The Evolution of Division of Labour in Social Insects



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Declaration

I declare that this thesis was composed by myself and that the work contained herein is my own except where explicitly stated in the text. The work has not been submitted for any degree or professional qualification except as specified.

Louis Bell-Roberts, Hilary 2024

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Abstract

The division of labour between queens and the various types of workers has helped eusocial insects achieve ecological dominance across many of Earth's terrestrial ecosystems. However, the form that division of labour takes, regarding the level of specialisation within each role in the colony, varies considerably across species. In this thesis, I examined both the causes and consequences of division of labour in social insects. First, I performed a phylogenetic comparative analysis across ants to test several factors that are predicted to explain variation in the level of non-reproductive division of labour. I examined how the levels of cooperation and conflict within colonies, influenced by the level of relatedness, affect the evolution of division of labour. In addition, I tested how ecological factors such as colony size and environmental variables—including measures of temperature, rainfall, and diurnal temperature range—may also influence the form that division of labour takes. Among the variables tested, I identified larger colony sizes as the only factor that consistently favours the evolution of increased division of labour. However, the evolution of more complex division of labour with greater levels of specialisation, may have permitted the colonisation of hotter and drier environments. Next, I examined the consequence of division of labour on the molecular evolution of the genome in social insects. The recent surge in the availability of genomic data has opened new opportunities to look for signatures of kin selection at the genomic level, the process which gave rise to the evolution of sterile castes. While I found that signatures of kin selection could be detected, results were mixed, and the availability of greater amounts of genomic data will aid future studies in this search.

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Introduction

Division of labour, where cooperating individuals specialise in different tasks (West and Cooper, 2016), is a unifying feature across all levels of biological organization (Fig. 1). Organisms are built by genes performing different functions, bacteria in clonal groups specialise in producing and secreting different factors essential for collective growth (Veening *et al.*, 2008), and in social insect colonies queens perform reproductive functions while workers forage and care for the brood (Oster and Wilson, 1978). If we want to understand how complex life evolved on Earth, we need to understand how and why division of labour evolves.

Division of labour is not just a consequence of the evolution of complex life but plays a crucial role in driving the evolution of biological complexity (West *et al.*, 2015). The hierarchical nature of life has been driven by a small number of major evolutionary transitions in which individuals form a new higher-level organism (Szathmáry and Smith, 1995; Queller and Strassmann, 2009; Bourke, 2011; West *et al.*, 2015). For example, collections of genes form genomes, individual organisms are made up of cells, and eusocial insect societies are groups of cooperating multicellular organisms. Division of labour has played a key role in these transitions because it leads to individuals only being able to reproduce as part of a larger group (mutual dependence) (West *et al.*, 2015). Therefore, to understand how complex life evolves, a major aim is to understand how division of labour evolves.

However, we understand relatively little about why division of labour only evolves on some branches of the tree of life and not others (Cooper and West, 2018). Furthermore, when division of labour does evolve how can we explain the form that it takes? Theoretical

advances have shown that division of labour will be favoured when there are efficiency benefits to specialisation and when the interests of individuals align, such as when individuals are related (kin selection) (Michod, 2007; Bourke, 2011; Cooper and West, 2018). These insights alone, however, cannot fully explain why division of labour has evolved only in certain species, nor can they account for the specific form that it takes. Therefore, we also need to identify the underlying empirical factors that vary across species to explain when division of labour has and hasn't evolved. For example, what are the ecological factors that influence the relative benefits of division of labour?

In this thesis, I examine the factors responsible for explaining variation in the form that division of labour takes among ant species. In addition, I will explore how genomic data can be used in a novel way to test the predictions of kin selection theory. This genomic approach has only just become possible due to the recent substantial increase in the amount of genetic data that is available for social insects. However, before describing the outline of my thesis, I introduce the concept of division of labour in social insects, highlighting several unresolved questions within this broad field of research and suggesting some potential solutions. I also explain the relevance of genomic data harvested from social insects for the first test of Hamilton's rule at the genomic level.

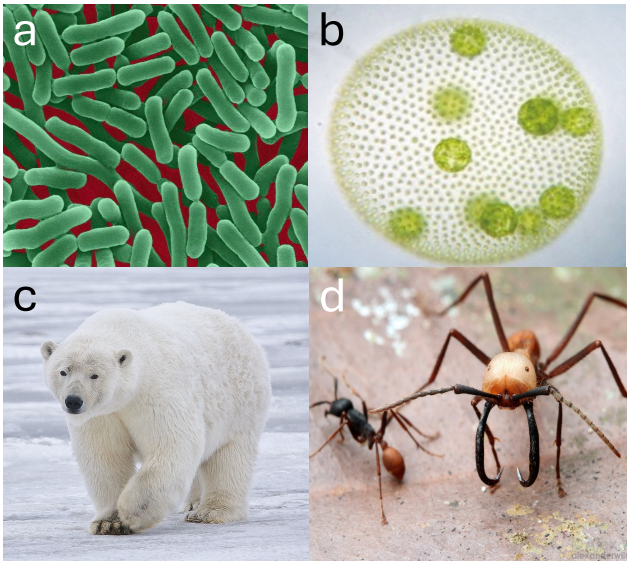


Figure 1. Division of labour and life on Earth. (a) *Salmonella enterica*—a portion of cells infiltrate the gut and initiate a response that eliminates competitors for the remaining cells (Ackermann *et al.*, 2008). (b) *Volvox carteri*—The somatic (small) cells keep the colony afloat by beating their flagella, while the germ cells (large) reproduce (Herron and Michod, 2008). (c) Multicellular organisms, such as polar bears, are made from different types of cells performing different functions. (d) In social insects, such as *Eciton burchelli*, the workers take on different functions within the colony such as forager (left) or soldier (right) (Oster and Wilson, 1978).

Division of labour in social insects

From an evolutionary perspective, the defining feature of eusocial insects, such as ants, termites, and social bees and wasps, is the presence of a reproductive division of labour between a reproductive and a sterile worker phenotype (castes) (Bourke and Franks, 1995; Crespi and Yanega, 1995; Queller and Strassmann, 1998, 2009; Boomsma, 2007, 2009, 2013; Bourke, 2011; Boomsma and Gawne, 2018). Reproduction in the colony is monopolised by queens who often possess physiological and morphological adaptations to aid their specialised role (Oster and Wilson, 1978). In contrast, workers are responsible for the non-

reproductive tasks such as caring for the brood, foraging and nest defence. Often, the workers in the colony are monomorphic, with limited variation in size and morphology, differing only in terms of their behavioural roles. However, in some species of ants, termites and stingless bees, workers may also vary in size and shape, forming distinct physical worker castes (hereafter "worker castes") (Oster and Wilson, 1978; Hölldobler and Wilson, 1990; Grüter *et al.*, 2012, 2017). The differences correspond to their functional roles in the colony, such as soldiers, living doors, prey couriers or food storage organs (Hölldobler and Wilson, 1990; Powell and Franks, 2005; Powell, Price and Kronauer, 2020).

The ecological dominance of the social insects arises from the division of labour between queens and the different types of workers (Oster and Wilson, 1978; Hölldobler and Wilson, 1990). Despite comprising only 2% of known insect species, eusocial insects account for over half of total insect biomass (Wilson, 2008). Therefore, if we want to understand how these complex societies have come to dominate Earth's terrestrial ecosystems, a major challenge is to understand the reasons why division of labour evolved, and what factors determine the form that it takes. While the initial evolution of sterile castes has garnered significant attention, there has been relatively less focus on understanding the factors that explain the variation in non-reproductive division of labour among species (Hamilton, 1972; Queller, 1994; Boomsma, 2009; Nonacs, 2011; Gardner, Alpedrinha and West, 2012; Alpedrinha, Gardner and West, 2014; Rautiala, Helanterä and Puurtinen, 2014, 2018; Olejarz *et al.*, 2015; Quiñones and Pen, 2017; Davies and Gardner, 2018). Chapters 2 and 3 will investigate why species vary in the form that non-reproductive division of labour takes.

How can we explain variation in the form of non-reproductive division of labour among species?

To explain variation in the level of non-reproductive division of labour in eusocial insects, measured by the number of worker castes present in the colony, multiple hypotheses have been proposed. These explanations can be grouped into one of three broad categories: (i) more worker castes could be favoured in species where there is greater cooperation and less conflict in colonies; (ii) the benefit of having different numbers of castes could depend upon the ecological conditions encountered by a species, with more castes being favoured in certain environments; (iii) various types of constraints, such as developmental or genetic, might limit the proliferation of the number of worker castes. The work contained within this thesis will examine the first two kinds of explanations.

Conflict-based explanations for variation in the number of worker castes

Intra-colony relatedness, via its effect on levels of conflict, is predicted to influence the evolution of the number of worker castes (Oster and Wilson, 1978; Frumhoff and Ward, 1992; Fjerdingstad and Crozier, 2006; Hall, Yi and Goodisman, 2013). However, contrasting hypotheses have been made for its predicted effects. One hypothesis proposes that when colonies are formed by a single monogamous pair, relatedness among colony members will be maximised, which may reduce conflict within the colony and favour the evolution of multiple worker castes (Oster and Wilson, 1978; Frumhoff and Ward, 1992).

In contrast, the opposite prediction could also be made in hymenopteran species, where multiple mating by the queen, via its effect on relatedness, selects for workers to remove (police) the eggs laid by other workers (Ratnieks, 1988). Policing could reduce conflict, leading to an association between the number of castes and low levels of intra-colony relatedness (Fjerdingstad and Crozier, 2006). Tests for the role of relatedness influencing the evolution of the number of worker castes have been limited to a small number of studies in

ants, finding mixed results. Across 35 ant species Fjerdingstad & Crozier, 2006 found that lower levels of relatedness were associated with greater division of labour in the worker caste. In contrast, two alternative studies in ants did not find consistent evidence supporting a relationship between division of labour and either relatedness or queen mating frequency (Ferguson-Gow, 2014; Lecocq de Pletincx, Dellicour and Aron, 2021). However, no study has yet investigated both the possible confounding factors and the underlying causality—whether changes in the level of intra-colony relatedness precede the evolution of multiple worker castes.

Ecological explanations for variation in the number of worker castes

The size-complexity hypothesis is one of the leading explanations for the evolution of increased division of labour, predicting that larger group size selects for increased division of labour (Oster and Wilson, 1978; Bell and Mooers, 1997; Reeve and Jeanne, 2003; Bonner, 2004; Bourke, 2011; Fisher, Shik and Boomsma, 2020; Boomsma, 2022). This prediction arises because groups formed by larger numbers of individuals have more tasks that require doing, and are better able to maintain the optimal ratio of specialised individuals performing each task (Oster and Wilson, 1978; Bourke, 2011; Ulrich *et al.*, 2018; Cooper *et al.*, 2022). Support for the size-complexity hypothesis has been provided by examining division of labour in both individual multicellular organisms and social insects. Across multicellular species, organisms with larger numbers of cells have more cell types (Bell and Mooers, 1997; Bonner, 2004; Fisher, Shik and Boomsma, 2020). Across social insects, species with larger colony sizes have more worker castes (Bonner, 1993; Anderson and McShea, 2001; Dornhaus, Powell and Bengston, 2012; Ferguson-Gow *et al.*, 2014; Wills *et al.*, 2018; Lecocq de Pletincx, Dellicour and Aron, 2021). However, despite these correlations, challenges to the size-complexity hypothesis remain, as it is uncertain whether large group size evolves before

the evolution of increased division of labour. Does the observed correlation reflect larger colony sizes selecting for greater division of labour, or greater division of labour facilitating the evolution of larger colony sizes?

Abiotic environmental conditions may also influence the costs and benefits of possessing different numbers of worker castes, with more castes being favoured in certain environments. For example, a recent study in ants showed that hotter and drier climates were associated with the evolution of worker polymorphism, where workers vary in size and morphology (La Richelière *et al.*, 2022). While a crucial first step in investigating the relationship between climate and division of labour in social insects, there remain several open questions following this study. For example, the analysis did not fully control for the effect of phylogeny. Therefore, it's unclear whether a correlation still exists between climate and non-reproductive division of labour when accounting for the shared ancestry of species. Furthermore, the order of evolutionary events regarding whether hotter and drier climates select for greater division of labour, or whether greater division of labour permits the colonisation of hotter and drier climates, was not investigated. Therefore, it remains uncertain whether division of labour is truly associated with hotter and drier climates and whether climate may indeed be driving changes in the number of worker castes.

Phylogenetic comparative analyses as a means of identifying the determinants of non-reproductive division of labour in social insects

To address each of these specific hypotheses, we need to perform a series of phylogenetic comparative analyses. Species share characteristics through common descent, and so cannot be considered independent data points when performing statistical tests, analogous to the problem of pseudoreplication in experimental studies (Felsenstein, 1985; Harvey and Pagel,

1991). Therefore, accounting for the phylogenetic relationships between species will be crucial to addressing the questions that I have outlined above. Furthermore, analyses of this kind not only permit tests of association between variables over evolutionary time, but they also allow the testing of causal hypotheses by investigating the evolutionary order of events (Garamszegi, 2014). For example, in Chapter 2, I test the hypothesis that larger colony sizes favour the evolution of more worker castes. Performing ancestral state reconstructions for both the ancestral number of worker castes and colony size may lend support to our hypothesis if we determine that increases in colony size occurred before the evolution of more worker castes. However, if multiple worker castes evolved before increases in colony size, we know that our hypothesis is false. Therefore, we can examine whether each of the factors proposed may be a potential causal determinant of non-reproductive division of labour, or a trait that has evolved secondarily.

Ants as a model system to test hypotheses regarding the evolution of non-reproductive division of labour

Ants provide an excellent opportunity to examine the factors responsible for influencing variation in the form of non-reproductive division of labour in eusocial insects. They are a single monophyletic clade in which the number of physical worker castes varies across species from one to four, with approximately 15% of ant genera possessing species with multiple castes (Oster and Wilson, 1978; Hölldobler and Wilson, 1990). Castes are determined during development and are irreversibly fixed for life (Boomsma and Gawne, 2018). I examine the number of physical worker castes as a measure of non-reproductive division of labour as they represent a form of extreme specialisation, involving mutual dependence. There is also considerable data in ants on factors that are predicted to influence the evolution of castes, such as: (i) mating systems, which will influence levels of intra-

colony relatedness (Hughes, Ratnieks and Oldroyd, 2008); (ii) colony size (Hölldobler and Wilson, 1990; Burchill and Moreau, 2016); and (iii) species ranges which can be used to estimate the climatic conditions that species experience (Guénard *et al.*, 2017; La Richelière *et al.*, 2022).

In Chapter 4, I move away from trying to identify the factors responsible for influencing variation in the form that non-reproductive division of labour takes. Instead, I focus on the consequences of division of labour on the molecular evolution of the genome in social insects.

How has division of labour influenced the molecular evolution of the genome in social insects?

The evolution of sterile castes once posed a special challenge for the theory of natural selection (Darwin, 1859). However, kin selection theory addressed the problem of how seemingly non-adaptive traits, such as a sterile helper phenotype, could be favoured by natural selection (Hamilton, 1963, 1964). A gene encoding a cooperative trait may be favoured by kin selection if the recipient of the social behaviour shares the same gene. With the recent explosion in the availability of genomic data it's now possible to look within the genomes of social insects to see if we can detect signatures (footprints) of kin selection, and test Hamilton's rule at the genomic level.

Theoretical work has shed light on the anticipated patterns of sequence diversity between genes under direct selection compared to those influenced indirectly by kin selection (Linksvayer and Wade, 2009, 2016; Hall and Goodisman, 2012). Genes associated with indirect fitness effects are predicted to experience weaker selection compared to genes with

direct fitness effects (Linksvayer and Wade, 2009). This prediction arises because as relatedness diminishes among social partners, the chance that a gene encoding a cooperative trait being shared between them also decreases. Therefore, the benefits of cooperation are more likely to be directed towards individuals lacking the gene for the cooperative trait. The dilution of kin-selected benefits of cooperation decreases the probability of advantageous mutations becoming fixed while increasing the likelihood of deleterious mutations becoming fixed.

Social insects are ideal candidates to test the theoretical predictions regarding the impact of social behaviours on molecular evolution, owing to the presence of reproductive and sterile castes. Division of labour means that queens can obtain direct fitness, while workers may only obtain indirect fitness. Therefore, genes which are differentially expressed in queens are expected to experience predominantly direct selection, while genes that are differentially expressed in workers will largely be subject to indirect selection. As a result, genes with queen-biased expression are predicted to experience relatively stronger selection than genes with worker-biased expression (Linksvayer and Wade, 2009). The relative difference in the strength of selection will depend on the level of relatedness between the workers and their reproductive siblings that they are helping to raise. The results of this analysis may help to disentangle the molecular causes and consequences for the evolution of sterile castes in social insects.

Thesis outline

In Chapters 2 & 3, I explore the factors responsible for influencing variation in the level of non-reproductive division of labour in ants. In Chapter 2, I examine whether colony size, queen mating frequency or the number of reproductive queens per colony influences the

evolution of the number of worker castes. In Chapter 3, I investigate the relationship between climatic conditions—temperature, rainfall, and diurnal temperature range—and non-reproductive division of labour. Finally, in Chapter 4, I investigate the consequences of reproductive division of labour on the molecular evolution of the genome in social insects. Specifically:

Chapter 2—Colony size, queen mating frequency, and the number of reproductive queens per colony have been hypothesised as potential evolutionary determinants of the number of worker castes in ants, but their role has been obscured by the fact that they might also influence each other. I carried out phylogenetically based analyses across 794 ant species, to determine causal relationships for the evolution of multiple worker castes. I found that: (i) larger colony sizes favour increased division of labour, resulting in more worker castes and greater variation in worker size; and (ii) that there is no consistent support for alternative hypotheses regarding either queen mating frequency or number of reproductive queens per colony explaining variation in division of labour. Overall, my results are consistent with the causal hypothesis that large colony size drives the evolution of multiple worker castes and provides strong support for the size-complexity hypothesis.

Chapter 3—In this chapter I examined additional ecological determinants suggested to influence the evolution of non-reproductive division of labour in ants. This work was motivated by a recent study that demonstrated an association between hotter and drier climates with the presence of worker polymorphism in ants (La Richelière *et al.*, 2022). However, the relationship between climate and worker polymorphism was not investigated while fully controlling for the effect of phylogeny, which could substantially impact results. While a key first step, this result is open to multiple possible explanations. Therefore, I

performed a phylogenetic comparative analysis across 474 species of ants. I tested for correlations between colony size, worker polymorphism and climatic conditions and used phylogenetic path analysis to disentangle the potential causal relationships between these variables. When accounting for phylogenetic relationships between species, I found an association between worker polymorphism and hotter and drier climates. When examining the causal relationship between worker polymorphism, colony size and climate, I found that worker polymorphism facilitates the colonisation of hotter and drier climates. However, this result was contingent on the geographical scale over which species were analysed. In some cases, worker polymorphism facilitates the colonisation of hotter and drier climates, while in other cases, I identified no causal relationship. In contrast, larger colony sizes were the only factor consistently associated with the evolution of worker polymorphism across all analyses.

Chapter 4—Division of labour, with reproductive and sterile castes, is a defining feature of eusocial insect species. However, following the transition to eusociality, what consequences has this had on the molecular evolution of the genome in social insects? Using phylogenetic comparative analyses across 8 eusocial insect species, I tested the predictions outlined by kin selection theory that genes with worker biased-expression are under relaxed selection relative to genes with queen-biased expression, in species where queens mate multiple times. I found mixed results in support of the hypothesis. However, the relatively small sample size used may have limited the statistical power available to detect any underlying effects.

Chapter 5—I summarise and discuss the main results presented in Chapters 2-4. I identify areas that merit further exploration.

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Chapter 2: Larger colony sizes favoured the evolution of more worker castes in ants

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Abstract

The size-complexity hypothesis is a leading explanation for the evolution of complex life on earth. It predicts that in lineages that have undergone a major transition in organismality, larger numbers of lower-level subunits select for increased division of labour. Current data from multicellular organisms and social insects supports a positive correlation between the number of cells and number of cell types, and between colony size and the number of castes. However, the implication of these results is unclear, because colony size and number of cells are correlated with other variables which may also influence selection for division of labour, and causality could be in either direction. To resolve this problem, we tested multiple causal hypotheses using data from 794 ant species. We found that larger colony sizes favoured the evolution of increased division of labour, resulting in more worker castes and greater variation

in worker size. In contrast, our results did not provide consistent support for alternative hypotheses regarding either queen mating frequency or number of queens per colony explaining variation in division of labour. Overall, our results provide strong support for the size-complexity hypothesis.

Main

Division of labour has played a pivotal role in the evolution of life on earth, by facilitating evolutionary transitions to more complex organisms[1–4]. In these transitions, individuals cooperate to form a new higher-level individual (organism). For example, cells formed multicellular organisms, with germline and soma, and insects formed complex colonies termed 'superorganisms', with queen and worker castes[3]. Division of labour is fundamental to these transitions because specialisation allows the individuals that form a higher-level organism to perform more diverse functions, and to become more reliant upon each other for reproduction (mutual dependence)[4]. Consequently, a major aim in evolutionary biology is to understand the factors that favour an increased level of division of labour[2,5,6].

The size-complexity hypothesis is one of the leading explanations for the evolution of increased division of labour because of the generality of its explanatory power across different levels of life. In lineages that have undergone a major evolutionary transition, such as to obligate multicellularity or superorganismality, the hypothesis predicts that larger numbers of lower-level individuals (subunits) selects for increased division of labour [1,2,6–9]. This prediction arises because organisms or superorganisms formed by larger numbers of individuals have more tasks that require doing, and are better able to maintain the optimal ratio of specialised individuals performing each task[2,6,10,11]. Support for the size-complexity hypothesis has been provided by examining division of labour in both individual

multicellular organisms and insect colonies. Across multicellular species, organisms with larger numbers of cells have more cell types[7–9]. Across ants, species with larger colony sizes have more worker castes[12–17]. However, despite these correlations, challenges to the size-complexity hypothesis remain.

Firstly, alternative factors have been suggested to explain variation in the level of division of labour. For example, in ants, variation in both queen mating frequency and number of queens per colony has been predicted to influence the evolution of number of worker castes[6,18,19]. When colonies are formed by a single monogamous pair, relatedness among colony members will be maximal, which may reduce conflict within the colony and favour the evolution of multiple worker castes[6,19]. Alternatively, the opposite prediction could also be made in hymenopteran species, where multiple mating by the queen selects for workers to remove (police) the eggs laid by other workers[20]. Policing could reduce conflict, leading to a positive correlation between the number of castes and queen mating frequency[18]. The problem of multiple hypotheses is exasperated by the fact that colony size can be correlated with queen mating frequency[13,14,21]. Consequently, the correlation between colony size and number of castes could just be the result of a relationship caused by variation in queen mating frequency.

Secondly, in superorganismal social insects, the correlation between colony size and number of worker castes is open to alternative causal explanations. For example, while larger colony sizes may favour the evolution of greater division of labour, it could alternatively be that increased division of labour favours the evolution of larger colony sizes. The problem of causal direction becomes even greater when we consider that there are multiple correlated variables. For example, it could be that an increase in colony size favours an increase in

queen mating frequency, and it is an increase in queen mating frequency that favours the evolution of a greater number of worker castes. In this case, colony size and the number of castes would be correlated. However, this pattern would lead to the false impression that larger colony size directly favours increased division of labour and misses the involvement of an intermediate factor. Ultimately, distinguishing between the role of different factors requires phylogenetically based analyses examining the causal direction and order of evolutionary changes[22].

We resolve this problem by performing a series of phylogenetic analyses on the number of physical worker castes in ants. We focus on physical castes which are irreversibly fixed for life and determined during development, rather than behavioural differentiation among workers. This is because extreme specialisation, involving mutual dependence, requires differences in size and morphology. Ants provide an excellent opportunity for testing the size-complexity hypothesis because they are a single monophyletic clade in which the number of worker castes varies across species from one to four, and there is considerable information on factors that could influence the evolution of castes[23–27]. We analysed data from 794 species to examine the influence of three possible factors: colony size, queen mating frequency and number of queens per colony. We performed phylogenetic regressions, to determine how these three traits are correlated with the number of castes and each other. This step provided an overview that helped to guide further causal analyses and allowed comparison with previous studies. We then examined the likely causal relationships between number of worker castes, queen mating frequency and colony size with three methods: (i) phylogenetic path analysis[28]; (ii) transition rate analysis between pairs of traits[29]; and (iii) ancestral state reconstruction[30]. These analyses allowed us to tease apart the different roles of each variable. Lastly, to assess the robustness of our results, we also tested if the same

patterns emerged when examining an alternative possible measure of division of labour – variation in worker size[12,13,18,31].

Results

Summary of ant social traits

Across different ant species, there was considerable variation in the characteristics of a colony. The number of discrete physical worker castes varied from one to four, although 76% of species had only one worker caste (Fig. 1a). Ancestral state reconstruction suggested that polymorphic workers, with more than one caste, had evolved from monomorphic ancestors at least 27 times (Fig. 2 and Supplementary Table 1). Colony sizes varied from 7 to 14,750,000, with a median of 300 (Fig. 1b). The effective number of males that inseminated each queen varied from 1-26, with 31% of species mating with a single male, 36% mating with between 1-2 males and 33% mating with more than 2 males. The median value for queen mating frequency was 1.1 mates (Fig. 1c). The number of queens per colony varied from 1-70, with 63% of species possessing a single queen, 18% possessing between 1-2 queens and 19% possessing more than 2 queens. The median number of queens per colony was 1 (Fig. 1d). The dataset was not complete for all traits for each species, and so for each analysis we used the maximum amount of data available. Therefore, sample sizes vary between analyses depending on the variables being analysed, as detailed within each section.

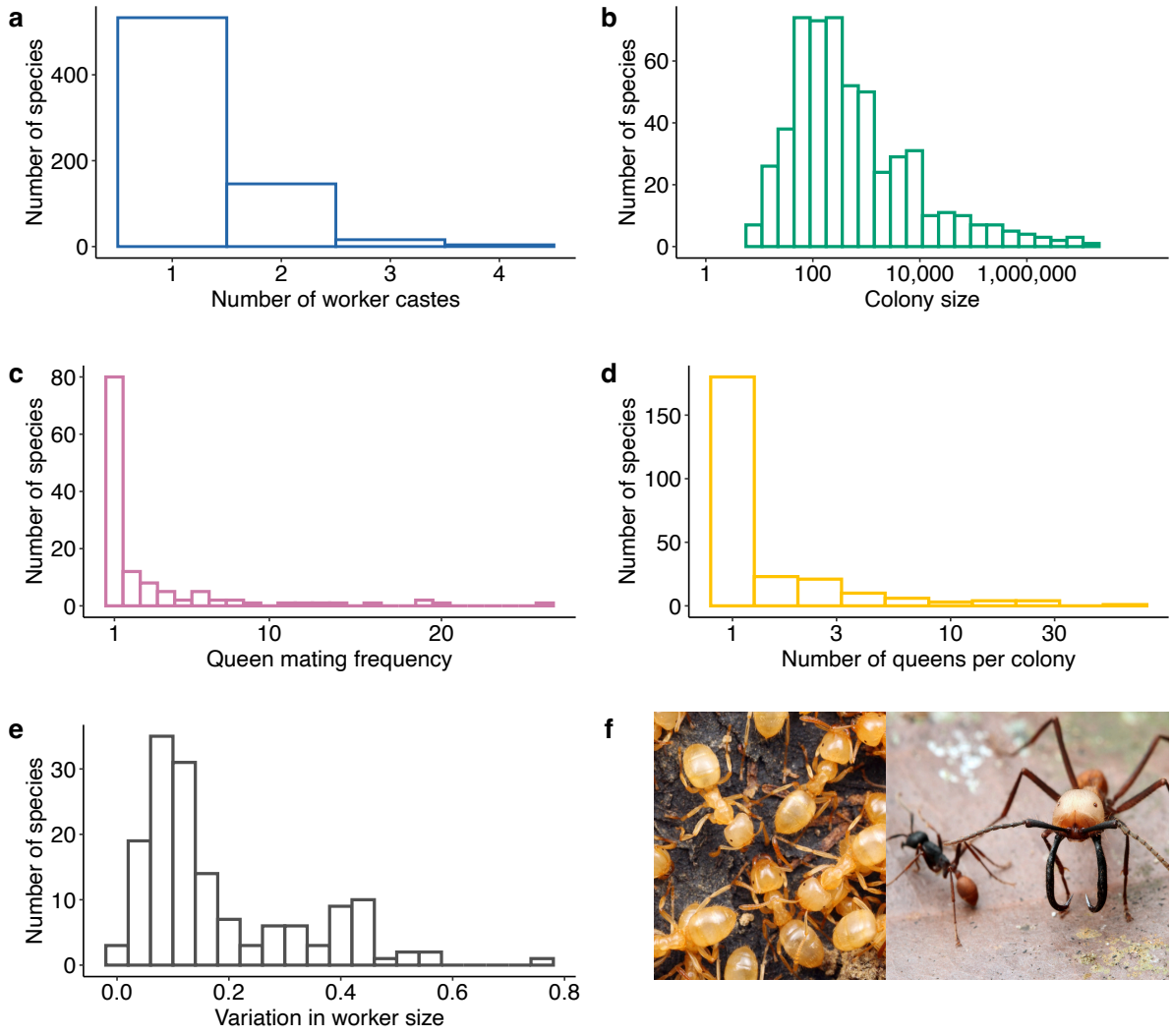


Figure 1. Distribution of colony characteristics across ant species. (a) Number of worker castes ($n_{species}$=699). (b) Colony size ($n_{species}$=541). (c) Queen mating frequency ($n_{species}$=126). (d) Number of queens per colony ($n_{species}$=252). (e) Variation in worker size quantified using the coefficient of variation for worker head width ($n_{species}$=152). (f) Ants vary in their number of physical worker castes. The left image displays *Lasius claviger*, a species characterized by having just a single physical worker caste. The right image displays two distinct physical worker castes of the same species, *Eciton burchelli*, representing one of the most extreme cases of morphological caste variation (photo © Alex Wild). Axes for colony size and number of queens per colony are plotted on a $\log_{10}$ scale.

