Based on the measurements taken, the major worker is simply an extension of the minor worker along the size-shape axis. The major caste, in other words, is simply a size increase of the minor worker using the same allometric growth rules – and the gyne is, in a sense, a size extension of the male using the same allometric growth rules – and surprisingly, workers and sexuals vary on parallel axes only differing in their starting size. This result suggests that, despite appearances, all castes differ only slightly in basic developmental scaling.

Clearly, including more genera and more body measurements is a logical next step to further evaluate the relationship between sexual dimorphism and worker polymorphism. Looking at more subfamilies of ant will likely also reveal how generalizable this pattern is and how other aspects of biology constrain or facilitate caste evolution. Fortunately, the growing database of Antweb.org is an amazing resource and it seems that the maintainers of the database have done a commendable job of representing species-level variation. Given the high quality and taxonomic breadth of the database it is sure that it will be useful for the evaluation of additional macroevolutionary patterns in ants. Additionally, the availability of three-dimensional data for ant morphology and anatomy promises to add significantly to our knowledge of how castes differ in complex elements of internal anatomy and shape.

## Acknowledgements

Add post-review

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