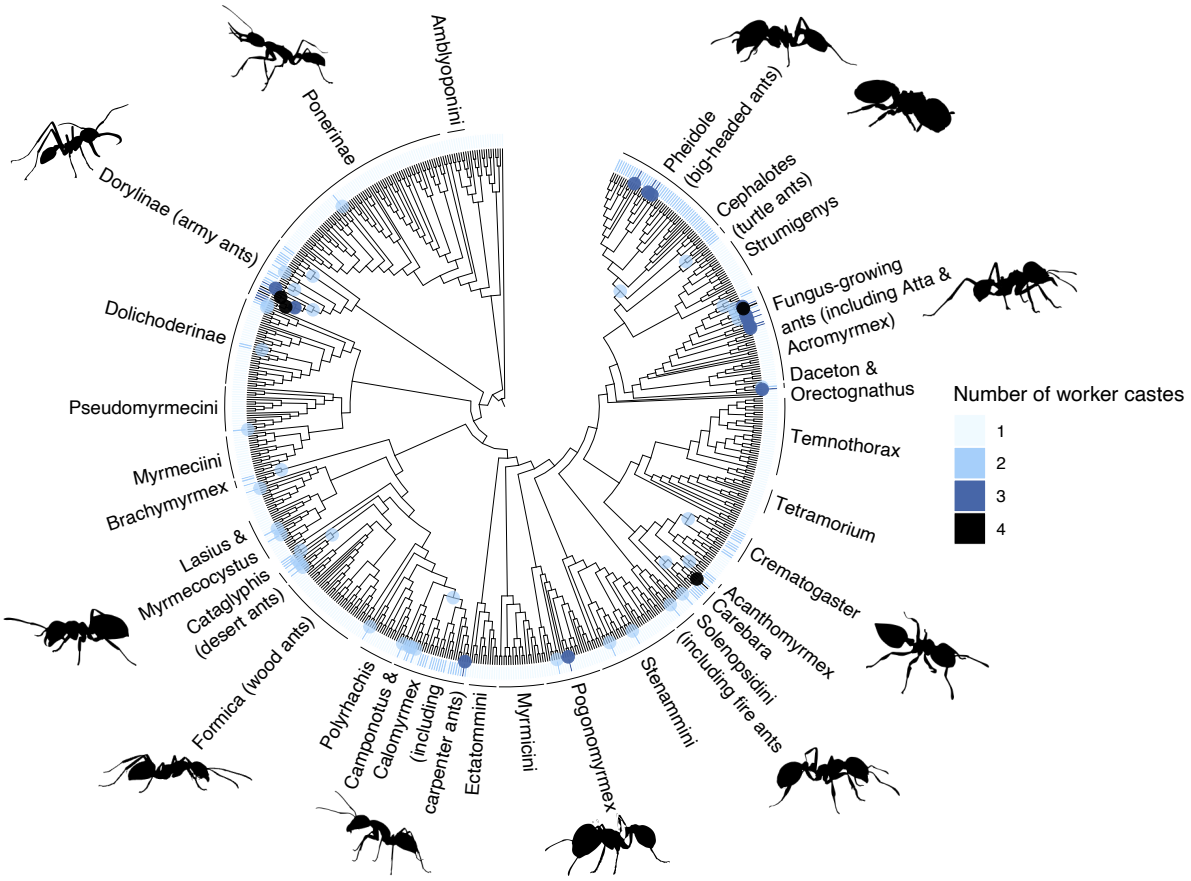


Figure 2. Evolutionary origins of multiple worker castes (worker polymorphism). The ancestral state at the root of the ant phylogeny is a single physical worker caste. Coloured circles represent evolutionary increases in number of worker castes, with colour corresponding to the number of castes that have evolved. The number of worker castes present in each species is also displayed around the outside of the phylogeny. Branch lengths are not to scale. Ant phylogenetic lineages, of varying levels, are displayed in black text around the outside of the phylogeny. Analyses were repeated over 400 phylogenetic trees, but we display the results of a single representative tree.

Phylogenetic correlations

We used Bayesian phylogenetic mixed effect models (BPMMs) to test for correlations between the different ant traits. We found that colony size and queen mating frequency were both positively correlated with number of worker castes (Fig. 3a and b; BPMMs: colony size:

$\beta = 0.17$, credible interval (CI) = 0.11 to 0.24, $n_{species} = 436$; queen mating frequency: $\beta = 0.47$, CI = 0.04 to 0.91, $n_{species} = 104$). In contrast, we found no relationship between number of queens per colony and number of worker castes (Fig. 3c; BPMM: $\beta = -0.14$, CI = -0.56 to 0.30, $n_{species} = 192$).

We also found that species with higher queen mating frequencies had larger colony sizes (Fig. 3e; BPMM: $\beta = 1.25$, CI = 0.50 to 2.03, $n_{species} = 109$, $R^2 = 0.08$). The significant correlations between queen mating frequency, colony size and number of worker castes emphasises the need to carry out analyses that can examine the underlying causality between these variables. In contrast, we found no relationship between number of queens per colony and colony size (Fig. 3f; BPMM: $\beta = 0.07$, CI = -0.37 to 0.49, $n_{species} = 195$, $R^2 = 0.00$). However, we did find a negative relationship, which was close to significance, between number of queens per colony and queen mating frequency (Fig. 3d; BPMM: $\beta = -0.20$, CI = -0.39 to 0.01, $n_{species} = 111$, $R^2 = 0.00$). Based on the results of our correlational analysis, we excluded the number of queens per colony from our causality analysis, except for a potential relationship between number of queens per colony and queen mating frequency, where a negative relationship was also observed previously[25].

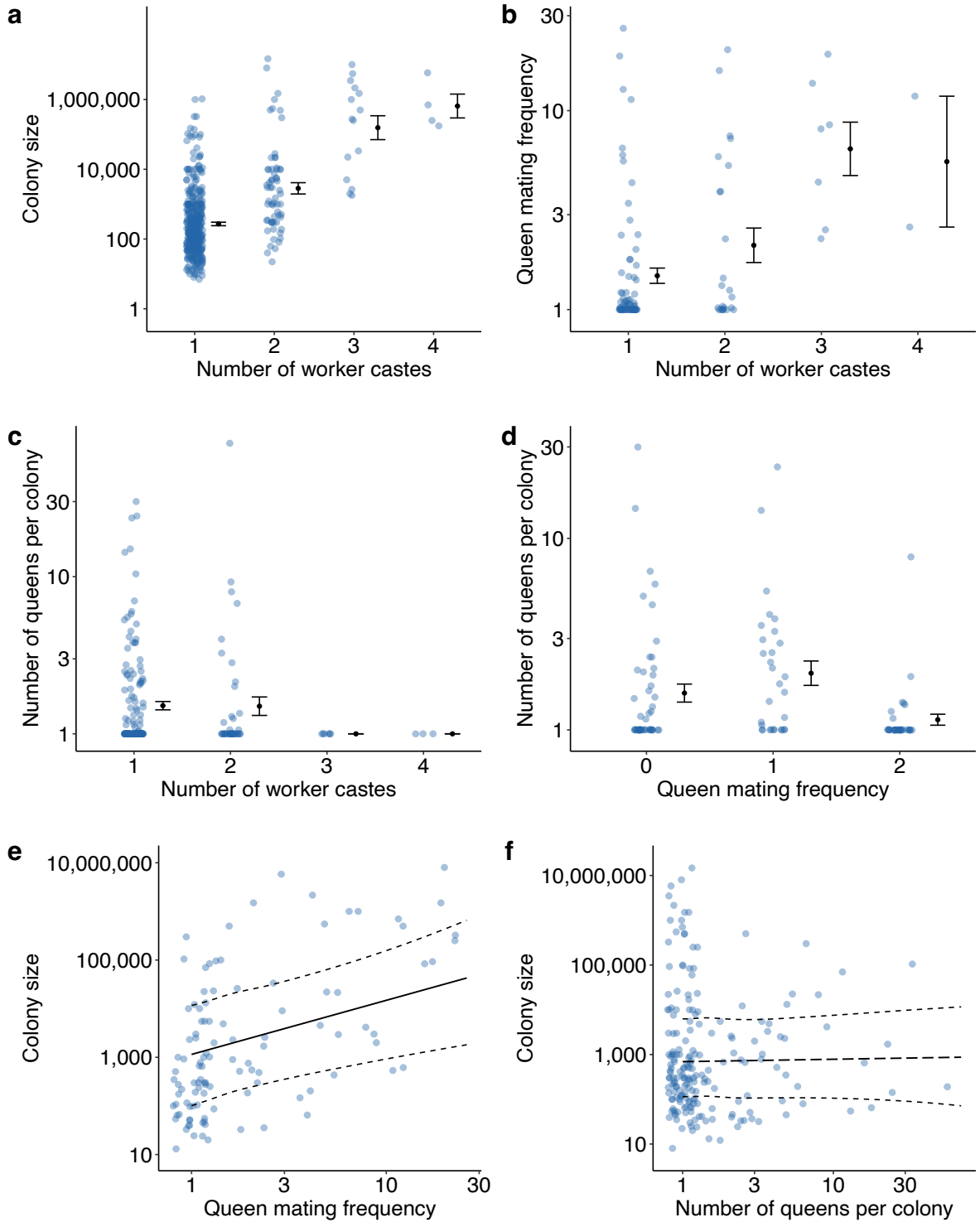


Figure 3. Relationships between colony size, queen mating frequency and number of queens per colony, both among themselves, and with number of worker castes. Species with larger colony sizes (a, $n_{species}$=436) and higher queen mating frequencies (b, $n_{species}$=104) had significantly more worker castes. We did not find a significant association between

number of queens per colony and either number of worker castes (c, $n_{species}$=192), queen mating frequency (d, $n_{species}$=111) or colony size (f, $n_{species}$=195). Species with higher queen mating frequencies had significantly larger colony sizes (e, $n_{species}$=109). (a-d) Data show mean ± standard error. (e-f) Fitted lines are mean regression slopes with 95% CIs from BPMMs using a single phylogenetic tree. Solid regression lines represent significant relationships, while dashed regression lines represent non-significant relationships. Dots represent species averages. Axes for colony size, queen mating frequency, and number of queens per colony are plotted on a $\log_{10}$ scale.

Causality Analyses

We then used three different methods to examine the causal relationships that underly these correlations: (i) phylogenetic path analysis, (ii) transition rate analysis and (iii) ancestral state reconstruction. These forms of analysis required number of worker castes to be modelled as a binary variable and so we classified species as possessing either a single (monomorphic) or multiple (>1: polymorphic) worker castes.

Phylogenetic path analysis

We used phylogenetic path analysis to test four alternative causal models of the relationships between: number of worker castes, colony size, queen mating frequency and number of queens per colony (Extended Data Fig. 1). We found strong evidence in favour of the size-complexity hypothesis. The analysis supported a single causal model, where large colony sizes favoured the evolution of multiple worker castes, and size was the only variable that directly did so (Fig. 4, Extended Data Fig. 2 and Supplementary Tables 2-3; ω (relative weight of support for a model within a set of competing models) = 0.67, $n_{species}$ = 94). We found no support for alternative causal models where colony size is influenced by number of

worker castes or where number of worker castes is influenced by queen mating frequency (Supplementary Table 2; $n_{species} = 94$).

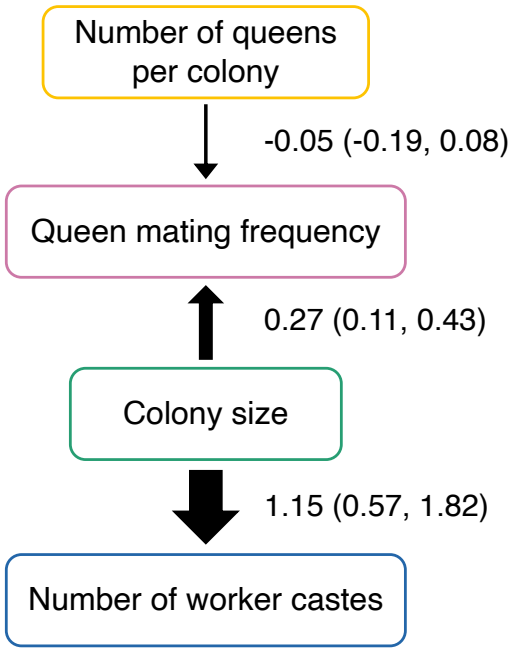


Figure 4. The best-supported causal model identified by phylogenetic path analysis. Larger colony sizes facilitated the evolution of multiple worker castes. We display the results from the analysis of one maximum clade credibility (MCC) consensus tree (Supplementary Tables 2-3). However, we repeated the analyses on three additional MCC trees, and in each case, found that the conclusions drawn remained unchanged due to the similarity in the presence, direction and magnitude of the causal relationships identified (Extended Data Fig. 2 and Supplementary Tables 2-3). Values represent standardised path coefficients with 95% confidence intervals (path coefficients describe the strength and direction of the relationship between two variables in terms of their correlated variance, after accounting for the effects of other variables in the model). Arrows indicate the direction of the relationship between variables, with heavier lines indicating larger coefficients. Number of worker castes was modelled as a discrete binary variable while colony size, queen mating frequency and number of queens per colony were modelled as continuous variables.

Transition rate analysis

We used transition rate analysis as a second method to test the hypothesised causal relationships between number of worker castes and both colony size and queen mating frequency. This method allowed us to test whether the evolution of two traits is correlated, as well as the underlying causal direction, but required all traits to be modelled as discrete binary variables. We classified species as having small or large colony sizes depending upon whether colony size was smaller (≤ 300) or larger (>300) than the median colony size. We classified species as having low or high mating frequencies depending on whether queens mated more (>2) or less than or equal to (≤2) two times.

We found very strong support for the size-complexity hypothesis, with correlated evolution between colony size and number of worker castes (Supplementary Table 4; Bayes Factor (BF) = 20.83, $n_{species}$= 436; BFs quantify the relative support for two competing models where BF > 2 offers positive evidence, > 5 provides strong evidence, and > 10 very strong evidence that the more complex model performs better than the simpler model[29]). Transitions to multiple castes only occurred in species with large colony sizes (Fig. 5). In species with only a single worker caste, transition rates from small colony sizes to large colony sizes were slightly elevated in comparison to species with multiple castes (Fig. 5). In contrast, we found that variation in queen mating frequency does not influence the evolution of number of worker castes, as queen mating frequency evolved independently of the number of worker castes (Supplementary Table 5; queen mating frequency and worker castes: BF = -1.39, $n_{species}$= 104).

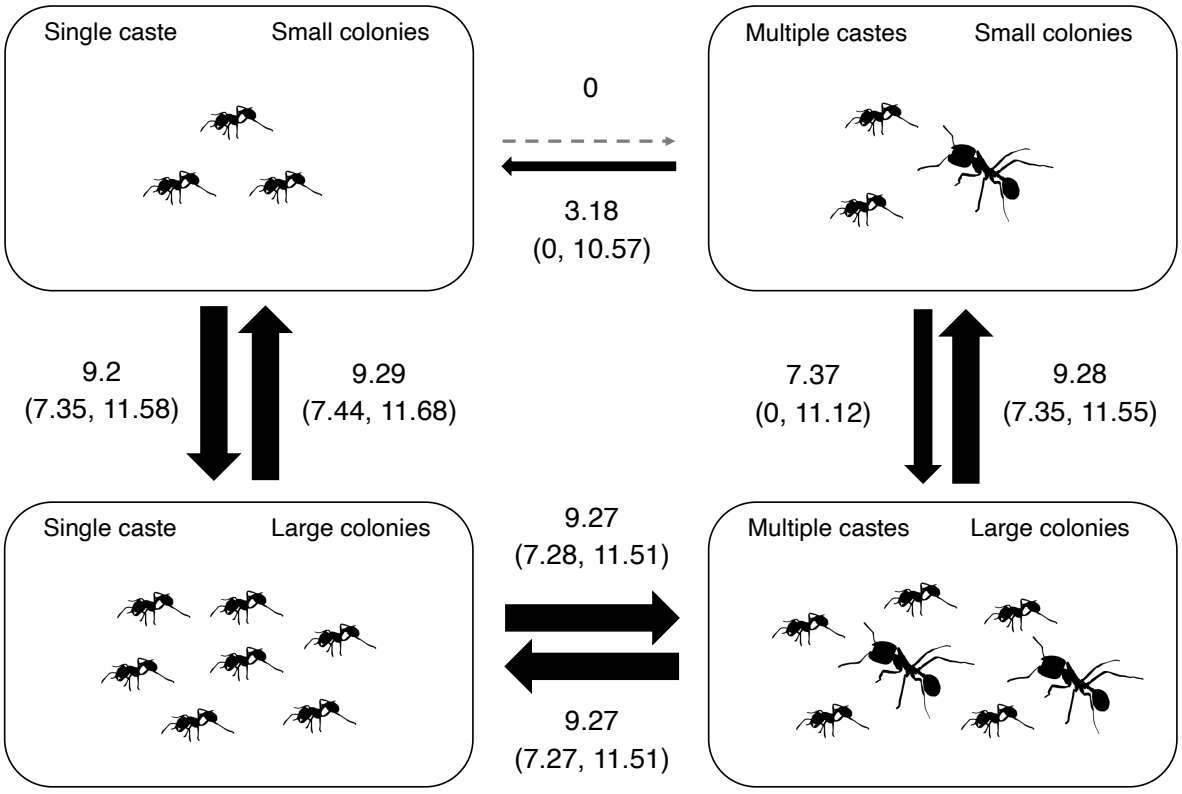


Figure 5. Evolutionary transitions between number of worker castes and colony size. Multiple worker castes only evolved in lineages with large colony sizes. Values represent the inferred transition rates between number of worker castes and colony size, accompanied by 95% credible intervals ($n_{species}$= 436). Transition rates were estimated using rjMCMC implemented in BayesTraits. Each possible state is shown as a box, and the direction of transitions between states are shown with arrows with higher rates of transition indicated by a heavier line. Silhouette images from phylopic, courtesy of Mattia Menchetti (large worker) and Richard Harris (small worker).

Ancestral state reconstruction

We used ancestral state reconstruction as a third method to examine the underlying causal relationships, and again found support for the size-complexity hypothesis. We found that the ancestors of species that evolved multiple worker castes had colony sizes that were more than 3.5 times larger compared to the ancestors of species that retained one worker

caste (Extended Data Fig. 3a; BPMM: ancestral colony size in species with one worker caste: β =281.67, CI = 40.04 to 1590.90; ancestral colony size in species with multiple worker castes: β = 1025.05, CI = 137.17 to 9064.82; $P_{single\ vs\ multiple} < 0.01$, $n_{species}$= 436). In contrast, queen meeting frequency did not differ significantly between ancestors that evolved multiple worker castes and those that did not (Extended Data Fig. 3b; BPMM: ancestral queen mating frequency in species with one worker castes: β = 1.99, CI = 0.95 to 3.90; ancestral queen mating frequency in species with multiple worker castes: β = 2.46, CI = 1.10 to 5.55; $P_{single\ vs\ multiple}$ = 0.16, $n_{species}$= 104). The phylogenetic signal present in both colony size and queen mating frequency across ants allowed us to estimate ancestral values relatively accurately in these traits (Supplementary Table 6; BPMMs: phylogenetic heritability of colony size = 72.61%, CI = 59.67% to 84.02%; phylogenetic heritability of queen mating frequency = 88.94%, CI = 74.01% to 99.86%).

Division of labour and worker size

Taken together, our analyses on the number of worker castes provide strong support for the size-complexity hypothesis. Larger colony sizes correlate with and appear to lead to the evolution of more worker castes (Figs. 3-5). However, most ant species do not possess more than one physical worker caste. Despite this, there can be considerable variation in worker size within castes, and there is some evidence that different-sized workers perform different roles even within ant species that have a single physical worker caste[32]. Consequently, an alternative method for testing the size-complexity hypothesis would be to examine the relationship between colony size and variation in worker size, rather than number of castes, as a measure of non-reproductive division of labour[12,13,18,31].

Using data obtained from AntWeb, we examined variation in the head width measurements of 1064 workers from 152 species and found mixed support for the size-complexity hypothesis (Extended Data Fig. 4). We found a positive correlation between colony size and variation in worker size, and that larger colony sizes may favour the evolution of greater variation in worker size (Fig. 6, Extended Data Fig. 5 and Supplementary Tables 7-8; BPMM: colony size: $\beta = 0.05$, CI = 0.03 to 0.08, $n_{species} = 122$, $R^2 = 0.14$; Path analysis: $n_{species} = 94$). However, there was some uncertainty regarding the causal direction of the relationship between colony size and variation in worker size. Among the three supported causal models identified by path analysis, larger colony sizes favoured the evolution of greater variation in worker size in two cases, and greater variation in worker size favoured the evolution of larger colony sizes in the third case (models within 2 CICc units of the best model were considered to have equal support; Supplementary Table 7; combined weight for the models supporting the hypothesis that variation in worker size is influenced by colony size: $\omega = 0.55$). Due to the uncertainty regarding the best-supported model, the confidence intervals for the path coefficients in the average model, linking both colony size and queen mating frequency with variation in worker size, were relatively large and overlapped with zero (Fig. 6b). When examining species with only a single worker caste, we found the contrasting result that greater variation in worker size favours the evolution of larger colony sizes. (Fig. 6a, Extended Data Fig. 6, and Supplementary Tables 9-10; BPMM: $\beta = 0.04$, CI = 0.01 to 0.07, $n_{species} = 84$, $R^2 = 0.08$; Path analysis: $\omega = 0.75$, $n_{species} = 60$).

In contrast, we did not find support for the hypothesis that variation in worker size is influenced by queen mating frequency. This was the case when analysing either all species, or just those with a single worker caste (Fig. 6b, Extended Data Figs. 5-7, and Supplementary Tables 7-11).

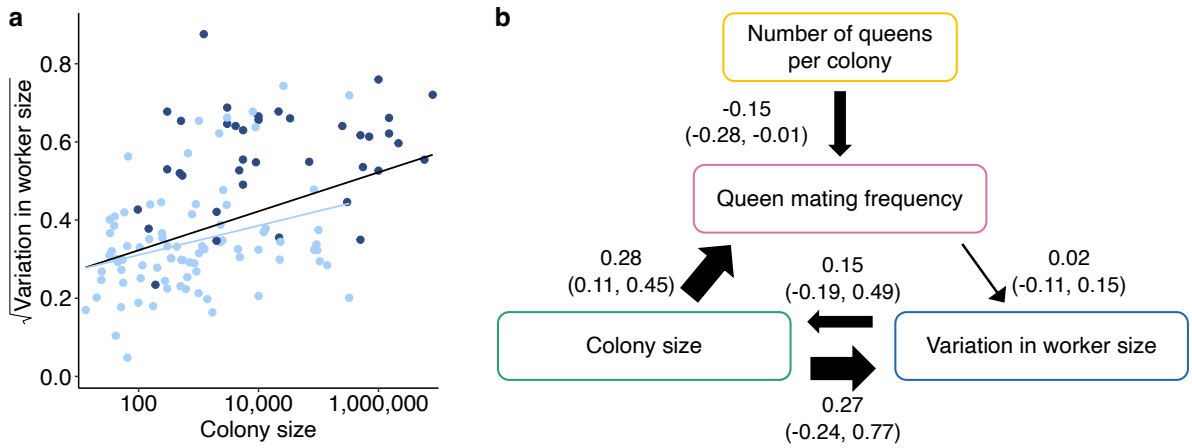


Figure 6. Larger colony sizes may favour the evolution of greater variation in worker size. (a) Species with larger colony sizes possess significantly greater variation in worker size when analysing either: (i) all species for which data were available (all dots and regression line in black, $n_{species}$=122); or (ii) only species which possess a single worker caste (dots and regression line in light blue, $n_{species}$ = 84). Fitted lines represent mean regression slopes from BPMMs. Dots represent species averages. Colony size is plotted on a $\log_{10}$ scale while variation in worker size is on a square root scale. (b) Averaged model from the three supported path analysis models (Supplementary Tables 7-8). Larger colony sizes may favour the evolution of greater variation in worker size, but opposite causation remains possible. Analysis performed using all species where data were available for variation in worker size, colony size, number of queens per colony and queen mating frequency ($n_{species}$ = 94). We display the results from the analysis of one maximum clade credibility (MCC) consensus tree. We repeated the analyses on three additional MCC trees, and in each case, found that the conclusions drawn remained unchanged due to the similarity in the presence, direction and magnitude of the causal relationships identified (Extended Data Fig. 5 and Supplementary Tables 7-8). Values represent standardised path coefficients with 95% confidence intervals. Arrows indicate the direction of the relationship between variables, with heavier lines

indicating larger coefficients. Variation in worker size, colony size, queen mating frequency and number of queens per colony were modelled as continuous variables.

Sensitivity analyses

We examined whether our results were robust to alternative methods of quantifying variation in queen mating frequency and number of queens per colony as categorical rather than continuous variables[21,25,33]. Detailed statistics for each analysis are presented in the supplementary material (Extended Data Figs. 8-10 and Supplementary Tables 11-19). In all cases, the size-complexity hypothesis was still supported, but some of the other relationships depended upon how data were categorised. Our conclusions regarding the phylogenetic correlations were robust, except for the finding that the number of worker castes did not significantly differ between singly-mated and facultatively multiply-mated species (Supplementary Table 19). The conclusions of our causal analyses were also robust, except when analysing the number of discrete worker castes using phylogenetic path analysis. We identified a single supported model where the evolution of multiple worker castes was favoured by both larger colony sizes and obligate multiple mating by queens. However, the confidence interval for the path coefficient associated with queen mating frequency overlapped with zero (Extended Data Fig. 8 and Supplementary Tables 12-13).

Discussion

We found strong and consistent support for the size-complexity hypothesis, with larger colony sizes appearing to favour the evolution of multiple worker castes (Figs. 3-6). In contrast, we did not find consistent support for the hypothesis that multiple mating favours the evolution of multiple worker castes (Figs. 3-4 and 6b). We found no evidence to suggest a

direct relationship between the evolution of number of worker castes and number of queens per colony (Fig. 3c). Our conclusions were robust to different analysis methods.

Our results suggest that larger colony sizes favour the evolution of multiple worker castes in ants. Previous studies had highlighted a positive correlation between increased division of labour and both cell number in multicellular organisms, and colony size in social insects, but these results were open to multiple explanations[7–9,12–14,18,21]. Our results reveal a distinct evolutionary pattern where larger colony sizes tend to evolve before multiple worker castes (Figs. 4-5). There are at least two reasons why larger colonies may promote the evolution of multiple castes. Firstly, as colony size increases, the number of tasks that need to be performed also increases[2]. For example, species with larger colonies may experience greater logistical challenges in transporting food or waste within or outside the colony. Secondly, species with larger colonies are better able to maintain the optimal ratio of the different castes [10,34]. Consequently, these species face a lower risk of losing essential worker functions if workers are lost.

Our analysis also suggests that larger colony sizes favoured the evolution of greater variation in worker size (Fig. 6). However, there is some uncertainty regarding the direction of this relationship, as causality could potentially be reversed. In addition, we found that this result only applied when analysing across species with variable numbers of physical worker castes. When analysing variation in worker size exclusively in species with a single caste, we found that greater variation in worker size preceded the evolution of larger colony sizes. Consequently, while our analyses of variation in worker size support the size-complexity hypothesis, they suggest that colony size is not responsible for the evolution of variation in worker size in species that lack discrete physical worker castes.

We did not find consistent support for the hypothesis that multiple mating favours the evolution of multiple worker castes. There is a positive correlation between both number of worker castes and variation in worker size with queen mating frequency, regardless of whether it's analysed as a continuous or categorical trait (Fig. 3b). However, when analysing the causal relationship between these variables, we found that higher mating frequency was not consistently associated with evolutionary transitions from single to multiple worker castes (Figs. 4 and 6b, Extended Data Figs. 8-9, and Supplementary Tables 5 and 18). This finding is consistent with the interpretation that queen mating frequency is positively correlated with number of worker castes due to both variables being positively associated with colony size (Figs. 3-4). Colony size and queen mating frequency may be positively correlated due to the advantages of increased genetic diversity that result from multiple mating[21,35–41]. If species with larger colony sizes are at greater risk of infection from pathogens, for example resulting from increased traffic of foragers into the nest, they may benefit more from the protection offered against disease by increased genetic diversity[40–42].

Excluding ants, possessing more than one physical worker caste is extremely rare in the social Hymenoptera. Multiple worker castes appear to be entirely absent in vespine wasps, while in bees, the presence of two castes has only been identified in some stingless bee species[31,43]. For example, *Tetragonisca angustula* possesses two castes – soldiers are both approximately 30% heavier than foragers and have a different morphology[43]. This contrasts with the much larger variation in ants – for example, major workers in *Atta colombica* leafcutter ants can be 60 times heavier than the smallest workers[44]. Why does the causal link between colony size and number of castes that we have identified in ants, seemingly not apply in superorganismal species of bees and wasps[31]? Multiple hypotheses have been

proposed, suggesting that factors such as the presence of a powerful sting or body size constraints imposed by having winged workers could have impeded the evolution of multiple worker castes in bees and wasps[6,24,45]. However, the phylogenetic power available to carry out statistical tests of these hypotheses is low. Other ways to test the size-complexity hypothesis include examining behavioural variation in bees and wasps, or the physical caste variation in termites[6,31,46–48].

To conclude, our results provide strong support for the size-complexity hypothesis in ants, suggesting that larger colony sizes have favoured the evolution of greater division of labour (more castes and greater variation in size). The generality of this hypothesis could be tested by carrying out similar analyses on other forms of division of labour, such as the number of cell types in multicellular organisms[9,49,50], or the difference between queens and workers in superorganismal social insects[48,51].

Methods

To test whether colony size, queen mating frequency or number of queens per colony explain variation in non-reproductive division of labour in ants, we performed a large-scale phylogenetic comparative analysis. We first tested which factors correlate with number of worker castes and then used three different methods to examine the causal relationships that underly these correlations: phylogenetic path analysis, transition rate analysis and ancestral state reconstruction. We examined causality in these three different ways as it also allowed us to test that our results are robust to different methods of analysis. We also examined whether our results were robust to alternative methods of quantifying variation in queen mating frequency and number of queens per colony. All the species used in the analysis are listed in the supplementary materials and we follow the taxonomic classification of ants listed in

Bolton's Catalogue available from AntCat.org (Supplementary Table 20)[52]. When analysed as continuous variables, colony size, queen mating frequency and number of queens per colony were $\log_{10}$ transformed for all analyses while variation in worker size was square root transformed.

Data collection

We collected data on five ant traits: (i) the number of discrete physical worker castes; (ii) colony size (number of workers in mature colonies); (iii) effective queen mating frequency[21,53] (estimated number of mates weighted by the proportion of offspring sired by each male; referred to as 'queen mating frequency'); (iv) observed number of queens per colony; and (v) variation in worker size. We started by gathering data from major reviews, comparative studies, and books, and then performed a topic search of the primary literature using Web of Science[24–27]. We also incorporated data collected by the Global Ant Genomics Alliance (GAGA) consortium[54]. In total, we collected data for 794 species from 160 different genera.

We collected data on queen mating frequency and number of queens per colony by searching papers published between 1 January 2008 and 2 May 2020. Among these papers, we included data from Hughes et al., 2008[25], which summarises the available data on these variables in the eusocial Hymenoptera. We assumed that all relevant data published before 1 January 2008 had been captured by Hughes et al., 2008. We used the following keywords: "Ant" AND (monandr* OR monogyn* OR polyandr* OR polygyn* OR effective-mating-frequenc* OR mating-frequenc* OR paternity-frequenc* OR mating-system* OR sociogenetic-structure*). We chose to use estimates of effective mating frequency, rather than number of copulations, because it more accurately reflects the genetic relatedness among workers in a

colony, considering the potential influence of biased sperm use by queens that have mated multiple times[21,55]. When multiple estimates of queen mating frequency were available, we calculated species-level averages for queen mating frequency using the harmonic mean. We used the harmonic mean because when mating frequency is low, increases in mating frequency will have greater effects on intra-colony relatedness. Therefore, harmonic means, which assign greater weight to small numbers, better represent the genetic effects of multiple mating on colony-wide relatedness than arithmetic means[56]. We collected data on the average observed number of queens per colony, rather than the effective number of queens per colony[57] (the estimated number of reproductive queens weighted by the respective contribution of each queen to the production of offspring) because this was the only available type of continuous data with a sufficient sample size for a large-scale phylogenetic comparative analysis. When multiple estimates of number of queens per colony were available, we calculated species-level averages. For queen mating frequency and number of queens per colony, we also collected categorical data with three factor levels for each trait from the papers identified in our literature search. These factor levels include obligate single mating (monandry), facultative multiple mating (facultative polyandry) where some monandrous queens still occur in the population and obligate multiple mating (obligate polyandry) where mature colonies are never monandrous. Similarly, for number of queens per colony, these levels were obligate single queen (monogyny), facultative multiple queens (facultative polygyny), and obligate multiple queens (obligate polygyny).

Species were classed as having 1-4 physical worker castes based on the following criteria: Species were classed as having a single worker caste if they showed limited variation in size or monophasic allometric scaling. Species were classified as having more than one caste if there was variation in body size with non-allometric scaling, where the number of castes

depended on the number of different scaling relationships between body parts. When morphometric data were absent, the number of castes was determined using direct estimates provided in the literature. We searched for published data up to and including the 13 July 2020 and we used the following keywords: "Ant" AND (worker polymorph* OR worker monomorph* OR (morphometric AND worker* AND caste*) OR subcaste* OR sub-caste* OR worker dimorph* OR major-worker* OR minor-worker* OR worker AND allometr*). We then performed two additional searches to collect missing data for number of worker castes. First, we performed a species-specific literature search for instances where data on queen mating frequency or number of queens per colony was available for a given species, but number of castes was not. Our search query included the species name along with the terms '(worker polymorph* OR worker monomorph* OR (morphometric AND worker* AND caste*) OR subcaste* OR sub-caste* OR worker dimorph* OR major-worker* OR minor-worker* OR worker AND allometr* OR soldier* OR replete*)'. Second, we performed a search at the genus level for genera where no caste data had been collected. The search query employed the same terms as mentioned above, in combination with the genus name.

To collect data on colony size, we performed a literature search focussing on species for which we already possessed data on queen mating frequency or number of queens per colony. Using Google Scholar, we searched for the following keywords in combination with the species name 'colony size OR colony collection OR worker number'. We adopted this approach based on the methodology outlined in Dornhaus et al., 2012[14]. Google scholar was chosen as it indexes the entire text of research papers. Notably, details regarding colony size are often not the primary focus of papers, resulting in brief mentions within the methods or results sections. Therefore, it is less likely to be recovered by search engines that rely solely

on keywords, titles, and abstracts to retrieve such information. When multiple measurements existed for a given species, we calculated arithmetic means weighted by sample size.

We collected data on variation in worker size by measuring the width of ant heads[12,13,18,31]. We quantified the relative variation in worker size for each species by calculating the coefficient of variation (CV), which is achieved by dividing the standard deviation of worker head width by its mean value ($CV_{worker\ size} = \frac{worker\ head\ width\ standard\ deviation}{worker\ head\ width\ mean}$). To obtain images of the species for which we already possessed data on colony size and queen mating frequency, we searched for scaled images on the AntWeb online database (antweb.org)[58]. We downloaded front-view images of the ants' heads and measured at the widest point excluding the eyes using the image-processing software ImageJ v1.53i[59]. In total, we measured 1,064 worker ant heads (Extended Data Fig. 4). The number of workers measured per species ranged from 2-33, with a mean of 7.

We excluded 112 species from our dataset for the analysis based on their distinct life history traits. These traits represent evolutionarily derived elaborations of the ancestral full-sibling-colony state at the transition to superorganismal colonies at the root of the ant clade. They included (i) species that formed supercolonies (vast networks of connected nests); (ii) were social parasites, lacking some or all of the worker castes (although temporary social parasites which only establish new colonies with the assistance of a host species were included in the analysis); (iii) that can reproduce parthenogenetically to produce queens and/or workers; (iv) that reproduce via gamergates (mated workers that reproduce sexually) following the evolutionary loss of the original queen caste; or (v) that use interlineage hybridisation for genetic caste determination (Supplementary Table 21). We gathered the information from papers that had summarised the available data on these five traits[60–64]. We excluded these

species from the analysis as they represent secondary reductions of complexity in social organisation and are likely experiencing different selection pressures for either the evolution of worker castes, colony size, queen mating frequency or number of queens per colony (Supplementary Table 22). This decision to remove species characterised by evolutionarily derived social systems improved our ability to detect which variables could influence the evolution of the number of worker castes.

Phylogeny

To control for shared evolutionary history in our comparative analyses, we used a posterior sample of 400 phylogenetic trees produced by Economo et al., 2018 which included 14,594 species and of which 731 overlapped with the species in our dataset[65]. However, since time-calibrated, molecular phylogenies are only available for ants at the genus level, the species topology within genera varied widely across the sample of 400 trees. Therefore, we took steps to account for phylogenetic uncertainty in each of our analyses (see each section below for details).

Statistical analyses

All analyses were performed in R v4.2.2 apart from transition rate models that were conducted in Bayestraits V4[29,66].

Bayesian phylogenetic models.

We fitted BPMMs with Markov chain Monte Carlo (MCMC) estimation using the MCMCglmm package v2.34[67]. Models were run for a minimum of 1,100,000 iterations, with a burn-in of 100,000 and thinning interval of 1,000. However, models were run for as long as necessary to obtain posterior effective sample sizes of at least 300 for all parameters. Overall,

the majority of estimates had an effective sample size of at least 1,000. To ensure model convergence, we used the coda package v0.19-4 to calculate the degree of autocorrelation between successive iterations in each chain. We fitted each model independently two times and used Gelman and Rubin's convergence test to compare within- and between-chain variance[68,69]. We modelled the number of worker castes as a discrete variable for our regression analyses and used a Poisson error distribution with log link function. We modelled colony size, queen mating frequency and number of queens per colony as Gaussian traits. The prior settings used for each analysis are specified in the supplementary R code. To select priors for random effects, we first checked model convergence using inverse-Wishart priors ($V = 1$, $\nu = 0.002$). However, in situations where the MCMC chain exhibited poor mixing properties, particularly in cases involving discrete response variables, we examined two different parameter expanded priors: the Fisher prior ($V = 1$, $\nu = 1$, $\alpha.\mu = 0$, $\alpha.V = 1{,}000$) and the χ^2 prior ($V = 1$, $\nu = 1{,}000$, $\alpha.\mu = 0$, $\alpha.V = 1$). We specified an inverse-Wishart prior for residual variances ($V = 1$, $\nu = 0.002$)[70]. For fixed effects, we used the default priors in MCMCglmm. We report parameter estimates from models as posterior modes along with 95% lower and upper credible intervals (CIs). For BPMMs with a Gaussian error distribution, we also calculated marginal R^2 values and report the median value for each analysis from a distribution of R^2 values estimated across a sample of 400 trees[71]. The reported P values, which assess differences between levels, such as species with a single worker caste vs species with multiple worker castes, represent the number of iterations where one level is greater than the other level divided by the total number of iterations.

Correlational analyses

Estimating phylogenetic correlations using MCMCglmm.

We used Bayesian phylogenetic mixed effect models (BPMMs) to test for correlations between four traits: number of worker castes, colony size, queen mating frequency and number of queens per colony. Additionally, we investigated the correlations between variation in worker size and colony size, queen mating frequency and number of queens per colony. When analysing variation in worker size, our analysis was split into two parts. The first part examined correlations across all species for which worker size data were available, while the second part specifically examined species that have a single worker caste. To account for phylogenetic uncertainty in our BPMMs, we repeated each analysis 400 times, each time with a different tree, and combined the posterior samples produced from each tree prior to parameter estimation. Model convergence was assessed as described in the section 'Statistical analyses.'

Causality analyses: examining colony size and levels of polyandry that preceded the evolution of multiple worker castes

Phylogenetic path analysis analysing number of discrete physical worker castes.

To test how the evolution of number of worker castes was influenced by colony size and queen mating frequency, we used phylogenetic path analysis[28,72]. This method compares alternative models of the causal relationships between traits, disentangling direct from indirect effects. After removing species with incomplete data for all variables, we analysed the data for a total of 94 species. By comparing four different causal models, we tested between the following possibilities: (i) variation in colony size directly influenced the evolution of number of worker castes, but queen mating frequency did not; (ii) variation in number of worker castes influenced the evolution of colony size, which then predicted queen mating frequency; (iii) variation in both colony size and queen mating frequency directly influenced the evolution of number of worker castes; and (iv) variation in queen mating

frequency directly influenced the evolution of number of worker castes, but colony size did not (list of full models in Extended Data Fig. 1). In each of the causal models tested, we assumed that the evolution of queen mating frequency was influenced by variation in both the number of queens per colony and colony size. We made these assumptions because (i) it has been suggested that the evolution of queen mating frequency has been influenced by variation in the number of queens per colony[21,25]; and (ii) larger colony sizes may select for multiple mating[40,41]. Larger colonies may be at greater risk of infection from pathogens and multiple mating may offer protection against disease because it increases genetic diversity within colonies[37,40–42]. However, multiple mating is a costly trait, and therefore, we hypothesised that larger colony sizes may evolve first, and then select for multiple mating if the benefits of multiple mating outweigh the costs[73]. We excluded the relationship between the number of queens per colony and either the number of castes or colony size in our models as we found no correlation between these variables in our regression analysis (see section '*Phylogenetic correlations*').

Based on the causal relationships presented in the alternative path analysis models, we generated conditional independence statements that can be formulated and tested as a set of phylogenetic generalised linear models. For each model, we calculated Fisher's C statistic and conducted the d-sep test[28]. P values less than 0.05 indicate that a proposed candidate model should be rejected. Next, we compared the fit of different models using CICc scores, where the lowest score represents the best candidate model[74]. Models that have $\Delta CICc < 2$ were considered to have equal support. We used a model averaging approach to estimate path coefficients that assigned weights to causal links based on the CICc weight (ω) of the supported models ($\Delta CICc < 2$). When a path was absent in a model, we assumed its coefficients and variance to be zero for the purpose of model averaging. We transformed the

number of castes into a binary variable (single worker caste/multiple worker castes) for the analysis as the phylopath package does not support discrete count data as response variables. We then used a Binomial family for binary response variables when modelling number of worker castes. To the best of our knowledge, there is currently no established method for combining results obtained across different trees to account for phylogenetic uncertainty in frequentist analyses. Therefore, we performed our analysis four times, each time using a different tree, from a sample of four MCC trees produced by Economo et al., 2018[65]. Each MCC tree was constructed from a sample of 100 trees. We then compared the results from each of the four analyses to determine whether our results remained consistent irrespective of the phylogeny that was used. In the main text, we present the results from a single tree as we found that the conclusions drawn across trees remained unchanged due to the similarity in the presence, direction and magnitude of the causal relationships identified. We present the results using the alternative MCC trees in the supplementary material (Extended Data Fig. 2 and Supplementary Tables 2-3). All phylogenetic path analyses were carried out using phylopath v1.1.3 in R[75].

Phylogenetic path analysis analysing variation in worker size.

We repeated our analysis, examining variation in worker size instead of number of worker castes. We used head width as a measure of worker size and modelled it as continuous variable. Models were constructed using Pagel's λ[76] for the associated error structure and we compared the same four causal models that were used in the previous path analysis (Extended Data Fig. 1). To investigate whether differences in the number of worker castes were driving the results when analysing worker size, we performed the analysis in two different ways: (i) with the full set of species for which we had data available for all four traits ($n_{species}$=94); and (ii) limiting our analysis to only species that possess a single worker caste (monomorphic;

$n_{species}$=60). To account for phylogenetic uncertainty, we repeated each analysis using the same four MCC trees used in the previous path analysis. In the main text, we present the results from a single tree as we found that the conclusions drawn across trees remained unchanged due to the similarity in the presence, direction and magnitude of the causal relationships identified. We present the results for both analyses using the alternative MCC trees in the supplementary material (Extended Data Figs. 5-6 and Supplementary Tables 7-10).

Testing models of dependent versus independent evolution and estimating evolutionary transition rates using BayesTraits

We tested for correlated evolution between number of worker castes and both colony size and queen mating frequency. We used the Discrete module with reverse jump MCMC estimation implemented in BayesTraits V4. This method requires pairs of binary traits, and so we transformed all traits into binary variables. We classified species as having either a single worker caste or multiple worker castes, to focus on the evolution of multiple castes. We classified species as having queens that mated with less than or equal to two males (≤2 males) or more than two males (>2 males), because: (i) the influence of larger numbers of matings on relatedness is diminishing; (ii) an effective mating frequency value of 2 leads to the average level of relatedness among workers in the colony being midway between the maximal and minimal values of 0.75 and 0.25 that are possible with a single queen; and (iii) mating with 2 males is the threshold at which worker policing is favoured[20]. We classified species as having either small or large colonies depending upon whether colony size was smaller (≤ 300) or larger (>300) than the median colony size. To assess the sensitivity of our results to different colony size classification thresholds, we repeated our analysis, but divided species into classes based on the $40^{th}/60^{th}$ and $60^{th}/40^{th}$ quantile boundaries. Across these

thresholds, we obtained the same result that large colony size was necessary for the evolution of multiple worker castes. Therefore, we present the results of the $40^{th}/60^{th}$ and $60^{th}/40^{th}$ only in the supplementary material (Supplementary Tables 23-24). In all cases, we tested the fit of the independent model of evolution against the dependent model of evolution using Bayes Factors (2 × (log(likelihood of the dependent model) – log(likelihood of independent model))). We estimated the marginal likelihood of both the independent and the dependent model using a stepping-stone sampler[77]. As recommended in the Bayestraits manual, we scaled tree branch lengths by a factor of 0.001. This helps prevent the estimated transition rates from becoming very small, which can make the values hard to estimate or search for. We accounted for phylogenetic uncertainty in our analysis by resampling each iteration from the posterior distribution of 400 trees to estimate parameters.

To reduce uncertainty over prior selection, we used a hyper prior approach to seed the mean and variance of an exponential prior[29]. The values for the hyper priors were drawn from a uniform distribution ranging from 0 to 100, based on the estimated range of transition rates determined by using analyses with maximum likelihood estimation. Additionally, we investigated the sensitivity of our models to the selection of priors by running models with gamma priors initialised using hyper priors. We found that the conclusions drawn between models using different priors remained the same. Therefore, we only present the results from the models using the gamma priors in the supplementary materials (Supplementary Tables 25-26). All models were run for at least 11,000,000 iterations with 1,000,000 iterations of burn-in and a thinning interval of 5,000. However, some models were run for 110,000,000 with 10,000,000 iterations of burn-in and a thinning interval of 50,000 iterations to improve chain convergence and mixing. Model convergence was assessed using the same approach described in ‘Statistical analyses.’

We identified correlated evolution between colony size and number of worker castes. Therefore, using the transition rates estimated by the model, we examined whether transitions to multiple worker castes were influenced by small or large colony sizes, as well as whether transitions to large colony sizes were influenced by number of worker castes. We found that queen mating frequency and number of worker castes evolved independently. Therefore, we did not explore the potential causal relationship between these traits. To determine the likelihood of transitions occurring, we examined the proportion of models visited by the reverse jump MCMC algorithm in which the rates were set to zero (Supplementary Tables 4-5, 18 and 23-26).

Ancestral state reconstructions.

We used ancestral state reconstruction to examine how colony size and queen mating frequency differed between the ancestors of lineages with a single caste and lineages with multiple castes[30]. First, we reconstructed the ancestral number of worker castes (single/multiple castes) to identify transitions between single and multiple worker castes. Second, we tested whether species that evolved multiple worker castes had greater estimated colony sizes and queen mating frequencies. To account for potential variation in the rate of caste evolution across the ant phylogeny, we used a hidden Markov model technique called hidden rate models (HRMs) to reconstruct the ancestral numbers of castes[78]. This method enabled us to incorporate heterogeneity in the loss and gain rates of our reconstruction. We used the R package corHMM v2.8 to examine HRMs with one to three rate classes and generated both equal rates (ER), and all-rates-different (ARD) models of evolution for each rate class[78]. To determine the best HRM model from the candidate set, we examined AICc values for each model across 400 trees using all available species with data on the number of

castes. We selected the equal rates model with 2 rate categories because it had the lowest AICc value over 57.5% of the 400 trees. We then ran the ER model with 2 rate categories for each analysis, examining the relationship between colony size and number of worker castes and queen mating frequency and number of worker castes. Next, we classified each node in the phylogeny depending on which state (single/multiple castes) had the highest likelihood, resulting in four possible classification categories for each node: (i) single caste with all descendants having a single caste; (ii) single caste with at least one descendant having multiple castes; (iii) multiple castes with all descendants having multiple castes; (iv) multiple castes with at least one descendant having a single caste.

The nodal classifications were entered as an explanatory variable in a BPMM and treated as a fixed factor with four levels. Depending on whether we were analysing the relationship between number of worker castes and either colony size or queen mating frequency, we specified either colony size or queen mating frequency as the response variable and used a phylogenetic covariance matrix that was linked to ancestral nodes as a random effect. We removed the global intercept and estimated the colony size and queen mating frequency values prior to the evolutionary origin of multiple worker castes (comparison of classification i versus ii). To account for phylogenetic uncertainty in our analysis, we repeated this process 400 times, once for each tree from our sample of 400 phylogenetic trees. We then combined the resulting posterior samples across the models to calculate parameter estimates. Accurate estimation of ancestral colony size and queen mating frequency values required phylogenetic signal in these traits. Using BPMMs, we estimated the amount of variation in each trait that can be attributed to shared ancestry between species, calculated as phylogenetic heritability ($phylo\ h^2 = \left(\frac{V_A}{V_A+V_R}\right) \times 100$, where V_A = Phylogenetic variance, V_R = Residual variance). For each trait, we repeated the analysis 400 times, each time with a different tree, and

combined the posterior samples of the variance components before phylogenetic heritability was estimated.

In addition, we estimated the ancestral number of discrete worker castes (1 to 4 worker castes) using HRMs. We ran each model over a sample of 400 trees and used the same model selection approach for HRMs as described above. The SYM model with 2 rate classes performed the best over 58.5% of trees. We then estimated the frequency of transitions between each number of castes, classifying each node based on the state with the highest likelihood estimate.

Sensitivity analysis

To assess the robustness of our findings to different methods of quantifying queen mating frequency and number of queens per colony, we performed a sensitivity analysis. We repeated our phylogenetic correlations analysis, treating queen mating frequency and number of queens per colony as categorical variables with three factor levels. This approach was chosen based on evidence indicating that these categorisations may reflect discrete reproductive strategies that may have evolved for different reasons and aligns with previous studies[21,25,33,79,80]. They were categorised as follows: obligate single mating (monandry), facultative multiple mating (facultative polyandry), and obligate multiple mating (obligate polyandry); and obligate single queen (monogyny), facultative multiple queens (facultative polygyny), and obligate multiple queens (obligate polygyny)[79,80].

We also repeated our phylogenetic path analysis and transition rate analysis. However, we transformed queen mating frequency and number of queens per colony into discrete binary variables, as these analytical methods cannot accept categorical variables with more than 2

factor levels. Species were classified as being obligately polyandrous or not, and obligately polygynous or not. We combined monandry with facultative polyandry, and monogyny with facultative polygyny to avoid excluding a large proportion of species from our analysis. We combined these categories because species that are obligately polyandrous or polygynous have made an evolutionary transition where mature colonies can no longer possess a monandrous or monogynous queen. In contrast, species that are facultatively polyandrous or polygynous may still exhibit monandry or monogyny. Detailed statistics for each analysis are provided in the supplementary material (Extended Data Figs. 8-10 and Supplementary Tables 11-19).

Data availability

All data are provided in Supplementary Table 20 and are made available, along with their associated references, via the public repository, Oxford University Research Archive (ORA) at https://ora.ox.ac.uk/objects/uuid:453278a3-61b9-4144-b08a-98d9379d5ce8[81]

Code availability

R code for analyses can be found at: https://github.com/LouisBell-Roberts/Larger_colony_sizes_favoured_the_evolution_of_more_worker_castes_in_ants

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Author information

Contributions

Conceptualisation: S.A.W., L.R. and G.D.A.W. Data collection: L.B.R., J.F.R.T. and L.R. Methodology: L.B.R., J.F.R.T., S.A.W., G.D.A.W. and P.A.D. Investigation: L.B.R., J.F.R.T., S.A.W. and G.D.A.W. Visualisation: L.B.R., J.F.R.T., S.A.W. Funding acquisition: S.A.W. Supervision: S.A.W. and G.D.A.W. Writing – original draft: L.B.R., J.F.R.T. and S.A.W. Writing – review and editing: L.B.R., J.F.R.T., S.A.W., G.D.A.W., P.A.D. and L.R.

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Ethics declaration

Competing interests

No competing interests.

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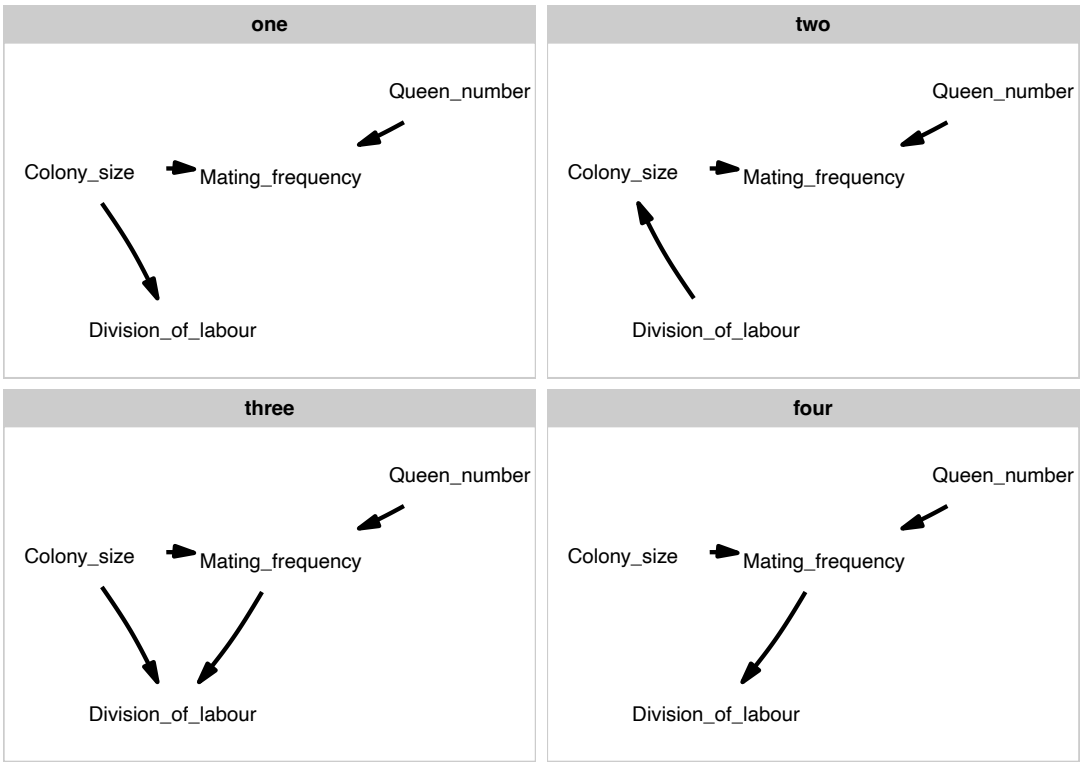
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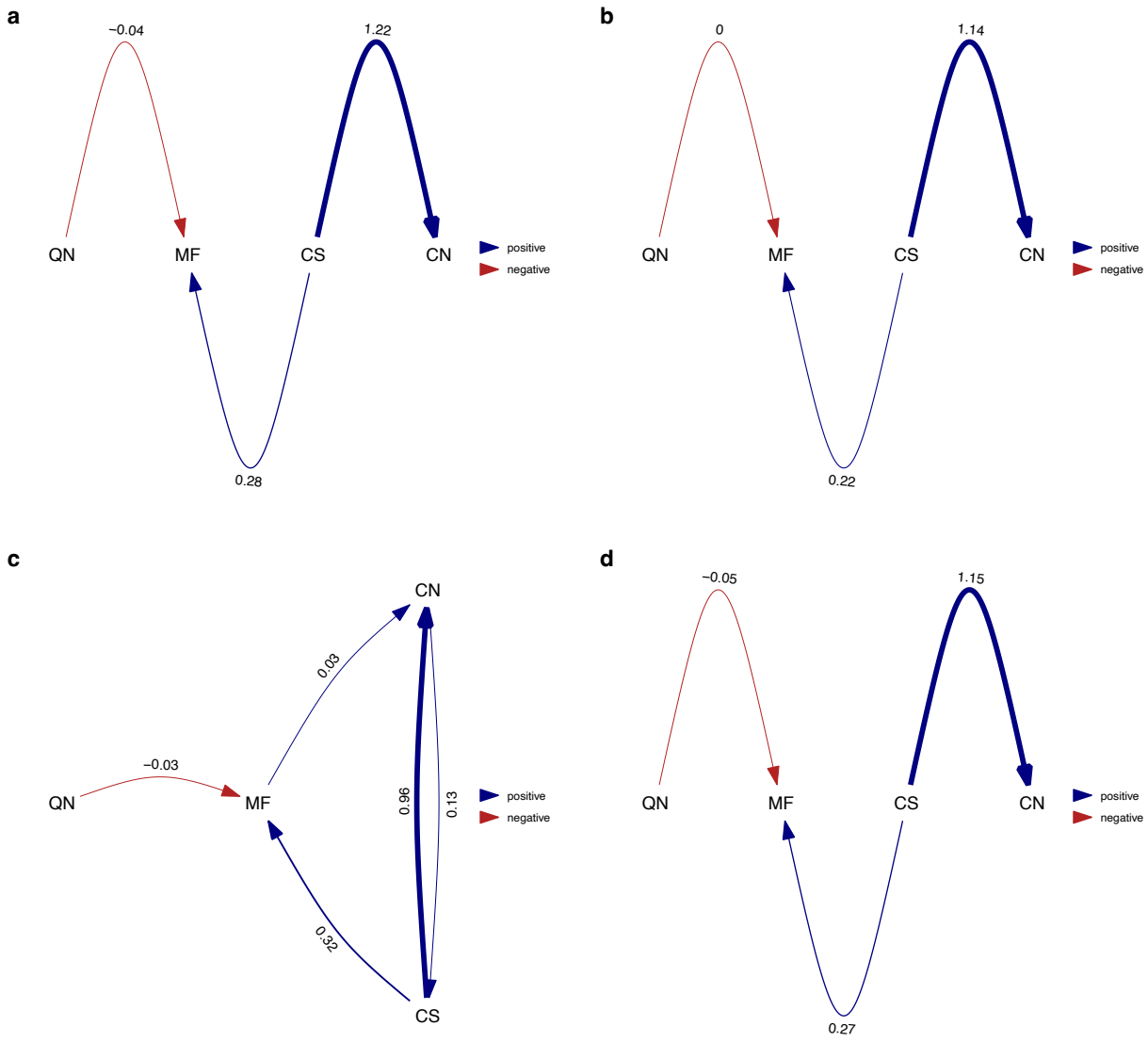
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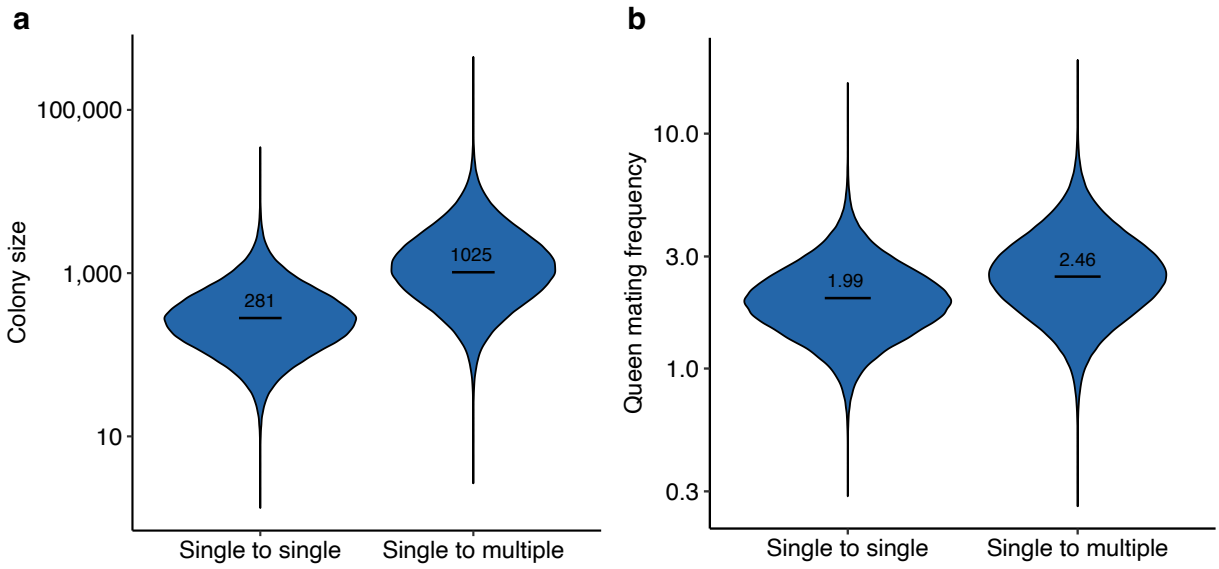
Extended data



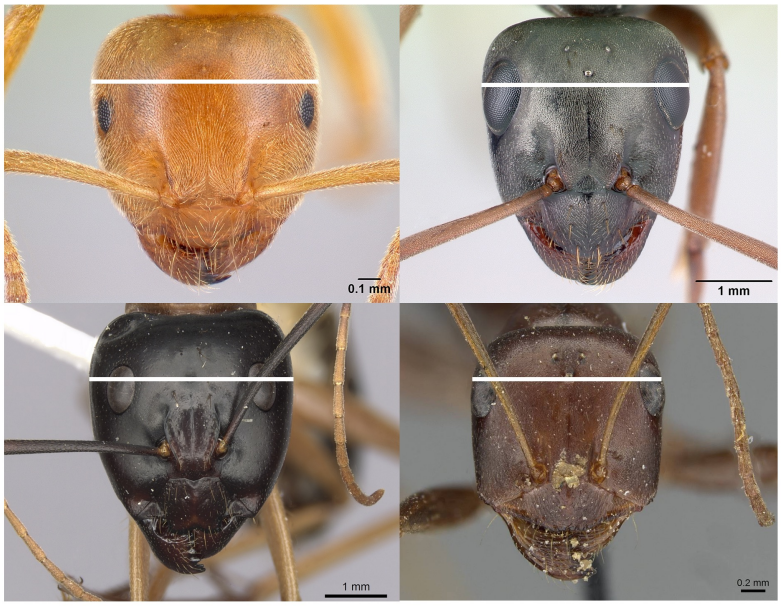
Extended Data Fig. 1 The full set of potential causal models tested in the phylogenetic path analyses illustrated using directed acyclic graphs (DAGs). In different path analyses, division of labour is modelled either as the number of discrete physical worker castes (Fig. 4) or as variation in worker size (Fig. 6b).



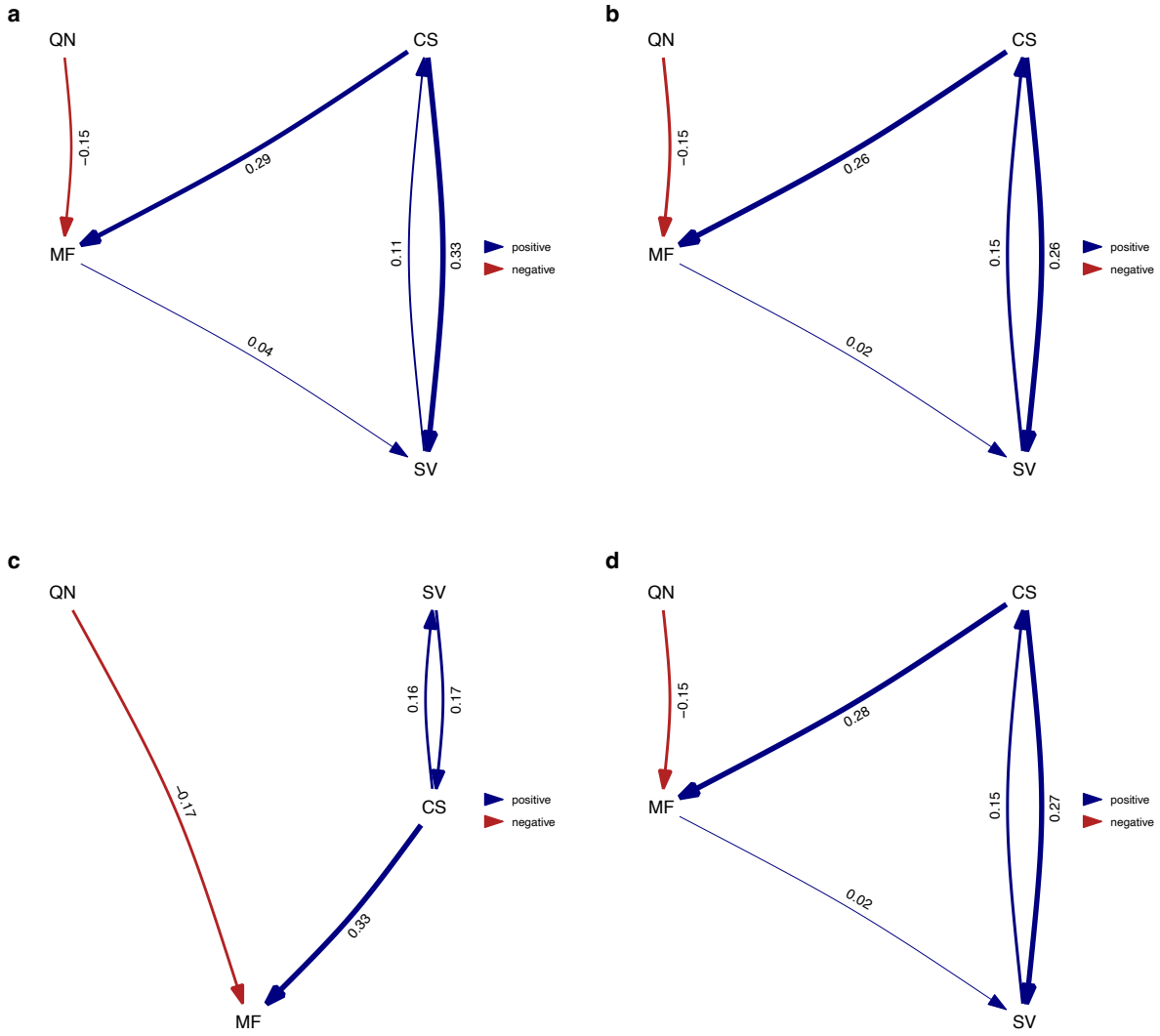
Extended Data Fig. 2 Average of the best-performing models where division of labour analysed as the number of physical worker castes (single caste/multiple castes). Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. CN = number of worker castes; CS = colony size; MF = queen mating frequency; QN = number of queens per colony.



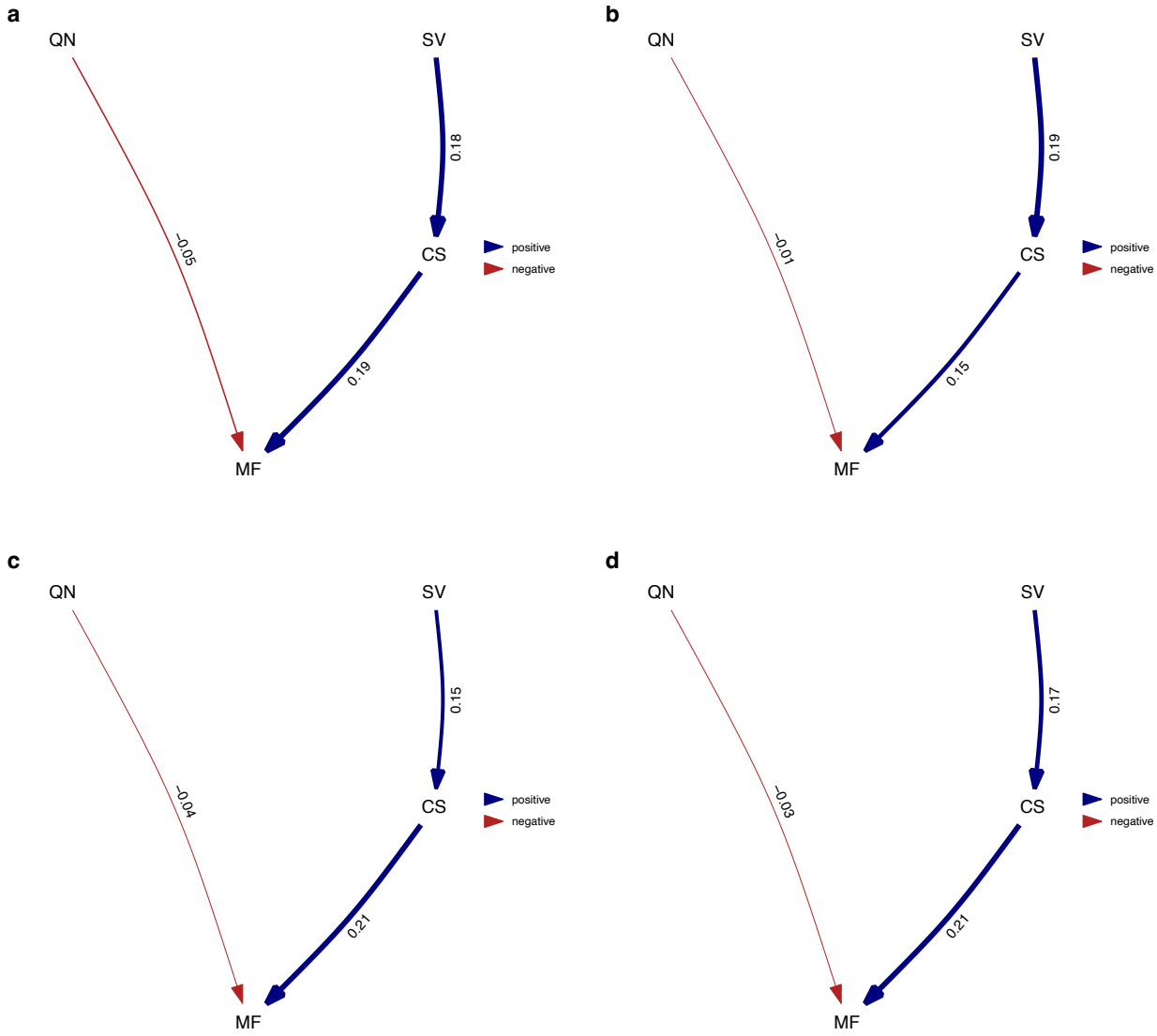
Extended Data Fig. 3 Ancestral state reconstruction: larger colony sizes favour the evolution of multiple worker castes. (a) The posterior distribution from a BPMM estimating colony size in ancestors with a single worker caste that led to descendants with a single caste (single to single) or multiple castes (single to multiple). Colony size is larger in the ancestors of lineages that evolve multiple castes, $P_{\text{single vs multiple}} < 0.01$ ($n_{\text{species}} = 436$). (b) The posterior distribution from a BPMM estimating queen mating frequency in ancestors with a single worker caste that led to descendants with a single caste (single to single) or multiple castes (single to multiple). Queen mating frequency was not significantly different in the ancestors of lineages that evolved multiple castes and the ancestors of lineages that retained a single caste, $P_{\text{single vs multiple}} = 0.16$ ($n_{\text{species}} = 104$).



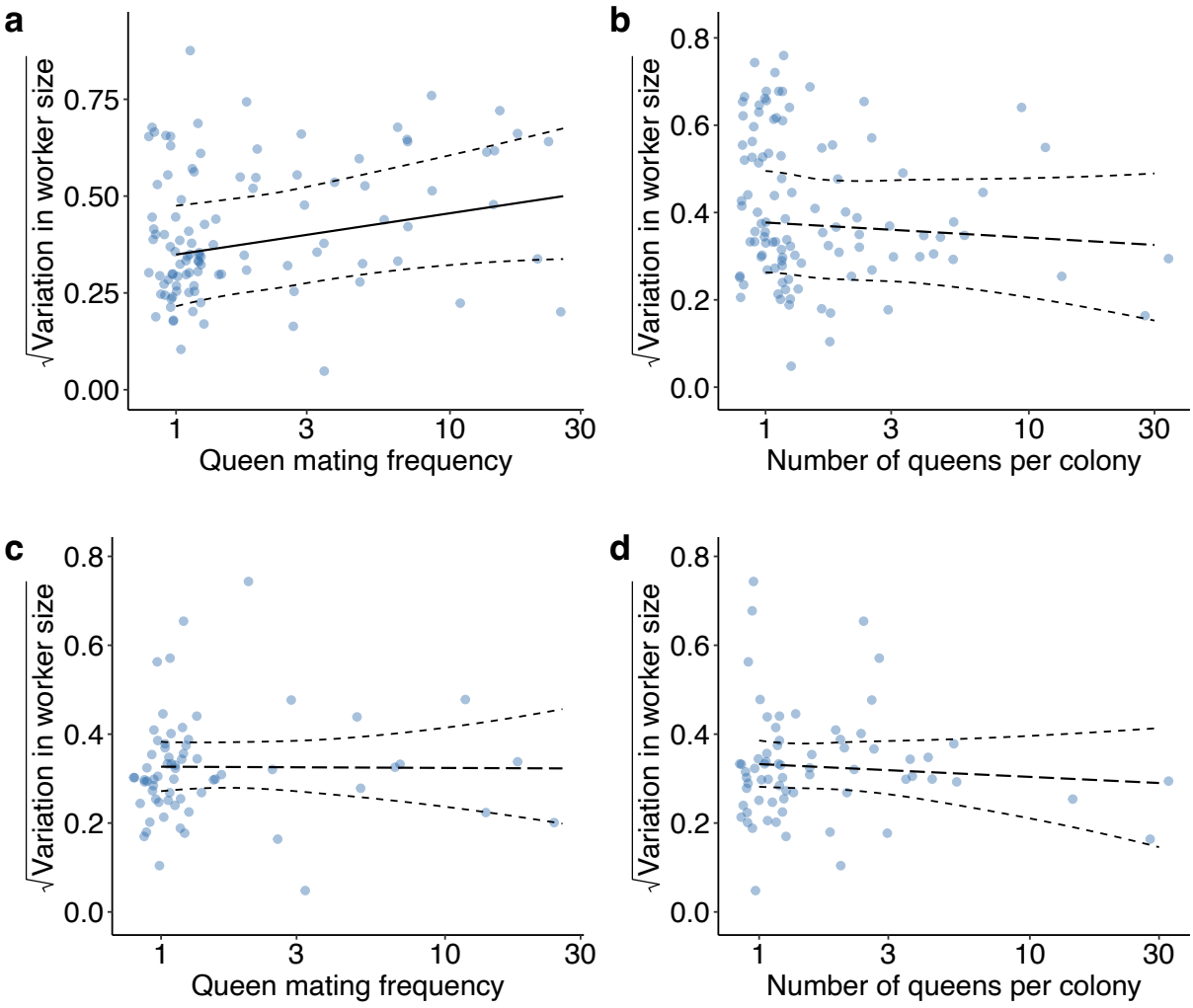
Extended Data Fig. 4 Measurement of ant head width. We downloaded front-view images of ant heads and measured at the widest point excluding the eyes (horizontal white line) using the image-processing software ImageJ.



Extended Data Fig. 5 Average of the best-performing models where division of labour analysed as variation in worker size. Analysis performed over all species for which data on all four traits was available. Analysis repeated over four different MCC ant trees from Economo et al., 2018 a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. SV = variation in worker size (degree of worker polymorphism); CS = colony size; MF = queen mating frequency; QN = number of queens per colony.

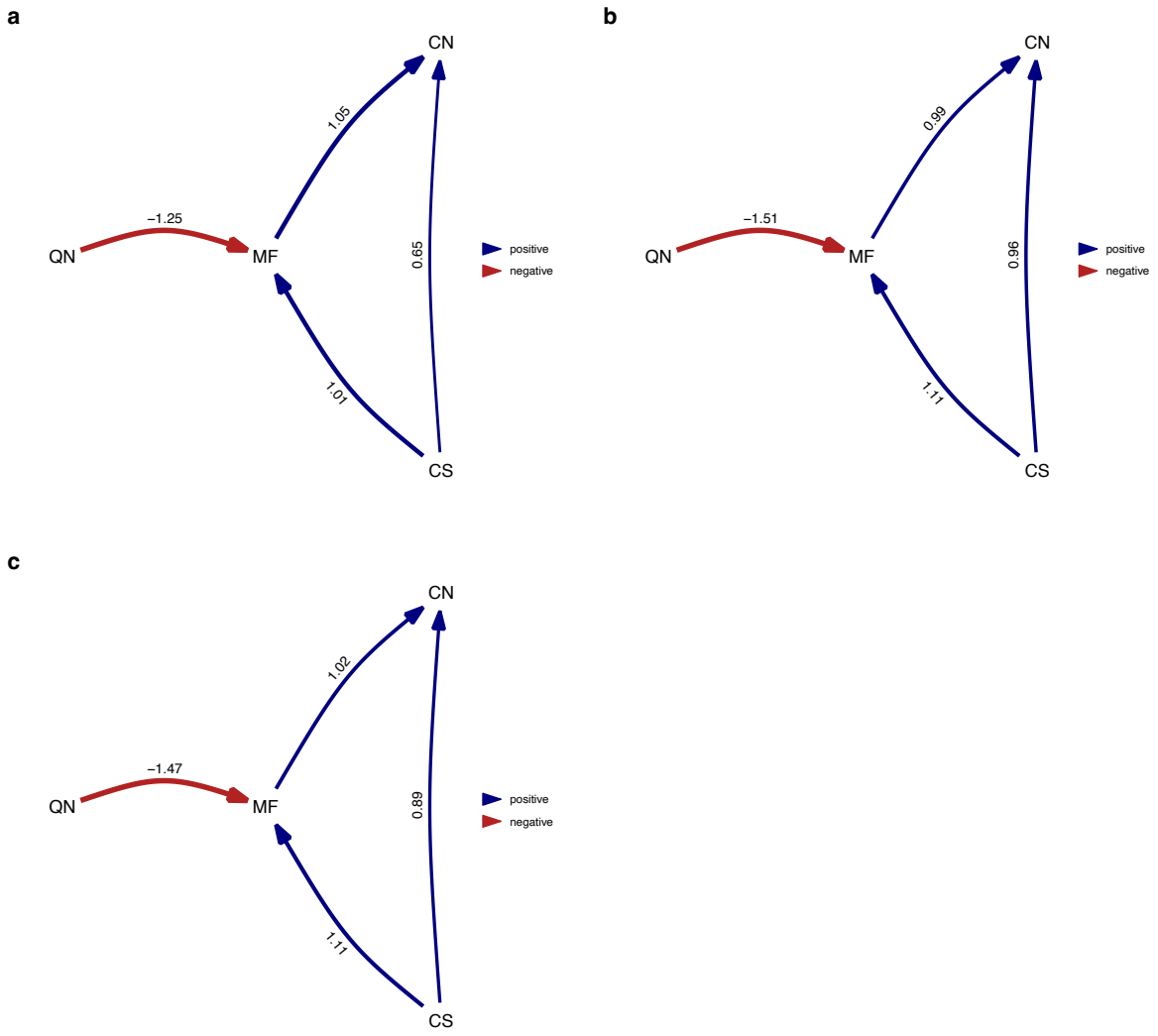


Extended Data Fig. 6 Average of the best-performing models where division of labour analysed as variation in worker size and only using species with a single physical worker caste. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. SV = variation in worker size (degree of worker polymorphism); CS = colony size; MF = queen mating frequency; QN = number of queens per colony.

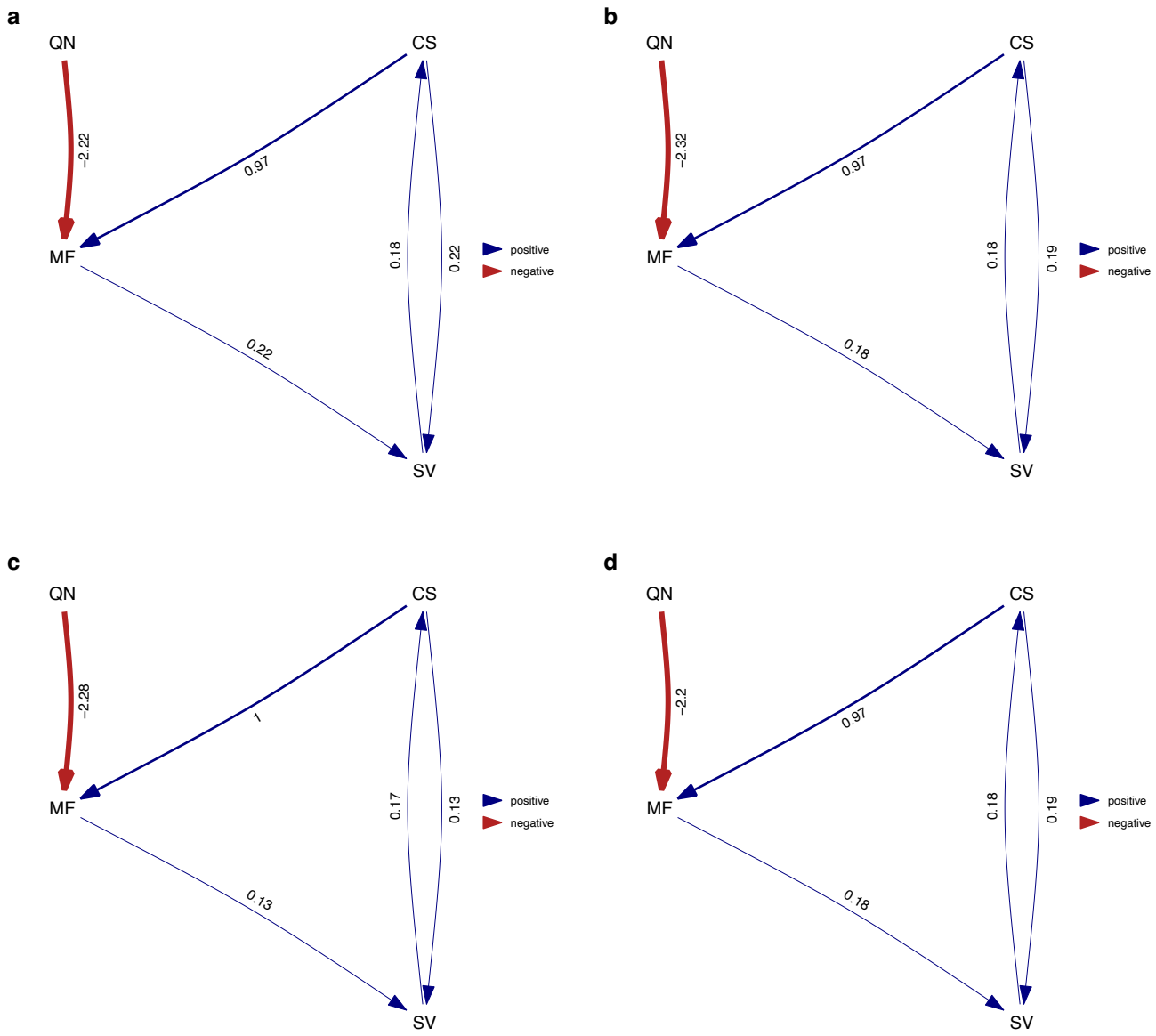


Extended Data Fig. 7 Relationships between queen mating frequency and number of queens per colony with variation in worker size. Species with higher queen mating frequencies exhibited significantly greater variation in worker size (a, BPMM: $\beta = 0.12$, CI = 0.01 to 0.23, $n_{species}=105$). We did not find a significant association between number of queens per colony and variation in worker size (b, BPMM: $\beta = -0.03$, CI = -0.12 to 0.07, $n_{species}=106$). When reanalysing our data so that we only included species with a single worker caste (c-d), we found no association between either queen mating frequency or number of queens per colony with variation in worker size (c, BPMMs: queen mating frequency: $\beta = -0.00$, CI = -0.10 to 0.10, $n_{species} = 65$; d, queen number: $\beta = -0.02$, CI = -0.12 to 0.07, $n_{species} = 68$). Regression lines are presented with 95% CI from BPMMs using a single phylogenetic tree. Solid regression lines represent significant relationships, while

dashed regression lines represent non-significant relationships. Dots represent species averages. Axes for queen mating frequency and number of queens per colony are represented on a $\log_{10}$ scale. Axes for variation in worker size are on a square root scale.

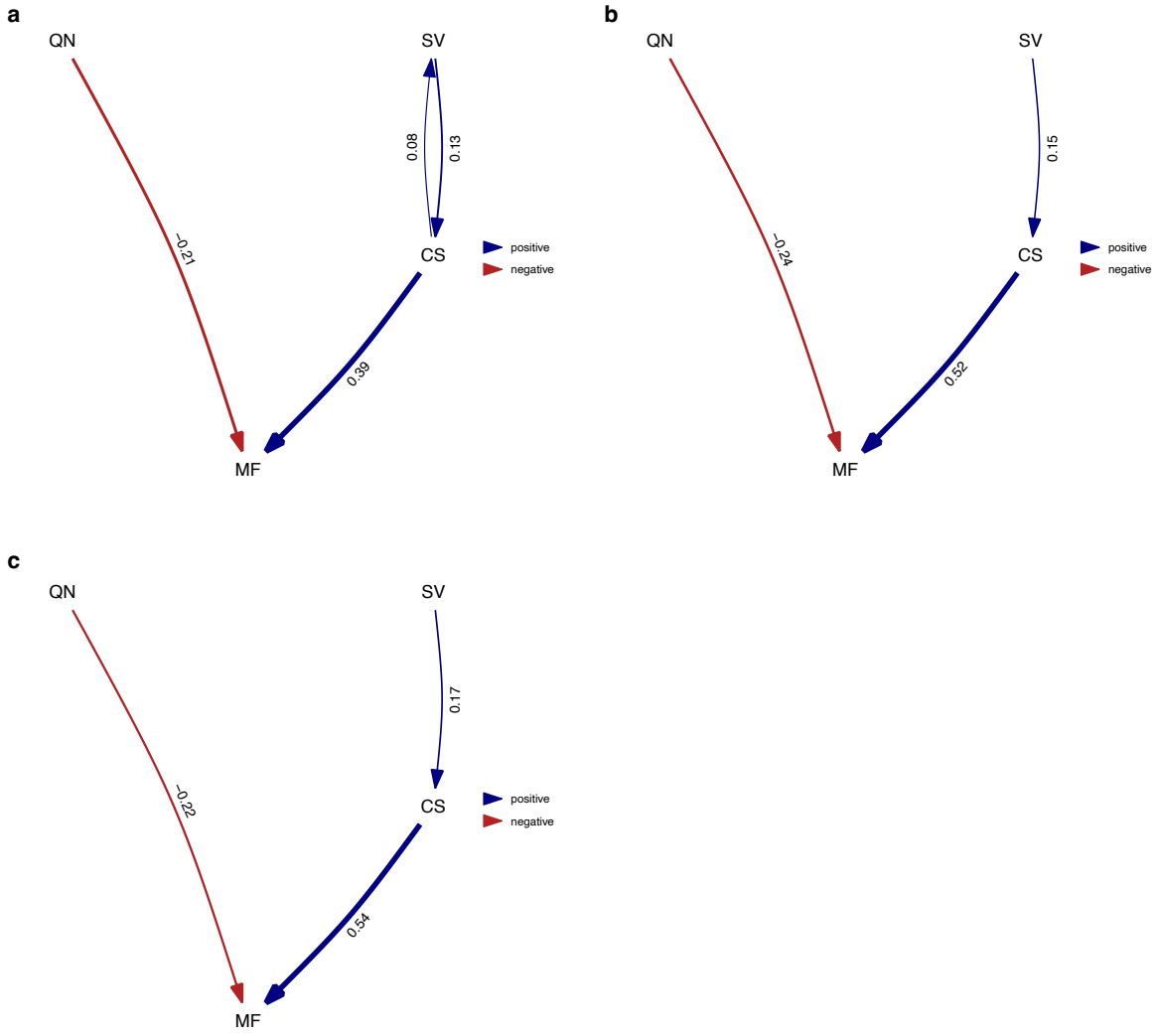


Extended Data Fig. 8 Average of the best-performing models where division of labour analysed as the number of physical worker castes (single caste/multiple castes). Queen mating frequency (obligately monandrous & facultatively polyandrous or obligately polyandrous) and number of queens per colony (obligately monogynous & facultatively polygynous or obligately polygynous) were analysed as binary traits. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. However, when using the FBD stem tree for the analysis, the model did not converge, and we exclude this result. Values represent standardised average coefficients. CN = number of worker castes; CS = colony size; MF = queen mating frequency; QN = number of queens per colony.



Extended Data Fig. 9 Average of the best-performing models where division of labour analysed as variation in worker size. Analysis performed over all species for which data on all four traits was available. Queen mating frequency (obligately monandrous & facultatively polyandrous or obligately polyandrous) and number of queens per colony (obligately monogynous & facultatively polygynous or obligately polygynous) were analysed as binary traits. Analysis repeated over four different MCC ant trees from Economo et al., 2018 a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent

standardised average coefficients. SV = variation in worker size (degree of worker polymorphism); CS = colony size; MF = queen mating frequency; QN = number of queens per colony.



Extended Data Fig. 10 Average of the best-performing models where division of labour analysed as variation in worker size and only using species with a single physical worker caste. Queen mating frequency (obligately monandrous & facultatively polyandrous or obligately polyandrous) and number of queens per colony (obligately monogynous & facultatively polygynous or obligately polygynous) were analysed as binary traits. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. However, when using the NCuniform stem tree for the analysis, the model did not converge, and we exclude this result. Values represent standardised average coefficients. SV = variation in worker size (degree of worker polymorphism); CS = colony size; MF = queen mating frequency; QN = number of queens per colony.

Chapter 3: Climate and the evolution of social complexity in ants

Abstract

Harsh and unpredictable environments are thought to favour the evolution of greater cooperation in animals. However, empirical studies testing this hypothesis—by examining the implicit assumption that greater cooperation evolves after the colonisation of harsh environments—have produced mixed results. We investigate the evolutionary order of events in ants, which differ from previously studied species due to the presence of fixed reproductive (queen) and helper (worker) castes. However, the nature of cooperation within colonies varies among species, such as in the number of workers or the degree of variation in worker size (worker polymorphism). We performed a phylogenetic comparative analysis across 474 species of ants, testing for correlations between both colony size and worker polymorphism with climatic conditions, and used phylogenetic path analysis to disentangle the potential causal relationships between these variables. We repeated our analyses over a range of different geographical scales, from global to smaller regional scales, to examine whether the same patterns hold across regions. Consistent with a previous study, we found that worker polymorphism is associated with hotter and drier climates, while larger colony sizes are associated with drier climates. However, in contrast to theoretical predictions, we found that worker polymorphism facilitates the colonisation of hotter and drier climates. Our results were dependent on the geographical scale over which species were analysed, suggesting that varying ecological conditions between regions play an important role mediating the relationship between social traits and climatic conditions. Overall, our results

support the central role of cooperation as a key factor in permitting ecological expansion into new niches.

Introduction

The relationship between climatic gradients and species traits has long been recognised, with traits such as body size and shape (Bergmann, 1847; Allen, 1877), as well as pigmentation showing patterns of adaptation to different climatic conditions (Gloger, 1833; Delhey, 2017, 2018). However, social behaviours, such as cooperation between individuals, could also play an important role influencing the environmental conditions under which organisms can survive and reproduce. For example, species that breed cooperatively are more commonly found living in harsh or unpredictable environments and has been demonstrated in groups such as birds (Jetz and Rubenstein, 2011; Griesser *et al.*, 2017), mammals (Lukas and Clutton-Brock, 2017), and wasps (Sheehan *et al.*, 2015). Theoretical work suggests that in these environments, individuals may be more likely to show helping behaviour towards relatives because independent breeding success is low (Emlen, 1982; Pen and Weissing, 2000; McLeod and Wild, 2014), or the benefit of helping is greater (Covas, Du Plessis and Doutrelant, 2008; Rubenstein, 2011; Griesser *et al.*, 2017; Kennedy *et al.*, 2018). Supporting these ideas, numerous within-species studies have shown that when environmental factors make independent breeding difficult, individuals are more likely to breed cooperatively (Pruett-Jones and Lewis, 1990; Komdeur, 1992; Bergmüller, Heg and Taborsky, 2005; Rubenstein and Lovette, 2007; Koenig and Dickinson, 2016) and that helpers reduce the risk of breeding failure during difficult years, which occur more frequently in harsh and unpredictable environments (Covas, Du Plessis and Doutrelant, 2008; Rubenstein, 2011; Koenig and Walters, 2015).

However, the idea that harsh and unpredictable environments may facilitate greater levels of cooperation may be incorrect. Instead, causality may be in the opposite direction, where greater levels of cooperation could facilitate the colonisation of harsh environments (Cornwallis *et al.*, 2017). Alternatively, an intermediate factor could link the relationship between cooperation and harsh environments. To date, the few studies that have investigated causality have yielded mixed results. For example, in Australian rodents, transitions to cooperative groups are associated with harsh environments characterised low rainfall and high temperature variability (Firman *et al.*, 2020). However, across birds, causality appears to be reversed, and cooperatively breeding species are better able to colonise harsh environments characterised by hotter temperatures and more variable rainfall (Cornwallis *et al.*, 2017). As a result, additional empirical studies in other taxonomic groups are required to examine how climatic conditions are associated with social traits, and their potential causal relationships.

Ants are an important group to examine how social traits are related to climatic conditions because they differ to cooperative breeders in the form that cooperation takes within groups and have been the focus of only a limited number of studies (Kaspari and Vargo, 1995; Kaspari, 2005; Geraghty, Dunn and Sanders, 2007; La Richelière *et al.*, 2022). Ants form obligate social groups with mutually dependent reproductive and sterile castes that are fixed for life and determined during development (superorganisms) (Boomsma and Gawne, 2018). Therefore, ant species differ from cooperative breeders in that individuals within ant colonies aren't faced with the decision to be a helper or a reproductive, and they can't change their role within the group, as caste is fixed. However, species do vary in terms of the complexity of cooperative traits at the level of the colony, and these traits have the potential to influence the climatic conditions in which a colony may be able to persist and reproduce. For example,

species vary in the number of colony members (colony size: from 3 to >20 million) (Raignier and van Boven, 1955; Jahyny, Delabie and Fresneau, 2002) and whether they possess workers that vary in size and morphology (worker polymorphism), which is associated with the overall level of division of labour in the colony (Fig. 1) (Oster and Wilson, 1978). How do these traits vary in relation to climatic conditions, and what might be the potential causal relationships between them?

A recent analysis identified that ant species were more likely to have polymorphic workers in hotter and drier climates. While a crucial first step, this result is open to multiple possible explanations. First, La Richelière et al., 2022 did not fully control for phylogeny in their analysis. While a test of phylogenetic signal in worker polymorphism at the genus level revealed that closely related genera are no more likely to be polymorphic than distantly related ones (low phylogenetic signal) (La Richelière *et al.*, 2022), issues of pseudoreplication may persist when excluding an estimate of phylogenetic relationships between lineages in further statistical analyses (Felsenstein, 1985; Harvey and Pagel, 1991). For example, this issue may occur if a genus contributes a disproportionately large number of polymorphic species to a particular climatic type. This scenario could generate a spurious association between climate and polymorphism if phylogeny is not properly accounted for. This problem is emphasised in their study by: (a) polymorphic genera such as *Myrmecosystus*, *Messor* and *Cataglyphis* comprising a large proportion of desert and scrubland species, and *Cephalotes* contributing a disproportionate number of polymorphic species to tropical dry forests and savannahs (La Richelière *et al.*, 2022); and (b) drier climates only being associated with a higher likelihood of worker polymorphism in 5/8 ant subfamilies despite finding an overall trend that worker polymorphism was negatively associated with the level of rainfall (La Richelière *et al.*, 2022). Second, no study has yet

examined the potential causal relationship between climate, worker polymorphism and colony size. While climatic conditions may directly influence the evolution of worker polymorphism and colony size, the direction of causality could be reversed, where colony size and polymorphism may instead facilitate the colonisation of new environments with different climates (Cornwallis *et al.*, 2017). Further complicating matters is that colony size has been shown to be a major determinant of worker polymorphism (Bonner, 1993; Anderson and McShea, 2001; Bourke, 2011; Dornhaus, Powell and Bengston, 2012; Ferguson-Gow *et al.*, 2014; Wills *et al.*, 2018; Lecocq De Pletincx, Dellicour and Aron, 2021; Bell-Roberts *et al.*, 2024). Therefore, it remains unclear, for example, whether environmental conditions directly affect worker polymorphism, or if environmental conditions influence colony size, which then affects worker polymorphism. Third, the relationship between climate and social complexity traits (colony size and worker polymorphism) have not been examined across separate geographic regions. Patterns at different geographical scales could potentially be obscured when examining species globally. For example, the ecology of tropical and temperate species can be very different and so it is possible that the same patterns do not hold across both regions.

We address these problems with a series of phylogenetic analyses, examining the evolution of social complexity traits in ants. We investigate how the occurrence of worker polymorphism and colony size vary with climatic conditions, and tease apart how these variables have influenced each other over evolutionary time. We also aim to address whether the observed patterns between ant social complexity traits and climatic conditions at a global scale persist when examined at smaller regional scales.

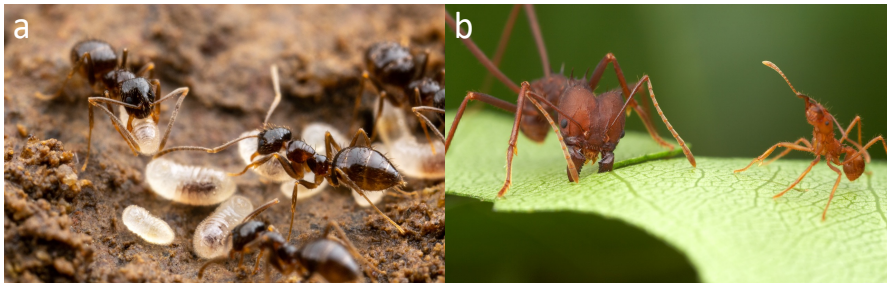


Figure 1. The diversity of social complexity in ants. Across species, ants display substantial variation in social complexity with large differences between species in both colony size and the degree of variation in worker size and morphology. *Nylanderia faisonensis* (a) forms colonies of approximately 120 workers, with limited variation in both size and morphology (monomorphic). In contrast, workers of *Atta sexdens* (b) display significant variability in size and morphology (polymorphic), with colonies consisting of millions of individuals.

Methods

We performed a series of phylogenetic comparative analyses across 474 species of ants to examine the relationship between climatic conditions and social complexity (Fig. 2). First, we used phylogenetic regressions to determine how three climatic variables (average temperature, average precipitation, and diurnal temperature range) were correlated with the presence of worker polymorphism (monomorphic versus polymorphic worker caste) and colony size. We also examined the relationship between the two measures of social complexity. Second, we used phylogenetic path analysis to examine the causal relationships between these variables. Including both biotic and abiotic factors enables the comparison of the relative strength of the causal relationships between these different types of variables. Third, we split our global ant data into tropical species, temperate species, and species with distributions that overlap both tropical and temperate regions. We reanalysed all three groups

to examine whether the global patterns that exist between ant social complexity traits and climatic conditions remain at smaller regional scales.

Social complexity data collection

We aimed to collect data on worker polymorphism and colony size from the literature for as many ant species as possible. We classified species as having monomorphic or polymorphic workers using data from La Richelière et al., 2022, which contains information on 15,518 ant species. We selected this dataset over alternative sources that use different methods to quantify the level of division of labour in the worker caste because it classifies the largest number of species to date and includes data on the vast majority of recognised ant species (Supplementary Table 10; an assessment of the alternative methods used to quantify division of labour is provided in the Supplementary Material).

We collected data on colony size (number of workers in a mature colony) from Bell-Roberts et al., 2024 and by performing a literature search. Bell-Roberts et al., 2024 summarises colony size data from previous literature reviews and comparative analyses, in addition to performing a literature search in 2020. We conducted our own literature search in May 2023 to capture any colony size data not contained within Bell-Roberts et al., 2024 using the search terms "ant" AND "collected" AND ("colony size" OR "colony collection" OR "worker number") in Google Scholar. Papers were read until 50 successive papers yielded no further novel colony size data. When multiple independent colony size estimates were identified for a species, we calculated the mean value. In total we collected colony size data for 763 species of ants.

Following Bell-Roberts et al., 2024, we excluded 112 ant species based on five life history traits that meant they were likely experiencing different selection pressures for the evolution of worker polymorphism or colony size (Supplementary Table 11). These traits were: (i) interlineage hybridisation for genetic caste determination; (ii) reproduction via gamergates (mated workers that reproduce sexually); (iii) the capacity for parthenogenetic reproduction to produce queens or workers; (iv) social parasitism, where species lack some or all of the worker castes (temporary social parasites, which only establish new colonies with the assistance of a host species, were included in the analysis); and (v) forming supercolonies (extensive networks of interconnected nests). We collected the information from papers that had summarised the available data on these five traits (Monnin and Peeters, 2008; Schwander *et al.*, 2010; Rabeling and Kronauer, 2013; Heikki Helanterä, 2022; Gray and Rabeling, 2023). The decision to remove these species improved our ability to detect which variables may have influenced the evolution of worker polymorphism or colony size.

Species distribution and climatic data collection

We collected monthly climate data from the climate research unit (CRUTS v4.06)(Harris *et al.*, 2020). We averaged each climatic variable over 1440 months (January 1901 to December 2020) for each longitude-latitude grid (0.5°x0.5°; ~55.5km north-to-south, east-to-west varies with latitude). We collected data on three climatic variables: (i) temperature; (ii) rainfall; and (iii) diurnal temperature range. We selected just these three climatic variables because climatic variables can be highly correlated and so adding additional climatic variables to analyses has limited explanatory power (Liu *et al.*, 2024). To characterise the climatic conditions that ant species experience, we used the geographic occurrence data supplied by La Richelière et al., 2022 and averaged across all spatial grids where a species was present.

We confirmed that collinearity between our chosen climatic variables did not pose an issue in our analysis (Supplementary Table 1).

Phylogenetic relationships between ant species

We controlled for the statistical non-independence of related species with phylogenetic analyses that incorporate an estimate of evolutionary relatedness. We used a posterior sample of 400 phylogenetic trees produced by Economo et al., 2018 which included 14,594 species. We then pruned the phylogenies to include the 474 species for which we had data for all traits as well as phylogenetic information (Economo *et al.*, 2018). These species were then used in the subsequent analyses. However, because time-calibrated molecular phylogenies are only available for ants at the genus level, there was considerable variation in species topology within genera across the sample of trees. Therefore, we accounted for phylogenetic uncertainty in all our analyses, with the specific details for each analysis provided below ('*Statistical analyses: phylogenetic regression analysis'* and '*Statistical analyses: phylogenetic path analysis'*).

Phylogenetic principal component analysis

We generated an index of climatic conditions using a phylogenetic principal component analysis (PCA) implemented in phytools v1.5-1 (Revell, 2012). We used mean estimates for only temperature and rainfall to generate this index, as diurnal temperature range was not correlated with either of our measures of social complexity. We used a maximum clade credibility tree (MCC) produced by Economo et al., 2018 that was constructed from 100 posterior tree samples. Prior to the analysis, temperature and rainfall were Z-transformed (mean centred with a standard deviation of 1). The first principal component (climatic PC1) showed a strong negative correlation with mean temperature (factor loading = -0.87) and

mean rainfall (factor loading = -0.76). The second principal component (climatic PC2) was positively correlated with mean temperature (factor loading = 0.50) and negatively correlated with mean rainfall (factor loading = -0.65). PC1 explained 67% of variation in the data, while PC2 accounted for the remaining 33%. We repeated this process three times using alternative MCC trees, producing three additional datasets (factor loadings for the alternative trees found in Supplementary Table 9). By repeating analyses, and comparing results across these datasets, we assessed the sensitivity of our analyses to phylogenetic uncertainty when using the PCA climatic indices.

Subsetting ant species by latitude

We examined whether the relationship between climatic conditions and measures of social complexity remained consistent on a global level as opposed to smaller regional scales. We classified species in three ways based on their geographical distributions, each experiencing distinct climates: (i) species found exclusively in tropical regions (within the range of 23.5 degrees North and South of the equator; $n_{species} = 116$); (ii) species exclusive to temperate regions (extending beyond 23.5 degrees either to the North or South; $n_{species} = 190$); and (iii) species with distributions in both tropical and temperate regions (Supplementary Figs. 1-2; $n_{species} = 168$). We conducted separate analyses for species within each of the three geographical categories, applying the same analytical methods to each regional scale as those used in the global analysis.

Statistical analyses: phylogenetic regression analysis

We used Bayesian phylogenetic mixed models (BPMM) to test for correlations between climatic conditions (temperature, rainfall, and diurnal temperature range) and social complexity traits (MCMCglmm v2.34 (Hadfield, 2010); a comprehensive description of the

methods, including model settings and assessments, is available in the Supplementary Material and R Code). Colony sizes were large and so we modelled it as a continuous variable with a Gaussian error distribution. We modelled the probability of worker polymorphism as a binary trait (monomorphic/polymorphic) with a logit link function. To account for phylogenetic uncertainty, we repeated our analysis 400 times, each with a different tree. We then combined the posterior samples produced from each tree for parameter estimation. We report parameter estimates from models as posterior modes along with 95% lower and upper credible intervals (CIs). We provide marginal R^2 values for each model, excluding those where worker polymorphism is the response variable, as it is binary, and estimates of R^2 can be misleading for binary variables (Cox and Wermuth, 1992; Nakagawa and Schielzeth, 2013). We provide the median R^2 value from a distribution of R^2 values estimated across a sample of 400 trees. We also estimated the amount of variation in colony size and worker polymorphism explained by shared ancestry between species. For colony size, we calculated this as phylogenetic heritability (H^2_{phylo} = phylogenetic variance/(residual + phylogenetic variance)), and for worker polymorphism, we calculated this as the intraclass correlation coefficient (phylogenetic variance/(phylogenetic variance + 1 + ($\pi^2/3$)) – an analogous measure, suitable for binary traits, quantifying the amount of variation explained by phylogenetic history. We repeated our regression analysis using alternative climatic measures (PC1 and PC2) generated by phylogenetic principal component analysis (PCA), which capture variation in both rainfall and temperature. Variation in PC1 is able to capture differences between hot and wet climates, or cold and dry climates, while variation in PC2 is able to capture differences between hot and dry climates, or cold and wet climates. Therefore, we analysed both PC1 and PC2 in order to capture all of the possible climatic types in our analysis, performing separate analyses for each variable.

Statistical analyses: phylogenetic path analysis

We used phylogenetic path analysis to investigate the causal relationships between climatic conditions and social complexity traits (phylopath v1.1.3 in R (Gonzalez-Voyer and von Hardenberg, 2014; Shipley, 2016; Van Der Bijl, 2018; R Core Team, 2020)). This method compares alternative models of the causal relationships between traits, disentangling direct from indirect effects, and resolving the direction of causal relationships. In each analysis, we compared four different causal models, assuming in each model that colony size directly influences the evolution of worker polymorphism (Supplementary Fig. 3) (Bell-Roberts *et al.*, 2024). It was not possible to analyse rainfall and temperature as separate variables in the same analysis because all causal models that did not specify a causal relationship between rainfall and temperature were rejected. We did not test causal models that specified a unidirectional relationship between temperature and rainfall, as hypothesising a direct causal relationship between these variables would be meaningless. Therefore, we modelled climatic variation using PC1 and PC2, performing separate analyses for each variable.

To identify the best-fitting models, we used CICc values, where models that have ΔCICc that differ by < 2 are considered to have equal support (Shipley, 2013; Gonzalez-Voyer and von Hardenberg, 2014). When our analysis indicated support for multiple models, we used a model averaging approach to estimate path coefficients that assigned weights to causal links based on the CICc weight (ω) of the supported models (ΔCICc < 2). Path coefficients describe the strength and direction of the relationship between two variables in terms of their correlated variance. Average path coefficients were calculated by considering only the path coefficients from models that contained that specific path. Therefore, missing paths were not included in the calculation of the average path coefficients ('conditional' method (Hardenberg and Gonzalez-Voyer, 2013)). When modelling worker polymorphism as a

response variable, we used a binomial distribution with a logit link function. To assess the robustness of our results to phylogenetic uncertainty, we performed our analysis three additional times, using a sample of three alternative MCC trees. Across trees, our results were mostly consistent. Therefore, in the main text, we present the results from a single analysis. However, we also highlight results that were sensitive to phylogenetic uncertainty. The additional analyses, along with detailed statistics for all models tested, are presented in the supplementary material (Supplementary Figs. 5-12 and Supplementary Tables 2-5).

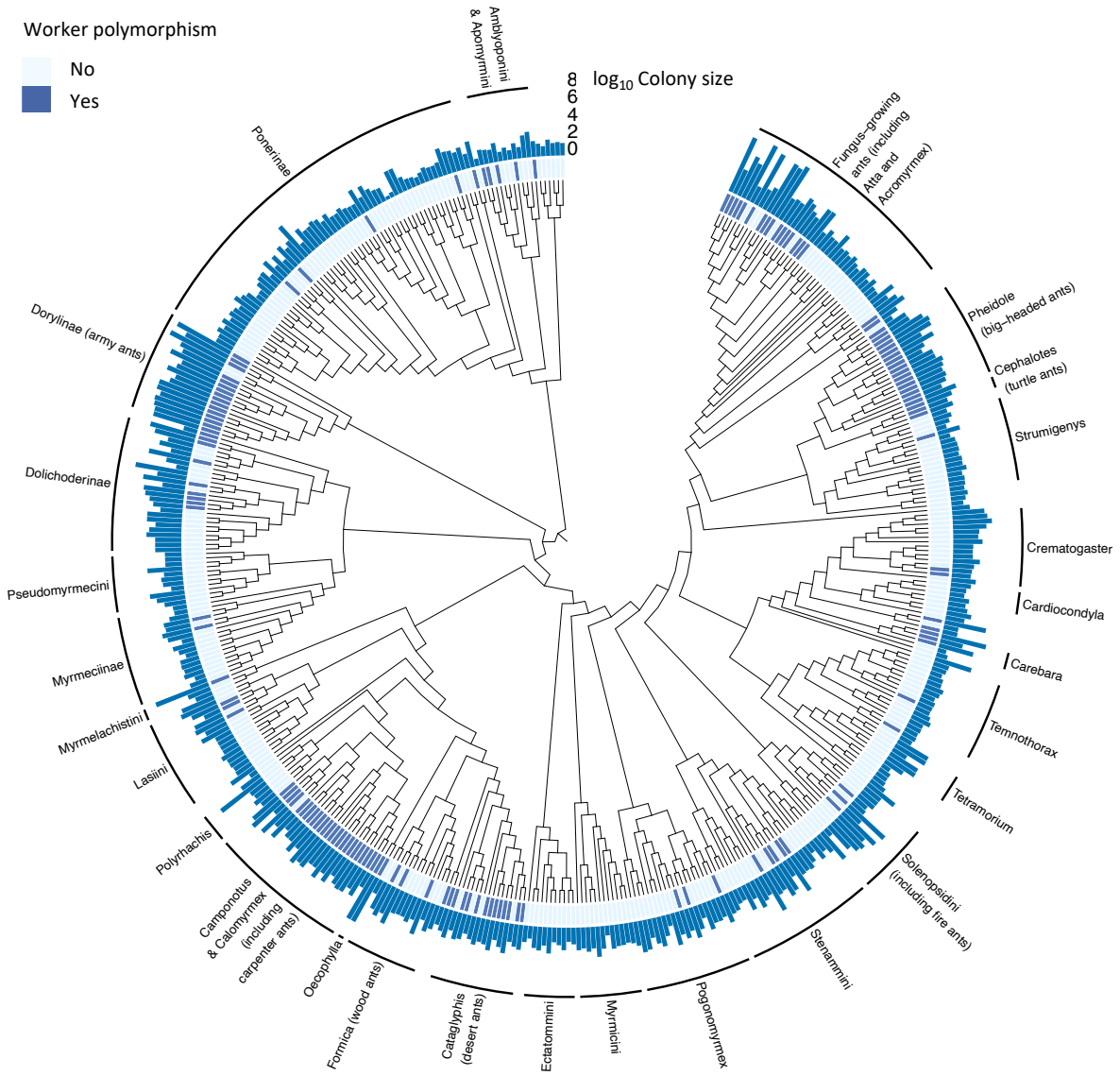


Figure 2. Phylogenetic distribution of worker polymorphism and colony size across 474 species of ants. Branch lengths are not to scale. Ant phylogenetic lineages, of varying levels, are displayed in black text around the outside of the phylogeny. Colony size represented by bars on a $\log_{10}$ scale.

Results

Phylogenetic signal

We found a strong phylogenetic signal in both worker polymorphism and colony size, supporting the need to account for shared ancestry between species analyses (BPMMs: Worker polymorphism$_{\text{phylogenetic heritability}}$ = 77.02%, CI = 64.77% to 85.43%; Colony size$_{\text{phylogenetic heritability}}$ = 72.26%, CI = 58.13% to 83.88%; n_{species} = 474; Supplementary Table 6).

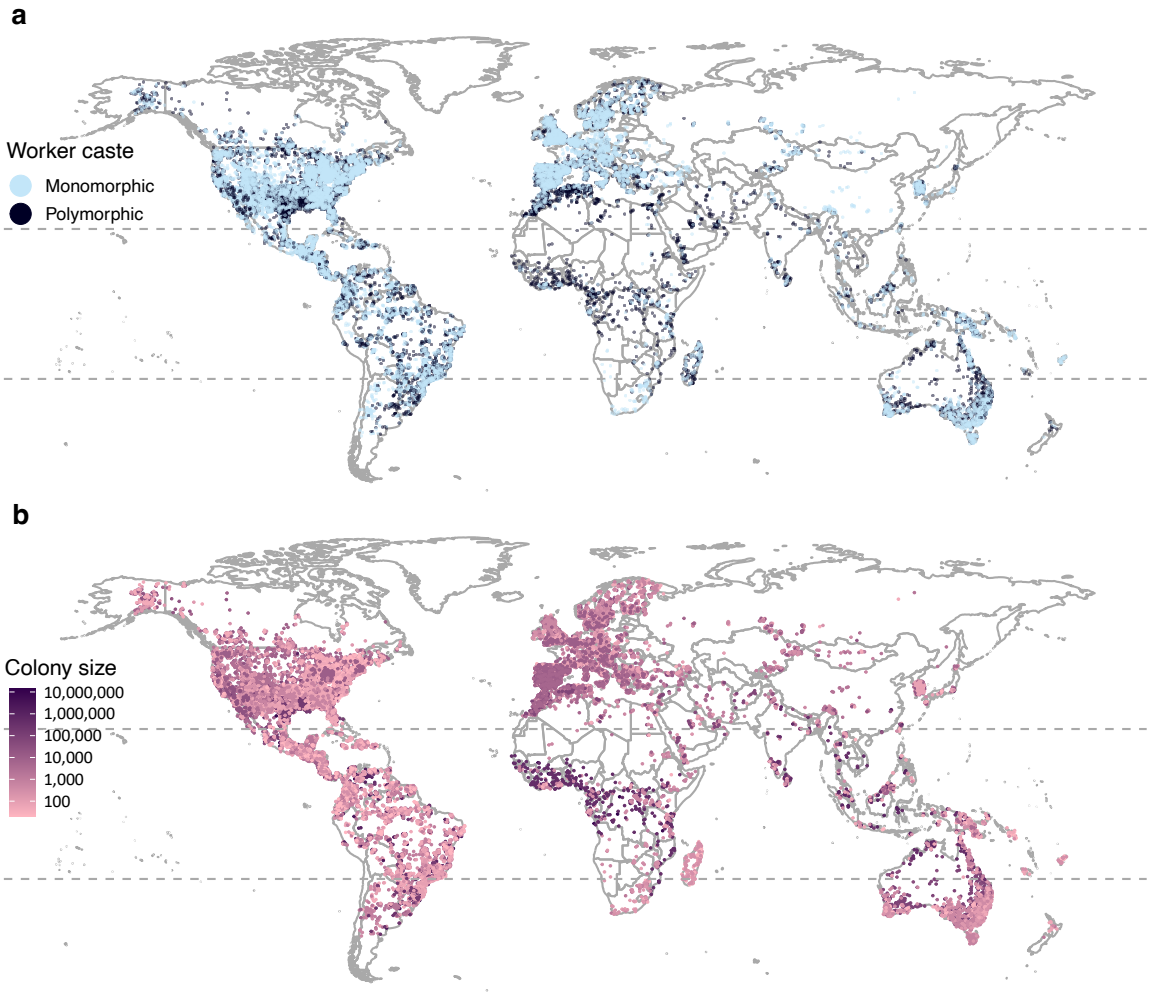


Figure 3. Global map displaying the geographical distribution of worker polymorphism and variation in colony size. Dotted lines delineate tropical (central region) and temperate regions (top/bottom regions) and represent the Tropic of Cancer and Capricorn.

Worker polymorphism associated with hotter and drier climates

At a global scale, we found that species with polymorphic workers are more likely to be found in hotter and drier climates (Figs. 3-4 and Table 1; $n_{species}$= 474). We then repeated this analysis examining species that occur in different regions separately. When analysing species with distributions that overlap with both tropical and temperate regions, we found the same pattern, that worker polymorphism was significantly more likely to be found in hotter and drier climates (Table 1; $n_{species}$ = 168). In contrast, when analysing species exclusive to either tropical regions or temperate regions, we found no correlation between worker polymorphism and any of the climatic variables (Table 1; Tropical: $n_{species}$ = 116; Temperate: $n_{species}$ = 190). There was no correlation between worker polymorphism and diurnal temperature range in any of our analyses (Table 1).

Larger colony sizes associated with drier climates

Globally, we found that species with larger colonies were more likely to be found in drier climates (Figs. 3-4 & Table 2; $R^2_{marginal}$ = 0.0080; $n_{species}$ = 474). We found the same pattern when analysing species that occur exclusively in temperate regions, as well as those with distributions that overlap both temperate and tropical regions (Table 2; Temperate: $R^2_{marginal}$ = 0.041, $n_{species}$ = 190; Temperate & Tropical: $R^2_{marginal}$ = 0.019, $n_{species}$ = 168). In contrast, for species with exclusively tropical distributions, we did not find a significant correlation between colony size and average precipitation (Table 2; $R^2_{marginal}$ = 0.0085, $n_{species}$ = 116). We found no correlation between colony size and average temperature or diurnal temperature range in any of our analyses (Table 2).

Worker polymorphism associated with larger colony sizes

We also examined the relationship between our two measures of social complexity: colony size and worker polymorphism. At a global scale, we found that species with larger colony

sizes were more likely to have polymorphic workers (Supplementary Table 7; $n_{species}$= 474). At all regional scales, species with larger colony sizes were more likely to have polymorphic workers (Supplementary Table 7; Tropical: $n_{species}$ = 116; Temperate: $n_{species}$ = 190; Temperate & Tropical: $n_{species}$ = 168).

Table 1. Parameter estimates for Bayesian phylogenetic mixed models (BPMMs) analysing the relationship between climatic variables and worker polymorphism. Bold text indicates the presence of a significant effect. Parameters estimated separately for the analysis of global species distributions, as well as for distinct geographical regions: (i) tropical; (ii) temperate; and (iii) species with distributions that overlap both temperate and tropical regions.

	Worker polymorphism							
	Global		**Tropical**		**Temperate**		**Tropical & Temperate**	
Predictor	*estimate*	*CI*	*estimate*	*CI*	*estimate*	*CI*	*estimate*	*CI*
Intercept	-1.1	(-5.9, 4.2)	-8.0	(-32, 14)	-1.6	(-7.7, 4.6)	-2.5	(-13, 6.7)
Temperature	**0.16**	**(0.050, 0.28)**	0.37	(-0.34, 1.19)	0.14	(-0.015, 0.32)	**0.29**	**(0.086, 0.54)**
Rainfall	**-0.016**	**(-0.029, -0.0053)**	-0.0035	(-0.019, 0.0089)	-0.019	(-0.048, 0.0039)	**-0.03**	**(-0.056, -0.0057)**
Diurnal temperature range	-0.23	(-0.56, 0.032)	-0.31	(-1.1, 0.27)	-0.15	(- 0.58, 0.21)	-0.078	(-0.69, 0.53)

Table 2. Parameter estimates for the Bayesian phylogenetic mixed models (BPMMs) analysing the relationship between climatic variables and colony size. Bold text indicates the presence of a significant effect. Parameters estimated separately for the analysis of global species distributions, as well as for distinct geographical regions: (i) tropical; (ii) temperate; and (iii) species occurring in both tropical and temperate regions (Both).

	Colony size							
	Global		**Tropical**		**Temperate**		**Tropical & Temperate**	
Predictor	*estimate*	*CI*	*estimate*	*CI*	*estimate*	*CI*	*estimate*	*CI*
Intercept	**2.9**	**(2.0, 4.1)**	2.1	(-3.8, 7.9)	**3.5**	**(2.3, 4.5)**	**3.8**	**(1.1, 6.3)**
Temperature	0.0097	(-0.013, 0.033)	0.027	(-0.17, 0.22)	-0.0092	(-0.038, 0.021)	0.035	(-0.014, 0.092)
Rainfall	**-0.0022**	**(-0.0041, -0.00047)**	-0.0016	(-0.0045, 0.0013)	**-0.0067**	**(-0.011, -0.0023)**	**-0.0064**	**(-0.013, -0.00011)**
Diurnal temperature range	-0.027	(-0.082, 0.023)	-0.068	(-0.10, 0.21)	-0.017	(-0.079, 0.049)	-0.083	(-0.25, 0.068)

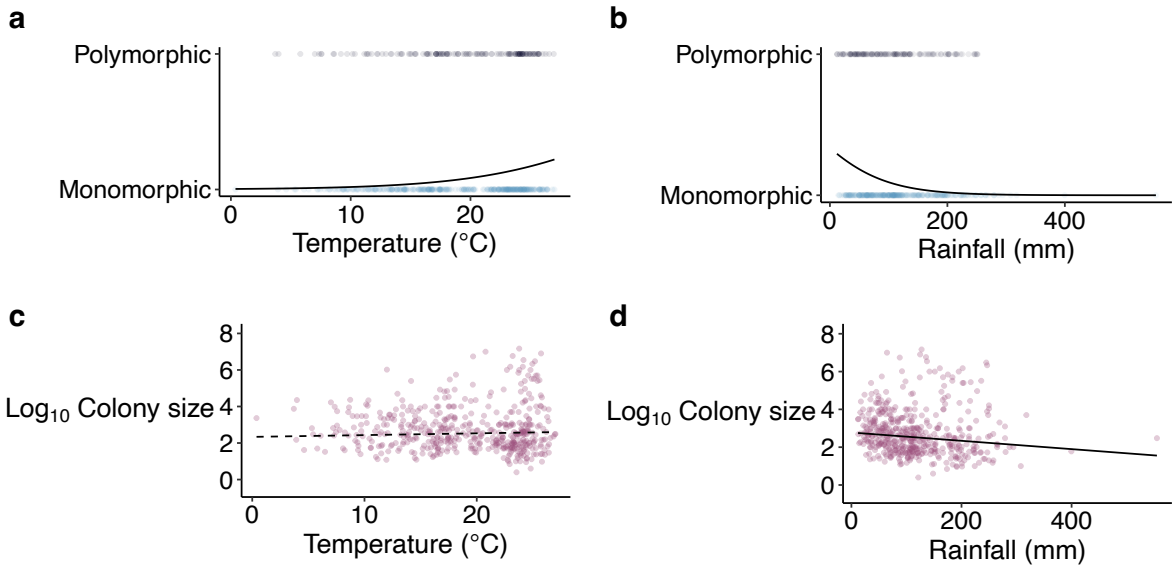


Figure 4. Global relationships between social complexity traits and both temperature and rainfall. We found that species with worker polymorphism are significantly more likely to be found in hotter and drier climates. We found that larger colony sizes are associated with drier climates, but no correlation between colony size and average temperature. We found no relationship between diurnal temperature range (DTR) and either of the social complexity traits. Solid regression lines represent significant relationships, while dashed represents non-significant relationships. Sample sizes = 474; each dot represents a species average.

Analysing climate as either PC1 or PC2

We reanalysed the relationship between social complexity traits and climate using measures of climatic variation (PC1 and PC2) generated by phylogenetic principal component analysis (PCA; Supplementary Table 9). This approach allowed us to combine climatic conditions, such as hot and dry climates, that were identified in the previous regression analyses as being associated with the evolution of greater social complexity. We excluded diurnal temperature range from the creation of PC1 and PC2, as it showed no significant correlation with either social complexity trait. PC1 represents climates on a scale from cold and dry to hot and wet, while PC2 represents climates on a scale from cold and wet to hot and dry.

We found that our results were robust to analysing climatic variables individually (rainfall, temperature, and DTR) or when grouped as PC1 or PC2 (Supplementary Table 8). When analysing PC2, we found that: (i) worker polymorphism was associated with hotter and drier climates; and (ii) larger colony sizes were associated with hotter and drier climates, but only at a global scale. When analysing PC1, we found that: (i) there was no association between climate and worker polymorphism; and (ii) larger colony sizes were associated with colder and drier climates, but only in species with temperate distributions. Regardless of analysing PC1 or PC2, we found no association between climate either worker polymorphism or colony size in tropical regions.

Phylogenetic path analysis

We used phylogenetic path analysis to test four alternative causal models of the relationships between colony size, worker polymorphism and climate (Supplementary Fig. 3). When analysing the rainfall and temperature climatic variables in the same causal model, but without hypothesising a causal relationship between them, we found that all possible model combinations were rejected. Therefore, we modelled climate as PC1 and PC2 to investigate the role of temperature and rainfall in the same path analysis models. We conducted two analyses, modelling climate as PC1 and PC2 separately.

Across all analyses, larger colony sizes favoured the evolution of worker polymorphism. When analysing climate using PC2, we found support for worker polymorphism facilitating the colonisation of hotter and drier climates, and mixed support for hotter and drier climates favouring the evolution of larger colony sizes. At a global level, we found evidence for worker polymorphism facilitating the colonisation of hotter and drier climates (Fig. 5a;

relative weight of support for a model within a set of competing models, ω, is 0.46; $n_{species}$ = 474). We also found support for a small but significant effect of hotter and drier climates favouring the evolution of larger colony sizes (Fig. 5a; ω = 0.49). When analysing species at a regional scale, we observed distinct patterns between regions: (i) In temperate regions, worker polymorphism facilitates the colonisation of hotter and drier climates (Fig. 5c; Temperate: ω = 0.38; $n_{species}$ = 190). (ii) In species with distributions that overlap both tropical and temperate regions, worker polymorphism also facilitates the colonisation of hotter and drier climates, and this was the only supported model (Fig. 5d; Tropical & Temperate: ω = 0.92; $n_{species}$ = 168). (iii) In tropical regions, we found no relationship between climate and either worker polymorphism or colony size (Fig. 5b; $n_{species}$ = 116).

When analysing climate using PC1, we found mixed support for an influence of climate on worker polymorphism or colony size. When analysing species in temperate regions, drier and colder climates sometimes favour the evolution of larger colony sizes (Supplementary Fig. 4c; ω = 0.71; $n_{species}$ = 190). However, sometimes the causal direction was uncertain, depending on phylogeny (Supplementary Fig. 7 and Supplementary Tables 2-3). We found no evidence supporting a relationship between climate and either worker polymorphism or colony size at any other regional scale (Supplementary Fig. 4; Global: $n_{species}$ = 474; Tropical: $n_{species}$ = 116; Tropical & Temperate: $n_{species}$ = 168).

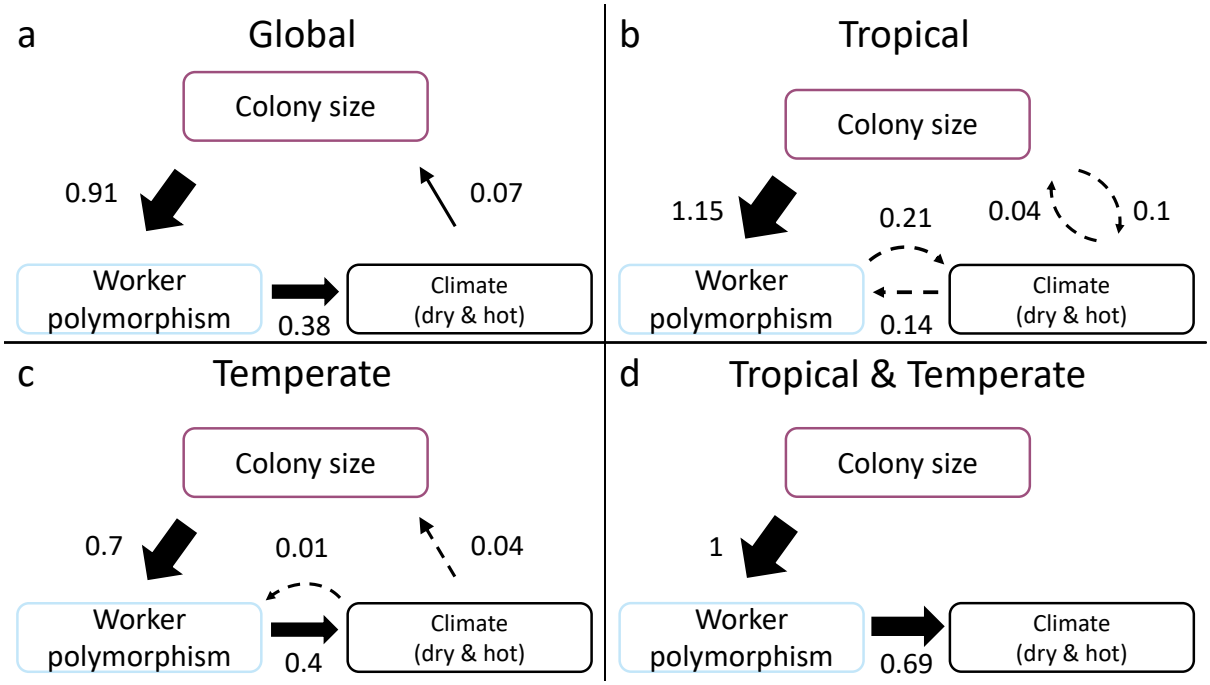


Figure 5. Average model from phylogenetic path analysis across different regional scales. Climate is modelled using PC2, where increasing values for this trait indicate hotter and drier climates, while decreasing values indicate colder and wetter climates. Globally, worker polymorphism facilitates the colonisation of hotter and drier climates, while hotter and drier climates also favour the evolution of larger colony sizes. In species that occur exclusively in temperate regions and species with distributions overlapping tropical and temperate regions, worker polymorphism facilitates the colonisation of hotter and drier climates. In tropical regions, we found no relationship between climate and either worker polymorphism or colony size. We analysed our species across four different geographical scales: (a) globally; (b) with distributions exclusive to tropical regions; (c) with distributions exclusive to temperate regions; and (d) with distributions that overlap both tropical and temperate regions. Solid arrows indicate the presence and direction of significant relationships between variables, with values representing the standardised average path coefficients. Path coefficients describe the strength and direction of the relationship between two variables in terms of their correlated variance. Larger coefficients are denoted by a heavier line. Dashed arrows represent non-significant relationships between variables. We

display the results from the analysis of one maximum clade credibility (MCC) consensus tree. However, we repeated the analyses on three additional MCC trees and found consistent results across trees (Supplementary Figs. 9-12 and Supplementary Tables 4-5). Worker polymorphism is modelled as a discrete binary variable (monomorphic/polymorphic) while colony size and climate are modelled as continuous variables.

Discussion

Consistent with previous studies, our results indicate that species exhibiting polymorphic workers are more commonly associated with hotter and drier climates (Figs. 4a-b) (La Richelière *et al.*, 2022), as well as larger colony sizes. We also found that species with larger colony sizes are more likely to be found in drier climates (Fig. 4d). Next, we investigated the potential causal relationships between social complexity traits and climate. To capture climatic variation, we used PC2, which represents climate on a scale from cold and wet to hot and dry. Our analysis revealed that worker polymorphism facilitates the colonisation of hotter and drier climates (Fig. 5). At a global scale, we also found support for hotter and drier climates favouring the evolution of larger colony sizes (Fig. 5a). Lastly, our results suggest that the relationship between climatic variables and social complexity traits is impacted by the geographical scale over which these traits are analysed, as contrasting results could sometimes be obtained across different regions (Fig. 5 and Tables 1-2).

In line with La Richeliére et al., 2022, we identified a global pattern indicating that species exhibiting worker polymorphism are more frequently found in hotter and drier climates (Figs. 4a-b). However, in contrast to theoretical predictions (Covas, Du Plessis and Doutrelant, 2008; Rubenstein, 2011; Griesser *et al.*, 2017; Kennedy *et al.*, 2018), our results suggest that this positive association is not explained by harsh environments selecting for greater levels of

cooperation. Instead, worker polymorphism evolves in relatively benign environments, due to increases in colony size (Bonner, 1993; Bourke, 2011; Ferguson-Gow *et al.*, 2014; Bell-Roberts *et al.*, 2024) and likely other additional factors (Oster and Wilson, 1978; Powell and Franks, 2006; Powell, 2008, 2009, 2016; Wills *et al.*, 2018), which then allows species to colonies harsh environments, such as those with hot and dry climates (Fig. 5). While the definition of a harsh environment depends on the traits of the organism in question, higher temperature and lower rainfall will create an environment where resources such as food and water may become scarce, and death due to desiccation is a serious concern. Therefore, worker polymorphism may facilitate the colonisation of these environments due to: i) the differential ability of workers of different sizes to forage under a range of climatic conditions (Lighton, Quinlan and Jr, 1994; Cerdá and Retana, 2000; Baudier *et al.*, 2015); ii) the colony's ability to exploit a wider range of resources when workers are more variable (Davidson, 1977; Heredia and Detrain, 2005); and iii) the advantages gained in competitive and defensive interactions if a species possesses a soldier caste (Passera *et al.*, 1996; Powell, 2009)(for a more detailed discussion of the potential benefits of worker polymorphism in hot and dry climates, see La Richeliére et al., 2022). Our findings align with a previous study on birds, which found that cooperative breeding evolves before the colonisation of harsh environments (Cornwallis *et al.*, 2017). Together, these results highlight the crucial role of cooperation in enabling adaptive breakthroughs that allow the colonisation of new ecological niches.

We also found that larger colony sizes are associated with drier climates at all geographical scales, except when analysing species with exclusively tropical distributions (Fig. 4d). Globally, our path analysis identified that the association between larger colony sizes and drier climates is driven by drier climates favouring larger colony sizes (Fig. 5a). Drier

climates may favour the evolution of larger colony sizes for several reasons. For example, if drier climates are limited in essential resources, such as food or water, larger colonies may be better at surviving through periods of starvation or limited access to water (Kaspari and Vargo, 1995), which may be frequent or prolonged. Larger colonies can store greater quantities of resources, as workers and brood represent a standing reserve of resources that can be catabolised in times of stress (Wilson, 1971; Sorensen, Busch and Vinson, 1983; Kaspari and Vargo, 1995). Alternatively, larger colony sizes may be advantageous because they reduce the variance in the expected amount of food that can be foraged over time (Wenzel and Pickering, 1991; Stevens, Hogendoorn and Schwarz, 2007). Therefore, larger colonies can more precisely match egg production to expected food intake, effectively reducing the potential costs associated with producing too many or too few brood. However, the strength of the relationship between rainfall and colony size is relatively weak (Globally: $R2_{marginal} = 0.0080$). This suggests that other factors are also important in shaping the evolution of colony size. For instance, colony size plays an important role in colony defence and in competitive interactions with other ant colonies for resources (Adams, 1990; Blanchard and Moreau, 2017; Champer and Schlenoff, 2024). As a result, biotic interactions may have a greater influence in determining colony size than climatic conditions.

Across all regions, we found strong support for larger colony sizes favouring the evolution of worker polymorphism (Fig. 5). However, the relationships identified between the climatic variables and both colony size and worker polymorphism varied depending on the geographical scale analysed (Figs. 4-5, Tables 1-2, and Supplementary Fig. 4). For instance, while we observed a relationship between social complexity and climate across all geographical scales, this relationship was absent in tropical regions (Fig. 5). The lack of a consistent pattern across geographical regions is likely due to their distinct ecological

characteristics, which directly influence the costs and benefits of various forms of cooperation (Kaspari and Vargo, 1995; Lancaster, Wilson and Espinoza, 2006; Paquet *et al.*, 2016; Cornwallis *et al.*, 2017; Lukas and Clutton-Brock, 2017; Wiedenová, Šumbera, and Jan Okrouhlík, 2018; Firman *et al.*, 2020; Guindre-Parker and Rubenstein, 2020; Martin *et al.*, 2020; Riehl and Smart, 2022). In tropical regions, abiotic factors may exert a weaker influence compared to biotic factors (Schemske *et al.*, 2009), where intense competition for resources and higher levels of predation play a more significant role (Jeanne, 1979; Kaspari and O'Donnell, 2003; Kaspari, 2005). For example, army ant raids are more prevalent in the tropics (Kaspari and O'Donnell, 2003), which may select for individuals specialised in defence against them. Alternatively, intense resource competition has been shown to drive the evolution of worker polymorphism in at least two ant genera (Powell and Franks, 2006; Powell, 2008, 2016). As a result, future studies should aim to address the relationship between climate and social complexity in ants across variable geographic and taxonomic scales (Purcell, 2011; Ferguson-Gow *et al.*, 2014), as different processes may vary in importance across scales.

Economo et al., 2018 identified that the latitudinal diversity gradient in ants—where tropical regions exhibit greater species diversity than temperate regions—can be attributed to the varying amounts of time that clades have had to diversify in different regions. Migrations of ant lineages from tropical regions into colder temperate regions began following the onset of the Oligocene, 34 million years ago (Economo *et al.*, 2018). This explains the higher species diversity in the tropics, as these clades have had more time to diversify. Combined with the results of our analysis (Fig. 5a), this suggests that the evolution of worker polymorphism is not able to explain how ants migrated from the tropics to the colder northern latitudes and adapt to this new ecological niche. Instead, other traits, such as leg length (Allen, 1877;

Sommer and Wehner, 2012), body size (Bergmann, 1847; Cushman, Lawton and Manly, 1993), or physiological adaptations might have been important in facilitating this geographical shift. However, given that temperate lineages are younger than tropical lineages (Economo *et al.*, 2018), this also opens the possibility that the association between worker polymorphism and hotter climates (Fig. 4a) could potentially be the consequence of evolutionary and biogeographic history. If worker polymorphism is a trait that evolves slowly and more often in older lineages, hotter climates may be associated with worker polymorphism because tropical lineages have had more time for polymorphism to evolve. However, this explanation seems unlikely as worker polymorphism is an evolutionarily labile trait that has evolved multiple times across a diverse number of ant clades (Fig. 2) (Blanchard and Moreau, 2017; Wills *et al.*, 2018; Bell-Roberts *et al.*, 2024). Given that worker polymorphism evolves frequently, we would not expect polymorphism to be associated with hotter climates solely because temperate species haven't had sufficient time to evolve this trait. Furthermore, we would expect that differences between lineages in the ability to evolve worker polymorphism would be captured and accounted for by the phylogenetic component in our statistical analyses.

Having identified that worker polymorphism facilitates the colonisation of hotter and drier climates (Fig. 5), future research should focus on explaining how this trait enables ant species to survive in harsh environments (Cerdá and Retana, 1997, 2000). For example, within-species studies could examine whether in harsh environments, worker polymorphism increases the overall food intake from foraging or reduces its variability (Wenzel and Pickering, 1991; Stevens, Hogendoorn and Schwarz, 2007; Kennedy *et al.*, 2018). To test this hypothesis, the amount of food collected by mature colonies with polymorphic workers could be compared against experimentally manipulated colonies with lower levels of

polymorphism, or incipient colonies where the full extent of worker polymorphism has not yet developed. Alternatively, studies could investigate how factors such as predation, diet preference, or resource competition are associated with social complexity traits (Heredia and Detrain, 2005; Kaspari, 2005; Powell and Franks, 2006; Powell, 2008, 2016; Dornhaus, Powell and Bengston, 2012; Wills *et al.*, 2018). Considering the fundamental role of social complexity traits in mediating interactions between ant colonies and their environment, identifying the selective forces that shape these traits, or the ecological benefits that they bring, is a crucial aim.

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Chapter 4: Looking for signatures of kin selection in the genomes of social insects

Abstract

Kin selection theory provides a framework for understanding the evolution of cooperation between relatives. However, prior to the recent explosion in the availability of genomic data, it was not possible to perform a test of Hamilton's rule at the genomic level. We tested the predictions made by kin selection theory that genes with indirect fitness effects experience relaxed selection relative to genes with direct fitness effects using a phylogenetic comparative analysis across 8 species of social insects. First, we identified genes evolving under direct or indirect selection, by identifying genes with caste-biased expression. Then, using phylogenetic regression analysis, we tested the hypothesis that species with lower levels of intra-colony relatedness harbour greater levels of sequence polymorphism and divergence (rate of protein evolution) in worker-biased genes relative to queen-biased genes. Overall, we found mixed results. Consistent with theoretical predictions, we found that species with higher queen mating frequencies harbour significantly greater levels of polymorphism at non-synonymous sites in worker-biased genes relative to queen biased genes. Contrasting with theoretical predictions, we did not find a significant relationship between queen mating frequency and levels of divergence in worker-biased genes relative to queen biased genes, at non-synonymous sites. We found no other relationships between queen mating frequency and other measures of polymorphism and divergence. Our analysis serves as a test of Hamilton's rule at the genomic level, and despite the use of a relatively small sample size, we are still able to detect some of the predictions made by kin selection theory. However, as more genomic data becomes available, future studies using a larger

sample size will be necessary to conclusively determine whether a signature of kin selection can be identified within the genomes of social insects.

Introduction

Cooperation between individuals is observed across the tree of life and at multiple levels of biological organisation, from single-celled organisms to complex societies of social insects (West, Griffin and Gardner, 2007). Bacteria rely on cooperative behaviours to produce essential "public goods" (Griffin, West and Buckling, 2004; Sandoz, Mitzimberg and Schuster, 2007; Xavier, Kim and Foster, 2011), while social insects, with their distinct reproductive and sterile castes, cooperate in tasks crucial for colony reproduction and survival, such as foraging and defence (Oster and Wilson, 1978; Hölldobler and Wilson, 1990). Kin selection theory provides a framework for understanding the evolution of cooperation at all biological scales, explaining how genes encoding cooperative traits can be favoured by selection and spread within populations (Hamilton, 1963, 1964). However, there is a growing recognition that as cooperative traits evolve, they may subsequently influence the molecular evolution of the genome itself (Linksvayer and Wade, 2009, 2016; Hall and Goodisman, 2012; Van Dyken and Wade, 2012; Ostrowski *et al.*, 2015; Vojvodic *et al.*, 2015; Noh *et al.*, 2018; Rubenstein *et al.*, 2019; Belcher *et al.*, 2022, 2023). What are the consequences of the evolution of social behaviours on patterns of genomic variation?

Theoretical work has clarified how patterns of sequence diversity are expected to differ between genes that are subject to direct selection compared to those that are shaped indirectly by the process of kin selection (Linksvayer and Wade, 2009, 2016; Hall and Goodisman, 2012). Genes with indirect effects on fitness are often expected to experience weaker selection compared to genes with direct effects on fitness (Linksvayer and Wade, 2009). This

prediction arises because genetic relatedness influences the inclusive fitness benefits derived by an actor performing a cooperative behaviour. For example, as relatedness decreases between social partners, the inclusive fitness benefits of preforming a cooperative behaviour will also decrease. The dilution of the kin-selected benefit of cooperation weakens the strength of selection and reduces the likelihood of beneficial mutations becoming fixed while increasing the likelihood of deleterious mutations becoming fixed (Linksvayer and Wade, 2009). As a result, genes with indirect fitness effects are expected to harbour greater levels of sequence polymorphism (genetic variation) relative to genes with direct fitness effects (Linksvayer and Wade, 2009). In addition, genes with indirect fitness effects are predicted to evolve faster, assuming an elevated fixation rate of alleles with mildly deleterious effects on fitness, resulting in greater levels of divergence (rate of protein evolution) (Linksvayer and Wade, 2009; Hall and Goodisman, 2012).

Although genes with indirect effects on fitness are generally expected to experience weaker selection compared to those with direct effects (Linksvayer and Wade, 2009; Hall and Goodisman, 2012), under certain social conditions, this prediction is not expected to hold. Specifically, under conditions of maximal genetic relatedness, the strength of selection acting on genes with direct fitness or indirect fitness effects is predicted to be equal (Hall and Goodisman, 2012; Linksvayer and Wade, 2016). Maximal relatedness can be found in clonal populations or in family groups with monogamous mating in non-clonal populations. This prediction arises from the presence of relatedness symmetries within these groups. In clonal populations, all individuals share the same set of genes. Therefore, inclusive fitness benefits may be accrued with the same efficacy by helping clonemates or reproducing independently, depending on the costs of these alternate strategies. In monogamous family groups, the level of relatedness between offspring and their siblings is equivalent to the level of relatedness

between a parent and their offspring. Therefore, in terms of inclusive fitness, it's equally beneficial for an offspring within the group to help its parents reproduce as it is for it to reproduce itself, given that the value of the potential costs associated with these two different strategies are the same. As a result, genes with indirect fitness effects are expected to harbour equivalent levels of polymorphism and divergence relative to genes with direct fitness effects, as the strength of selection acting on these genes is predicted to be the same (Hall and Goodisman, 2012; Linksvayer and Wade, 2016).

Social insects are ideal candidates to test theoretical predictions regarding the impact of social behaviours on molecular evolution. This is due to the presence of reproductive and sterile castes which influence the way in which natural selection operates: queens obtain direct fitness by reproducing, while workers may obtain indirect fitness by helping the queen. Therefore, genes upregulated in queens (queen-biased genes) are expected to experience predominantly direct selection, while genes upregulated in workers (worker-biased genes) will largely be subject to indirect selection (Linksvayer and Wade, 2009). This dynamic predicts that worker-biased genes will experience weaker selection compared to queen-biased genes, but only in species with multiply mating queens or multiple reproductive queens per colony (Fig. 1; i.e. any factor that might reduce intra-colony relatedness) (Linksvayer and Wade, 2009, 2016; Hall and Goodisman, 2012). The relative decrease in the strength of selection acting on worker-biased genes will result in greater levels of polymorphism and divergence.

Despite clear theoretical predictions (Linksvayer and Wade, 2009; Hall and Goodisman, 2012), a definitive test to determine whether a signature of kin selection can be detected in the genomes of social insects has yet to be performed. Although several studies have explored differences in the strength of selection or rates of protein evolution between genes

with caste-biased expression (Hunt *et al.*, 2010, 2011; Hall and Goodisman, 2012; Harpur *et al.*, 2014; Vojvodic *et al.*, 2015; Warner, Mikheyev and Linksvayer, 2017; Imrit *et al.*, 2020; Harrison, Chernyshova and Thompson, 2021), these studies alone are insufficient to resolve this question. For instance, Vojvodic et al., 2015 identified that genes associated with indirect fitness effects evolved more rapidly than those associated with direct fitness effects. While this result is consistent with kin selection theoretical predictions, an alternative interpretation is that these genes may be subject to elevated levels of positive selection, which could also generate increased rates of gene evolution (Belcher *et al.*, 2022). Therefore, to distinguish between the effects of kin selection and other selective pressures, such as positive or balancing selection, it is crucial to estimate both genetic divergence and polymorphism within social insect genomes, as each type of selection is expected to produce distinct patterns in these genetic measures (Table 1). Furthermore, studies must account for intra-colony genetic relatedness, as theory only predicts elevated divergence and polymorphism in worker-biased genes relative to queen-biased genes in species with multiply mated queens or multiple queens per colony (Hall and Goodisman, 2012; Linksvayer and Wade, 2016). Therefore, to test whether a signature of kin selection can be detected in the genomes of social insects, it is necessary to examine both the relative levels of polymorphism and divergence between caste-biased genes across a range of social insect species that vary in their level of intra-colony relatedness, controlling for their shared evolutionary history.

We performed a phylogenetic comparative analysis examining whether signatures of kin selection can be detected within the genomes of social insects. We tested the hypothesis that species with lower levels of intra-colony relatedness harbour greater levels of polymorphism and divergence in worker-biased genes relative to queen-biased genes. We focussed on species which have evolved morphologically distinct queen and worker castes, which are fixed for life and determined during development (superorganisms) (Crespi and Yanega,

1995; Boomsma and Gawne, 2018). Previous studies indicate that the strength of selection acting on species varies as a function of the level of social complexity, emphasising the importance of controlling for this factor in comparative analyses (Imrit *et al.*, 2020). Therefore, we analysed data across eight species of ants and bees which have morphological castes and for which the required genetic sequence data was available. We began our analysis by identifying genes with caste-biased expression, followed by estimating the levels of polymorphism and divergence for each of these caste-biased genes. Last, we examined the relationship between intra-colony relatedness and relative polymorphism and divergence using phylogenetic regression analysis. Only recently have tests of this kind become possible due to the increasing availability of genomic data and may help to disentangle the molecular causes and consequences for the evolution of superorganismal social insects.

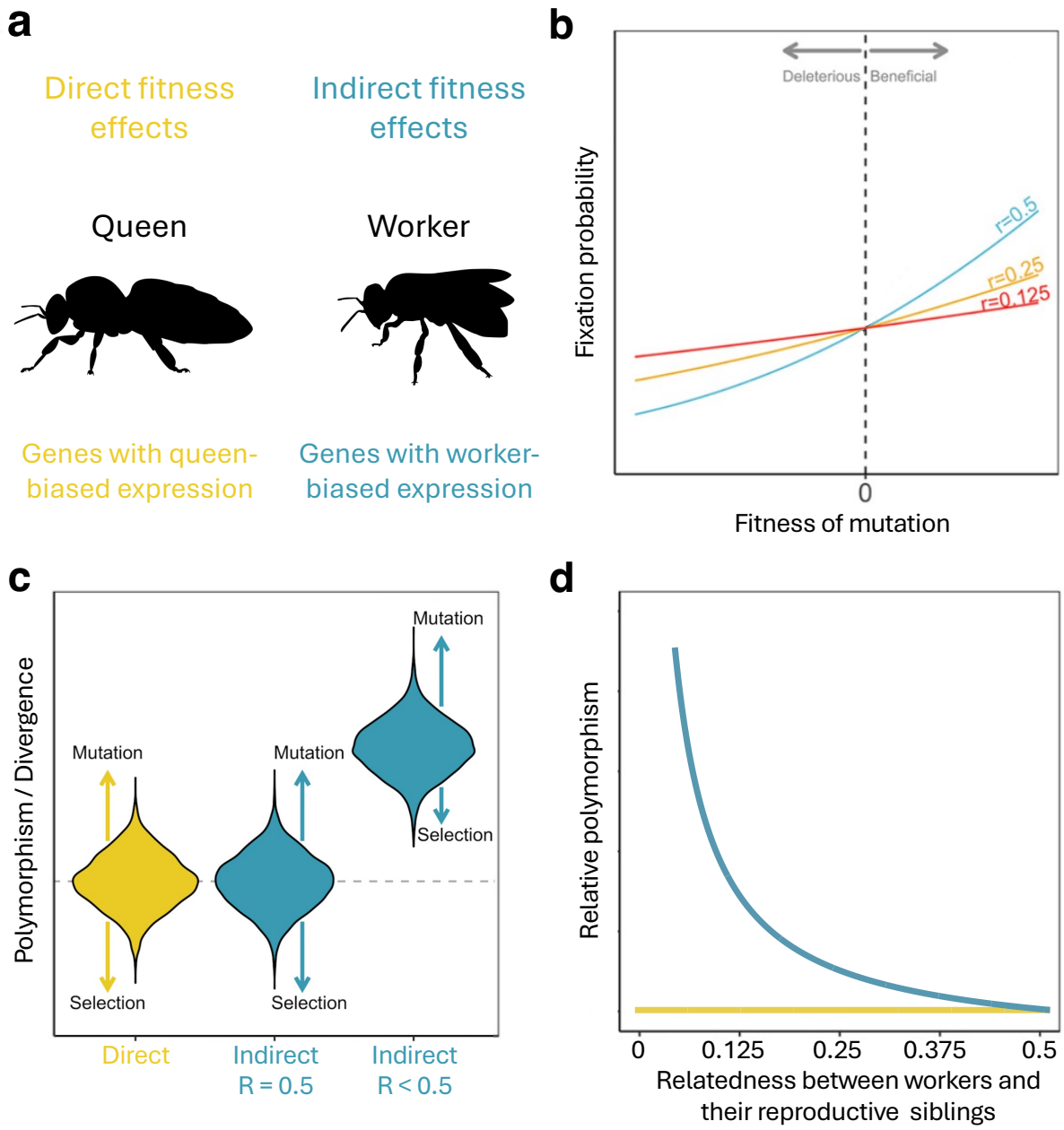


Figure 1. Population genetic theory for genes evolving under kin selection (Linksvayer and Wade, 2009; Hall and Goodisman, 2012). (*a*) Representation of how genes are categorised as possessing either direct or indirect fitness effects. In social insect species that possess reproductive and sterile castes, queens obtain direct fitness by reproducing, while workers may obtain indirect fitness by helping the queen. Therefore, genes with indirect fitness effects are those that have worker-biased expression, while genes with direct fitness effects are those that have queen-biased expression. (*b*) Probability of fixation for deleterious or beneficial mutations of varying effect as relatedness between workers and their

reproductive siblings (new gynes and drones) changes. In colonies with one singly mated queen, where the relatedness (*r*) between workers and their reproductive siblings = 0.5, the prediction is the same for mutations influencing genes with direct and indirect fitness effects (blue line). As relatedness diminishes, the prediction shifts regarding mutations that impact genes with indirect fitness effects (yellow and red lines). Beneficial mutations become less likely to fix and deleterious mutations become more likely to fix. Therefore, in colonies with either multiple reproductive queens or a multiply mated queen, there is relaxed selection on genes with indirect fitness effects relative to those with direct fitness effects. (c) Prediction for relative polymorphism and divergence for genes with direct fitness effects (yellow) compared to genes with indirect fitness effects (blue). r = relatedness between workers and their fully reproductive siblings. If relatedness, r = 0.5, then genes with indirect fitness effects (middle blue violin) should have the same polymorphism and divergence as genes with direct fitness effects (left yellow violin). In contrast, if $r < 0.5$, then genes with indirect fitness effects (right blue violin) should show greater polymorphism and divergence than genes with direct fitness effects. Polymorphism and divergence increase due to the increased likelihood of fixation for deleterious mutations and decreased likelihood of fixation for beneficial mutations. (d) The predicted level of polymorphism for genes with direct fitness effects (queen-biased genes; yellow) and indirect fitness effects (worker-biased genes; blue) as relatedness varies. In queen-biased genes, the level of polymorphism is independent of relatedness (yellow). In worker-biased genes, the expected level of polymorphism relative to queen-biased genes is inversely proportional to relatedness (blue). For colonies which possess a single once-mated queen, resulting in the level of relatedness between workers and their reproductive siblings = 0.5, there is no expected difference in polymorphism between queen-biased and worker-biased genes (intersection of the two lines). When relatedness = 0,

cooperation is no longer favoured, so relatedness no longer predicts the level of polymorphism observed. Adapted from (Belcher *et al.*, 2022, 2023).

Table 1. Levels of divergence and polymorphism are shown as values for genes with indirect fitness effects (worker-biased) relative to genes with direct fitness (queen-biased). For positive, balancing, and purifying selection, the predictions refer to scenarios where these types of selection disproportionately affect genes upregulated in workers. Table modified from Belcher et al., 2022.

Selection type	Divergence	Polymorphism
Positive/directional	High	Low
Kin	High	High
Balancing	Low	High
Purifying	Low	Low

Methods

We investigated whether signatures of kin selection can be detected in the genomes of social insects. We aimed to examine as many superorganismal species of social insects as possible, given the genomic and life history data available in the literature. We examined eight species because these were the only species where the necessary data was available to test our hypothesis. First, we identified genes with direct or indirect fitness effects by examining which genes had biased expression in queens or workers. Then, we calculated estimates for divergence and polymorphism for each of the caste-biased genes. Last, we performed a phylogenetic comparative analysis comparing the relative strength of selection acting on

worker-biased genes in comparison to queen-biased genes, as a function of intra-colony relatedness.

SNP calling

We identified single nucleotide polymorphisms (SNPs) from raw sequencing reads obtained for each of the 8 species (Fig. 2) and their respective outgroups. To accurately estimate population genetic parameters, we aimed to sample 20 individuals from a single population for each species, although using eight individuals has been shown to be sufficient to calculate accurate estimates of genetic diversity (Nazareno *et al.*, 2017). We analysed 20 individuals for all species except for *Camponotus floridanus* (19), *Formica exsecta* (7) and *Acromyrmex echinatior* (9). We downloaded sequence reads from the European Bioinformatics Institute's European Nucleotide Archive (EBI; www.ebi.ac.uk/ena; Supplementary Tables 1 & 2). For each species, we trimmed reads to remove adapters and low-quality reads using Trimmomatic v0.39 (Bolger, Lohse and Usadel, 2014). We removed leading and trailing reads with quality scores less than 3 and removed reads if the average quality score in a four base sliding window was less than 20. We then used BWA v0.7.17 to map and align reads for each species to their respective reference genome (Li and Durbin, 2009). Next, we used SAMtools v1.17 to sort and convert the resulting SAM files to BAM files (Li *et al.*, 2009). We called variants for all species and converted the results to a VCF file for analysis using BCFtools v1.17 (Li, 2011). Last, we performed quality filtering by removing variants with quality scores lower than 20 and INDELs, and used the vcfutils Perl script in SAMtools, applying default settings (Li *et al.*, 2009). These settings included criteria such as a minimum read depth (=2) and a minimum p-value for strand bias (=0.0001).

Identifying caste-biased genes

We identified caste-biased genes using gene expression datasets for each species. We prioritised the use of transcriptomic data from brain tissue for analysis because the brain is the centre of behavioural regulation, and behaviour is a defining feature of castes. When brain tissue was not available, we used alternative tissue types (Supplementary Table 3). However, the type of tissue that we used was kept consistent between queens and workers within the same species. We aimed to use samples from at least 3 queens and 3 workers for each species. However, data was only available for one queen and two workers for *Camponotus floridanus* and two queens and two workers for *Apis mellifera* and *A. cerana* (Supplementary Table 3).

We started by trimming RNAseq reads to remove adapters and low-quality reads using the same settings described in '*SNP calling'*. Using Salmon v1.9.0, we quantified transcript expression levels from the RNAseq reads, calculating relative estimates of abundance for each transcript in the sample (Patro *et al.*, 2017). Using tximport v1.26.1, we aggregated the quantified expression data into gene-level expression estimates and imported the resulting read count matrix into DESeq2 v1.38.3 (Love, Huber and Anders, 2014; Soneson, Love and Robinson, 2016). We compiled a list of genes exhibiting caste-biased expression using DESeq2, considering genes as differentially expressed if they exhibited at least twice the level of expression in one caste compared to the other and if their adjusted p-value was less than 0.05. When using a two-fold change in expression, DESeq2 maintains a very low false discovery rate (~0%) and a true positive rate of ~75% when identifying differentially expressed genes even at low sample sizes of 3 replicates per treatment (Schurch *et al.*, 2016). At these small sample sizes, however, using a lower fold-change threshold may result in a decrease in the true positive rate (Schurch *et al.*, 2016).

Population genetic analysis

For each species, we calculated estimates of nucleotide diversity (polymorphism) for protein-coding sequences and rates of protein evolution (divergence) at genes identified as having caste-biased expression. We used the PopGenome v2.7.7 package to calculate overall estimates of polymorphism, as well as individual estimates for synonymous and non-synonymous sites, for each gene (Pfeifer *et al.*, 2014). All estimates of polymorphism were scaled according to their relevant mapped length, which was the proportion of the gene identified as containing high-quality SNPs, to obtain measures of polymorphism per site. To calculate rates of divergence, quantified as the number of substitutions per site, we compared a reference genome to an outgroup from a separate population. We extracted SNPs for the outgroup from the VCF file generated during SNP calling and inserted them into the sequence of the reference genome using the 'FastaAlternateReferenceMaker' tool available in the GATK v4.5.0.0 suite (McKenna *et al.*, 2010). Using the seqinR v4.2-36 package, we compared this pseudo-genome sequence to the original reference genome sequence and calculated estimates for divergence at non-synonymous sites, synonymous sites, and the ratio of divergence at non-synonymous sites to synonymous sites (referred to as k_A, k_S and k_A/k_S, respectively) for each gene (Charif and Lobry, 2007). Genes that were not aligned between the two sequences were removed from the analysis.

Phylogenetic comparative analysis

For each species, we collected data on effective queen mating frequency and intra-colony relatedness (Supplementary Table 4). Data for effective queen mating frequency (hereafter referred to as queen mating frequency) accounts for the impact of biased sperm use by queens that have mated multiple times (Pamilo, 1993; Boomsma and Ratnieks, 1996). Therefore, it provides a more accurate reflection of how mating multiple times affects the level of intra-

colony relatedness. This data was sourced from (Bell-Roberts *et al.*, 2024) which summarised all available data in the literature on queen mating frequencies in ants. For non-ant species, we searched Web of Science using the keywords: 'Species name' AND (monandr* OR polyandr* OR effective-mating-frequenc* OR mating-frequenc* OR paternity-frequenc* OR mating-system* OR sociogenetic-structure*). We collected data on intra-colony relatedness from (Wenseleers and Ratnieks, 2006) which summarised data in the literature on intra-colony relatedness.

We controlled for the phylogenetic relationships between species in our analysis, as species traits cannot be considered independent as they share characteristics through common descent (Felsenstein, 1985; Harvey and Pagel, 1991). We constructed a phylogeny for the species under study from published sources (Johnson *et al.*, 2013; Nelsen, Ree and Moreau, 2018). However, combining phylogenetic trees resulted in the loss of branch length information. Therefore, we standardised branch lengths using Grafen's method, so that all branches were of equal length between the root and the tips, with the 'compute.brlen' function from the R package ape v5.7-1 (Grafen, 1989; Paradis and Schliep, 2019).

We performed a phylogenetic comparative analysis comparing the relative strength of selection acting on worker-biased genes in comparison to queen-biased genes, as a function of intra-colony relatedness. For each species, we computed average values for polymorphism and divergence from gene-level estimates, calculating values for worker-biased and queen-biased genes separately. Next, we calculated relative estimates of polymorphism and divergence by comparing worker-biased genes against queen-biased genes ($\text{relative polymorphism} = \frac{\text{average nucleotide diversity}_{\text{worker}}}{\text{average nucleotide diversity}_{queen}}$; $\text{relative divergence} = \frac{\text{average divergence}_{\text{worker}}}{\text{average divergence}_{queen}}$). We calculated values for relative polymorphism and relative

divergence to produce simplified metrics for each species, rather than using numerous estimates of polymorphism and divergence based on the number of caste-biased genes identified. This approach facilitated easier comparison in a comparative analysis (Supplementary Figures 3-6).

We then tested whether relative polymorphism or relative divergence correlated with queen mating frequency or intra-colony relatedness. Estimates of intra-colony relatedness capture the influence of both the number of reproductive queens per colony as well as the number of times that the queen mates. However, given that estimates of relatedness were available for only 6 species, we chose to also analyse queen mating frequency, as data was available for all 8 species in our analysis. We modelled these relationships using Bayesian phylogenetic mixed models (BPMMs) with Markov chain Monte Carlo (MCMC) estimation in the MCMCglmm package v2.34 (A detailed summary of the model settings and assessments is available in the Supplementary Material) (Hadfield, 2010). All variables were modelled as continuous traits, and we applied a Gaussian error distribution. We report parameter estimates from models as posterior modes along with 95% lower and upper credible intervals (CIs).

Results

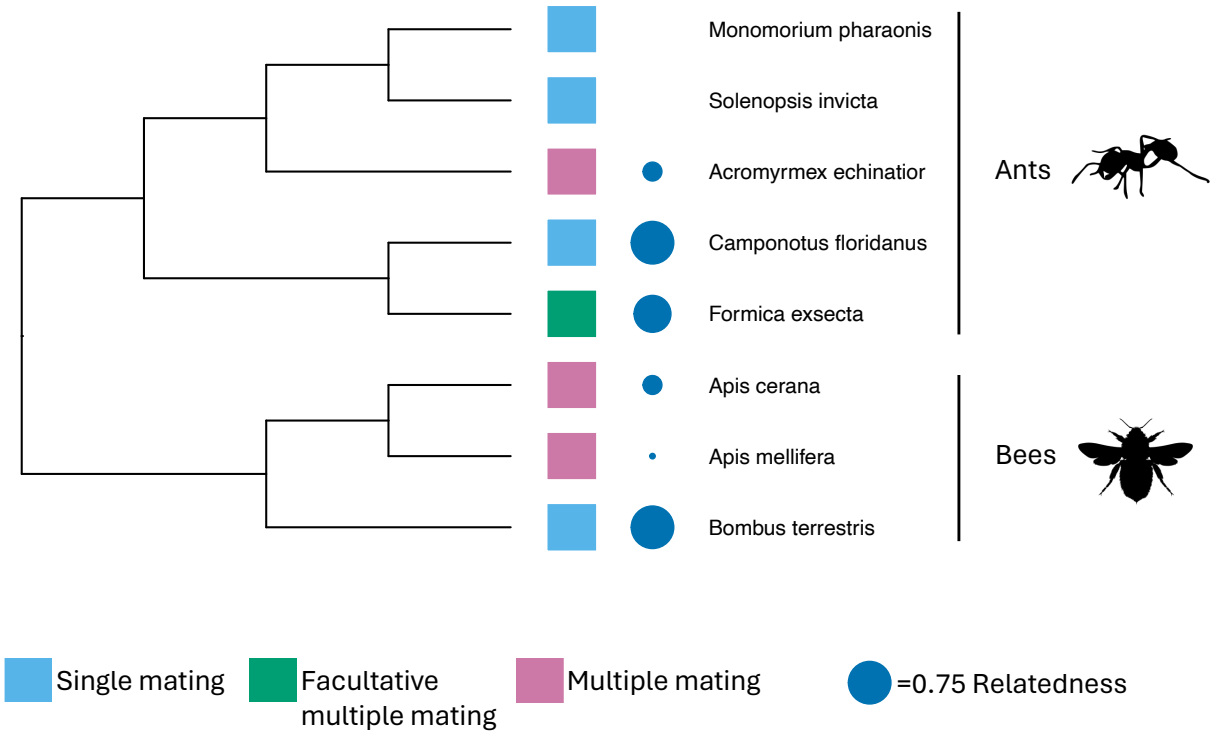


Figure 2. Phylogenetic relationships between the species under study, where branch lengths have been transformed using Grafen's method. Species with lower levels of intra-colony relatedness, such as *A.* echinatior and the *Apis* species, are predicted to harbour greater levels of polymorphism and divergence in genes with worker biased expression relative to genes with queen-biased expression. Estimates are presented for the number of times a queen mates as well as the level of relatedness present between nestmate workers for each species. Using direct estimates of relatedness captures the influence of both queen mating frequency and the number of reproductive queens per colony. A relatedness value of 0.75 indicates that a colony possesses a single once-mated queen. The size of each circle is proportional to the level of relatedness estimated for that species. Estimates of relatedness are unavailable for *Monomorium pharaonis* and *Solenopsis invicta*.

In total, we identified 11,797 genes with caste-biased expression (Table 2). The average number of differentially expressed genes per species was 1475, ranging from 110 genes in *Bombus terrestris* to 6908 in *Apis cerana*.

Table 2. Number of genes identified with caste-biased expression.

Species	Queen	Worker	Total caste-biased genes	Percentage of genes with caste biased expression in the genome
Acromyrmex echinatior	156	55	211	1.72%
Apis cerana	3570	3338	6908	52.55%
Apis mellifera	257	353	610	4.80%
Bombus terrestris	77	33	110	0.82%
Camponotus floridanus	372	917	1289	9.19%
Formica exsecta	101	65	166	1.21%
Monomorium pharaonic	592	1407	1999	11.68%
Solenopsis invicta	369	135	504	2.96%

Polymorphism

When examining relative polymorphism, we found mixed support for the hypothesis that genes with worker-biased expression are under relaxed selection relative to genes with queen-biased expression in species where the offspring of the queen are not full siblings. Consistent with our theoretical prediction, we found that species with higher queen mating frequencies exhibit significantly higher levels of relative polymorphism, when examining non-synonymous sites (Fig. 3b; BPMM: $\beta = 0.42$, credible interval (CI) = 0.04 to 0.70, pMCMC = 0.04, $n_{species} = 8$, $R^2 = 0.25$). We also found no relationship between relative polymorphism and queen mating frequency, when examining synonymous sites (Fig. 3c;

BPMM: β = -0.03, CI = -0.78 to 0.69, pMCMC = 0.99, $n_{species}$ = 8, R^2 = 0). In contrast to our predictions, we found no relationship between queen mating frequency and relative polymorphism when considering overall polymorphism, including synonymous and non-synonymous sites, or the ratio of non-synonymous sites to synonymous sites (Fig. 3a and d; BPMMs: overall polymorphism (π): β = 0.14, credible interval (CI) = -0.28 to 0.48, pMCMC = 0.48, $n_{species}$ = 8, R^2 = 0.03; polymorphism ratio (π_N/π_S): β = 0.61, CI = -0.57 to 1.74, pMCMC = 0.23, $n_{species}$ = 8, R^2 = 0.08).

We reanalysed our data, comparing relative polymorphism to direct estimates of intra-colony relatedness (r) rather than using estimates of queen mating frequency. In contrast to our theoretical prediction, we found no relationship between relatedness and relative polymorphism, when examining non-synonymous sites (Supplementary Fig. 1b; BPMM: β = -0.97, credible interval (CI) = -2.50 to 0.51, pMCMC = 0.18, $n_{species}$ = 6, R^2 = 0.13). Furthermore, we found no relationship between relatedness and relative polymorphism when considering overall polymorphism, including synonymous and non-synonymous sites, or the ratio of non-synonymous sites to synonymous sites (Supplementary Figs. 1a and d; BPMMs: overall polymorphism (π): β = -0.15, credible interval (CI) = -1.67 to 1.19, pMCMC = 0.58, $n_{species}$ = 6, R^2 = 0.02; polymorphism ratio (π_N/π_S): β = -1.31, CI = -6.53 to 2.79, pMCMC = 0.35, $n_{species}$ = 6, R^2 = 0.05). Consistent with theoretical predictions, we found no relationship between relatedness and relative polymorphism, when examining synonymous sites (Supplementary Fig. 1c; BPMM: β = 0.36, credible interval (CI) = -2.26 to 3.64, pMCMC = 0.89, $n_{species}$ = 6, R^2 = 0).

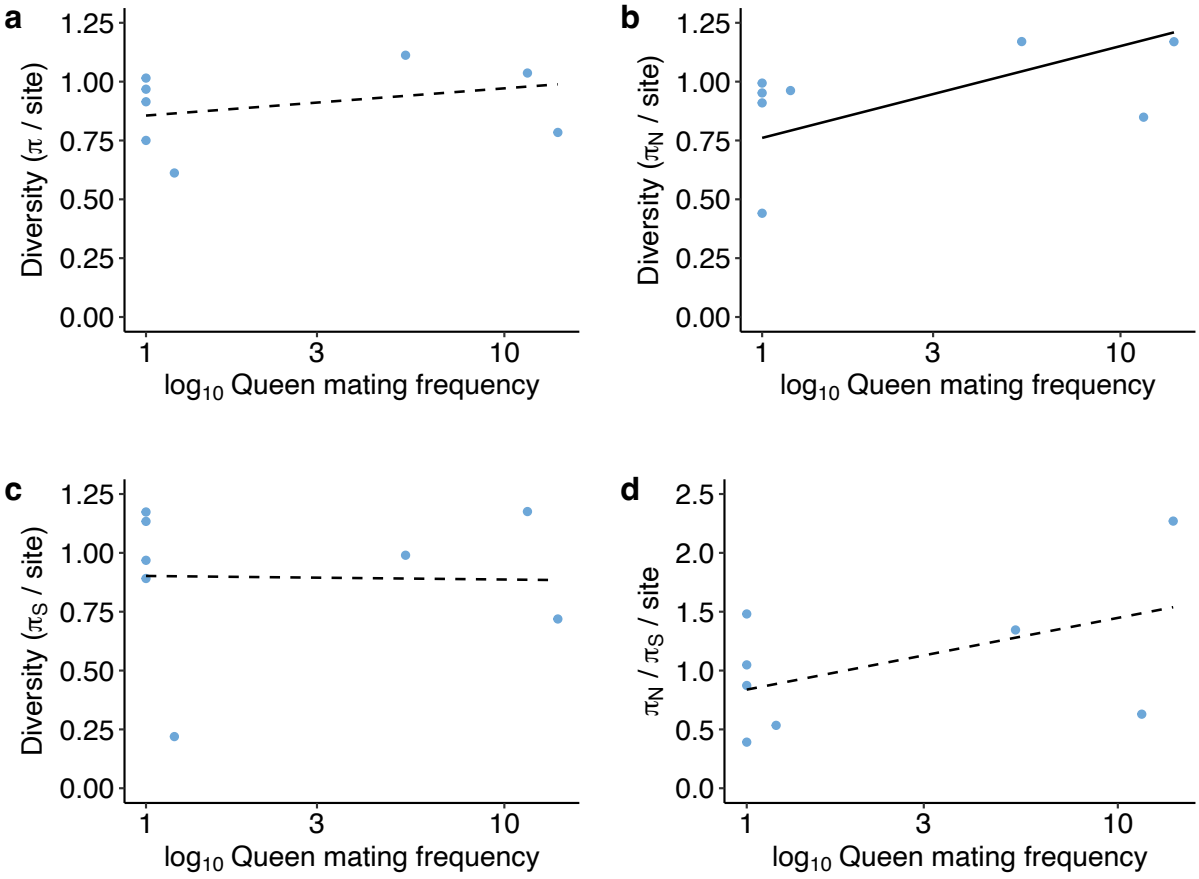


Figure 3. Relationship between queen mating frequency and relative nucleotide diversity (polymorphism) in worker-biased genes compared to queen-biased genes. (b) When examining non-synonymous sites, we found that species with higher queen mating frequencies exhibit significantly higher levels of relative polymorphism. Solid regression lines represent significant relationships, while dashed lines represent non-significant relationships. Sample sizes = 8; each dot represents a species. Axes for queen mating frequency are represented on a $\log_{10}$ scale.

Divergence

When examining relative divergence, our results did not support our hypothesis regarding the relative strength of selection acting on genes with worker-biased expression. In contrast to our theoretical prediction, we did not find a significant relationship between queen mating

frequency and relative divergence, when examining non-synonymous sites (Fig. 4a; BPMM: $\beta = 0.22$, CI = -0.45 to 0.95, pMCMC = 0.34, $n_{species} = 8$, $R^2 = 0.05$). However, the direction of the effect did match our theoretical prediction. In addition, we found no relationship between queen mating frequency and the ratio between divergence at non-synonymous and synonymous sites (Fig. 4c; BPMM: $\beta = 0.06$, CI = -0.46 to 0.59, pMCMC = 0.74, $n_{species} = 8$, $R^2 = 0.01$). Consistent with theoretical predictions, we did not observe a relationship between mating frequency and relative divergence, when examining synonymous sites (Fig. 4b; BPMM: $\beta = -0.43$, CI = -1.70 to 0.74, pMCMC = 0.52, $n_{species} = 8$, $R^2 = 0.03$).

We reanalysed our data, comparing relative divergence to direct estimates of intra-colony relatedness (r). Our findings remained consistent regardless of whether we analysed relatedness or queen mating frequency. Contrasting with our theoretical prediction, we found no relationship between relatedness and relative divergence, when examining non-synonymous sites (Supplementary Fig. 2a; BPMM: $\beta = -0.34$, credible interval (CI) = -2.56 to 2.03, pMCMC = 0.69, $n_{species} = 6$, $R^2 = 0.01$). Furthermore, we found no relationship between relatedness and the ratio between divergence at non-synonymous and synonymous sites (Supplementary Fig. 2c; BPMM: $\beta = -0.47$, CI = -1.83 to 1.34, pMCMC = 0.48, $n_{species} = 6$, $R^2 = 0.03$). Consistent with theoretical predictions, we found no relationship between relatedness and relative divergence, when examining synonymous sites (Supplementary Fig. 2b; BPMM: $\beta = 2.33$, credible interval (CI) = -1.68 to 4.99, pMCMC = 0.21, $n_{species} = 6$, $R^2 = 0.11$).

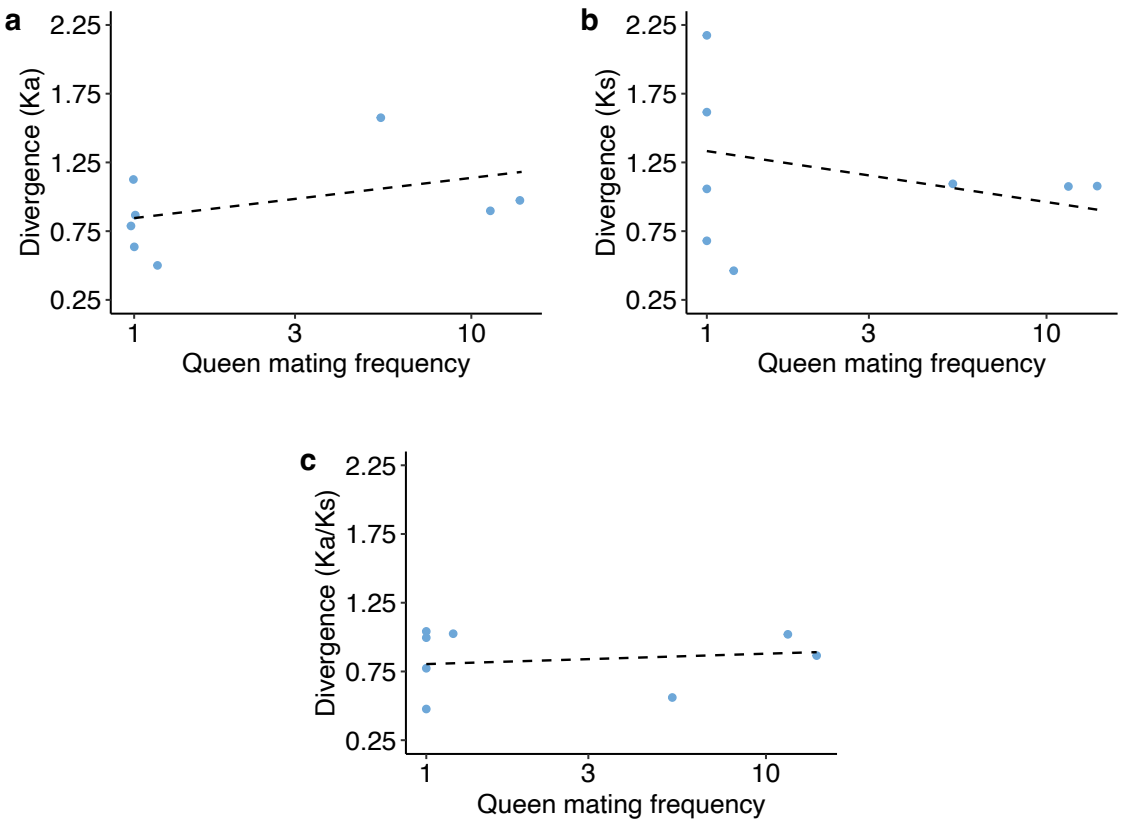


Figure 4. Relationship between queen mating frequency and relative divergence (measured as rates of protein evolution) in worker-biased genes compared to queen-biased genes. We found no significant relationships between relative divergence and queen mating frequency. Dashed lines represent non-significant relationships. Sample sizes = 8; each dot represents a species. Axes for queen mating frequency are represented on a $\log_{10}$ scale.

Discussion

We found mixed support for the hypothesis that signatures of kin selection can be found within the genomes of social insects. Consistent with our theoretical predictions, species with higher queen mating frequencies exhibit increased levels of polymorphism in worker-biased genes relative to queen-biased genes at non-synonymous sites (Fig. 3b). However, when examining overall polymorphism, combining polymorphism at non-synonymous and

synonymous sites, and the ratio of polymorphism at non-synonymous and synonymous sites, we failed to detect the same pattern (Figs. 3a & d). When examining relative divergence, we did not find evidence supporting our theoretical predictions. Contrary to our hypothesis, we found no relationship between queen mating frequency and relative divergence, when examining non-synonymous sites (Fig. 4a). Furthermore, we failed to detect a significant relationship between queen mating frequency and the ratio of divergence at non-synonymous and synonymous sites (Fig. 4c).

Tests of kin selection theory using estimates of polymorphism

Consistent with the predictions made by kin selection theory (Linksvayer and Wade, 2009; Hall and Goodisman, 2012), we found that species with higher queen mating frequencies harbour increased levels of polymorphism at non-synonymous sites in worker-biased genes relative to queen-biased genes (Fig. 3b and Supplementary Figs. 3b & 5b). This result may reflect a signature of kin selection in the genomes of social insects owing to relaxed selection on genes with indirect fitness effects (Linksvayer and Wade, 2009). However, contrary to our theoretical prediction, we found no relationship between queen mating frequency and relative divergence at non-synonymous sites (Fig. 4a and Supplementary Figs. 4a & 6a). Given that the predicted trend in relative divergence is not observed, it's possible that an alternative explanation, other than kin selection, may explain the trend found in relative polymorphism. For example, worker-biased genes may experience relatively stronger levels of balancing selection, which could generate the same pattern of increased polymorphism (Table 1) (Belcher *et al.*, 2022). Balancing selection could maintain genetic polymorphisms in worker-biased genes, such as those related to foraging or nursing, if an allele exists in the population that improves performance foraging while another allele improves performance caring for the brood. However, an additional factor would need to link stronger balancing selection with

species that have higher queen mating frequencies, as relative polymorphism increases in species with multiply mated queens (Fig. 3b). One possible explanation could be that multiple alleles conferring different advantages are more likely to persist in species with multiply mating queens, as the worker population is more likely to benefit from containing both allelic variants simultaneously due to contributions from multiple paternal lineages (Anderson *et al.*, 2008). This could, therefore, also generate a pattern where relative polymorphism is positively correlated with queen mating frequency across species because of the influence of balancing selection. To resolve this problem, future studies should calculate additional estimates for divergence rates in caste-biased genes across a broader range of social insect species. This could help to discern between the underlying selective forces driving the observed pattern in relative polymorphism, as the non-significant relationship identified between queen mating frequency and relative divergence may have been obtained due to a lack of statistical power ($n_{species}$=8).

Not all our analyses examining relative polymorphism supported the predictions made by kin selection theory. For example, when examining overall polymorphism and the ratio of polymorphism at non-synonymous and synonymous sites, we did not identify a significant relationship with queen mating frequency. The non-significant trend observed in both overall polymorphism and the ratio between polymorphism at non-synonymous and synonymous sites could be attributed to the relatively small sample size used in this analysis ($n_{species}$=8), as the direction of their effects matched theoretical predictions. Therefore, with the availability of more genomic data, future analyses should revisit the relationship between these variables with increased statistical power. Since the inception of this project, genomic data has become available for several more social insect species, including *Vespa crabro*, *V. velutina*, *Camponotus vicinus*, and *Tetragonisca angustula* (Favreau *et al.*, 2023; Ferrari *et al.*, 2024;

Ward *et al.*, 2024). Furthermore, additional novel genomes are expected to be released soon through the efforts of the Global Ant Genomics Alliance (GAGA) (Boomsma *et al.*, 2017). These recent additions emphasise the speed at which new data is becoming available and will facilitate more robust tests of the hypotheses explored in this thesis.

Tests of kin selection theory using estimates of divergence

While small sample size may explain why we found no relationship between queen mating frequency and relative divergence at non-synonymous sites, an alternative explanation also exists. Relaxed selection on worker-biased genes can exert a contrasting influence on rates of divergence in queen and worker-biased genes. Under certain conditions, relaxed selection on worker-biased genes can result in relatively greater levels of divergence in worker-biased genes compared to queen-biased genes (Linksvayer and Wade, 2009; Hall and Goodisman, 2012). However, under alternative conditions it can also result in relatively greater levels of divergence in queen-biased genes compared to worker-biased genes (Hall and Goodisman, 2012). Whether relaxed selection in worker-biased genes leads to greater divergence in worker or queen-biased genes depends on the effective population size (Hall and Goodisman, 2012). In larger populations, selection will dominate the effects of drift, leading to a decrease in the likelihood of substitution for mildly deleterious alleles. In this scenario, queen-biased genes will evolve at a faster rate under multiple mating, as only beneficial and neutral alleles will reach fixation. In smaller populations, worker-biased genes will evolve at a faster rate under multiple mating, given that alleles with mildly deleterious effects on fitness will accumulate faster and will avoid being purged by selection. Therefore, if relaxed selection in worker-biased genes results in greater divergence in worker-biased genes in some species, and greater divergence in queen-biased genes in other species, it may not be possible to detect a relationship between queen mating frequency and relative divergence at non-

synonymous sites when analysing these two groups of species together. To resolve this problem, future studies could control for the effect of effective population size by including it as a covariate in analyses with an interaction term with queen mating frequency.

Presence of an outlier

F. exsecta was an outlier, in that it was found to harbour very low levels of polymorphism at synonymous sites in worker-biased genes relative to queen-biased genes. This result may be attributed to the type of samples used in the differential gene expression analysis, and which made *F. exsecta* unique in our analysis. Our dataset comprised individuals sampled from distinct life stages: newly emerged workers and queens, as well as older workers and queens, where workers were approaching the end of their lives following overwintering. The inclusion of samples spanning a wide range of ages, specifically at the beginning and end of their adult lives, could have influenced the outcome of the differential gene expression analysis. This interpretation is supported as other studies have found that emerging workers have fewer caste-biased genes in comparison to adult workers (Morandin *et al.*, 2015). This may be because these individuals might not be expressing their usual repertoire of behaviours at these life stages. As a result, this will have influenced the identity of the caste-biased genes which were used to estimate the relative levels of polymorphism. Future studies should aim to identify caste-biased genes while consistently using individuals from the same life stage, both within species, and across species.

Data collection and quality

Several other aspects of data quality in this study could also be improved and may have impacted our results. First, we aimed to identify caste-biased genes using transcriptomic data generated from brain tissue. However, this type of data was not available for all species

analysed, and therefore, other tissues were used for *M. pharaonis*, *C. floridanus*, *A. mellifera*, and *A. cerana* (Supplementary Table 3). Ideally, brain tissue should consistently be used for all species, as tissue type might influence the number and identity of caste-biased genes identified. Second, in our differential gene expression analysis, we used tissues samples taken from a range of different worker castes, within and between species (Supplementary Table 3). For example, we used minor workers and major workers in *C. floridanus* but only minor workers in *S. Invicta*. This may have also influenced the number and identity of caste-biased genes identified. Additionally, the analysis would have benefitted from a larger number of samples to accurately identify caste-biased genes, although we were also constrained by the availability of data (Supplementary Table 3). For instance, *C. floridanus*, *A. mellifera*, and *A. cerana* had particularly low sample sizes, with fewer than three individuals per caste. Third, when estimating polymorphism, the geographic scale over which individuals were sampled was not consistent across species in the analysis (Supplementary Table 1). In particular, samples for *M. pharaonis* and *S. invicta*, were taken from a broader geographic range than samples for other species. The increased geographic scale over which individuals were sampled may lead to higher estimates of polymorphism in *M. pharaonis* and *S. invicta* if individuals sampled from more distant locations exhibit greater genetic divergence. In future studies, this problem may be addressed by using transcriptomic data to identify SNPs, rather than whole genome sequence data, if individuals can be sampled from a more appropriate geographic scale. In addition, transcriptomic data may be useful if available sample sizes for whole genome sequence data are too small. Fourth, various studies recommend using a minimum depth of sequence coverage of 10-15x for accurate SNP identification (Altmann *et al.*, 2012; Song, Li and Zhang, 2016). While most of the sequence data we used conformed to this threshold, *B. terrestris* (2.5x) and *A. mellifera* (8x) had low depth of coverage, and the depth for *M. pharaonis* was unknown. Therefore, some SNPs in the data may have been

missed, or potentially erroneously introduced into our dataset for these two species. Therefore, future studies should ensure that all species have a minimum depth of coverage of at least 10-15x for all species where SNPs are being identified. When performing comparative analyses, we are sometimes constrained by the availability and quality of data available. However, by consciously trying to maintain the standards set out above, this will improve the quality and reliability of results of future studies attempting to address the questions set out in this paper.

Tests of kin selection theory using non-superorganismal species

An interesting alternative test of kin selection theory at the genomic level would involve replicating our analysis using social insect species that have not made the major transition to superorganismality. Although these species exhibit reproductive division of labour, and workers cooperate to raise relatives, all individuals in the colony retain the ability to reproduce, and workers may transition into queens at some point in their life. While we still predict that genes with indirect fitness effects experience weaker selection compared to genes with direct fitness effects under conditions of reduced relatedness (Linksvayer and Wade, 2009; Hall and Goodisman, 2012), detecting this pattern may be more challenging in non-superorganismal species. This difficulty arises because simply identifying a gene as having caste-biased expression may not be enough to detect whether it has direct or indirect fitness effects, as worker-biased genes could still be linked to attempts to gain direct fitness. Additionally, genes may be more likely to have pleiotropic effects, impacting both direct and indirect fitness (Linksvayer and Wade, 2009), further complicating the detection of a signal of kin selection in the genome. Therefore, to test kin selection theory in non-superorganismal species, it would be more effective to focus on genes with known functions related specifically to queen or worker roles. Nonetheless, such a test would be valuable, as a

previous study in wasps indicates that both non-superorganismal and superorganismal species share genes that regulate queen and worker phenotypes (Wyatt *et al.*, 2023). However, lineages that have transitioned to superorganismality have also evolved novel genes associated with caste regulation (Feldmeyer, Elsner and Foitzik, 2014; Sumner, 2014; Wyatt *et al.*, 2023). Therefore, a test on non-superorganismal species would serve as a focused examination of the genetic regulation of caste phenotypes across the evolution of this major transition.

Conclusions

In conclusion, we found mixed support for the predictions made by kin selection theory, despite analysing only a limited number of species. To conclusively demonstrate support for or against the hypothesis that signatures of kin selection can be detected within the genomes of social insects, future studies should address two areas. First, they should analyse genomic data from a larger number of species with varying levels of queen mating frequency, including effective population size as a covariate. One approach to increasing sample size could be to use transcriptomic data, either in addition to or instead of whole genome sequence data, to identify SNPs and estimate polymorphism (Adetunji *et al.*, 2019). This may permit the inclusion of a greater number of species in the analysis. Overall, increasing sample size will improve statistical power, provide a more representative sample of social insect species, and enable the detection of any weaker effects that might be present.

Second, alternative approaches could be used to identify genes with direct or indirect fitness effects that will be used for downstream analyses of polymorphism and divergence. For example, genes which exhibit at least twice the level of expression in one caste compared to the other are commonly considered to be ‘differentially expressed genes,’ and we applied this

threshold within our own analysis. However, using this threshold is relatively arbitrary. Therefore, analyses could explore the effects of classifying genes using higher or lower differential expression thresholds. Using a higher threshold may help to limit subsequent analyses to fewer genes that are more likely to be experiencing either only direct or indirect fitness effects. It's possible that many genes may be simultaneously experiencing both types of effects, reducing the ratio of signal-to-noise in the data when trying to detect if worker-biased genes are under relaxed selection relative to queen-biased genes (Linksvayer and Wade, 2009, 2016). Alternatively, using a lower threshold may help to increase the sample size of the number of caste-biased genes that can be used in analyses. Alternative methods could also be used to classify genes as experiencing primarily direct or indirect fitness effects. For example, methods that use machine learning classification tools, which have been shown to effectively classify genes as being differentially expressed in queens or workers (Taylor *et al.*, 2021; Wyatt *et al.*, 2023), or methods that investigate only orthologous genes of known function that are expected to influence exclusively queen or worker phenotypes. Examples of suitable genes could include those associated with egg or wing production, as well as those involved in developmental pathways leading to the formation of soldier morphologies. Using alternative approaches to classify caste-biased genes may help to improve the detection of any patterns in molecular sequence variation by optimising the signal-to-noise ratio for any underlying effects. This improvement could occur even if sample sizes in future studies remain relatively small.

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Discussion

As an integrated thesis, each chapter stands alone as a self-contained unit, with its own discussion. In this chapter, I reflect on the results that arise from considering these chapters collectively. I also explore potential future directions that this work could take.

Summary and discussion of results

Identifying the determinants of non-reproductive division of labour

In Chapters 2 & 3, I used phylogenetic comparative analyses to test the role of multiple variables that have been hypothesised to influence the evolution of the number of worker castes in ants. These variables were predicted to influence the number of castes via their effect on either conflict and cooperation within colonies or how they influenced the benefits of having different numbers of castes, which depends on the ecological conditions encountered by a species. Larger colony sizes emerged as the only factor that consistently favoured the evolution of more worker castes and greater variation in worker sizes. In contrast, I found no consistent support for the hypothesis that variation in queen mating frequency or the number of reproductive queens per colony influenced the evolution of the number of worker castes via their effect on levels of intra-colony relatedness. In addition, while hotter and drier climates are associated with worker polymorphism, where workers vary in size and morphology within a colony, I found that worker polymorphism evolves prior to the colonisation of hotter and drier climates. Therefore, hotter and drier climates cannot be a causal determinant of greater levels of non-reproductive division of labour.

To assess the generality of these findings concerning the evolution of non-reproductive division of labour, similar analyses could be performed in other social insect taxa, like

termites. Alternatively, analyses could be performed at different levels of biological organisation, such as within multicellular organisms. Similar to ants, the sterile caste in many termite species exhibits multiple morphological phenotypes within colonies (Oster and Wilson, 1978; Noirot and Pasteels, 1987), and multicellular organisms consist of numerous cell types not directly involved in reproduction (Bell and Mooers, 1997; Bonner, 2004; Fisher, Shik and Boomsma, 2020). While previous comparative studies have found a positive correlation between cell number and the number of cell types (Bell and Mooers, 1997; Bonner, 2004; Fisher, Shik and Boomsma, 2020), the evolutionary order of these traits has yet to be investigated. Therefore, outside of ants, it is currently an open question regarding whether larger group size is either a cause or a consequence of the evolution increased non-reproductive division of labour.

Identifying the determinants of reproductive division of labour

Throughout the course of this thesis, I have not explored the evolution of reproductive division of labour. While it's an active area of research, it would have been beyond the scope of this thesis to have additionally investigated the factors influencing the evolution of reproductive division of labour. However, there are several important and unresolved questions regarding the evolution of this trait in social insects.

While kin selection theory can explain how a division of labour between a reproductive and a sterile helper phenotype may evolve (Hamilton, 1963, 1964), it remains unclear why eusociality has only evolved on some branches of the tree of life and not others. The controversy centres around the relative importance of two factors hypothesised to influence the evolution of sterile helpers. The first hypothesis proposes that eusociality has only ever evolved in species which exhibit life-time monogamy (Boomsma, 2009). Under this

condition, relatedness between siblings equates to 0.5, identical to the level of relatedness between parent and offspring. Therefore, selection will favour the evolution of a sterile helper caste if there is an efficiency benefit to helping raise siblings rather reproducing. The second hypothesis predicts that haplodiploidy predisposes species to evolve eusociality as relatedness among siblings can be increased beyond 0.5 (Hamilton, 1964, 1972). Therefore, a helper may be more closely related to its siblings than to its offspring, thus facilitating the evolution of a sterile helper caste. To resolve this debate, a phylogenetic comparative study across all major insect clades is required to determine the necessary and sufficient conditions for the evolution of eusociality.

Investigating this question requires a taxonomically broader investigation beyond ants, as eusociality only evolved once at the root of the ant clade. However, ants would be a good system to explore variation in the level of specialisation that has evolved to accompany the reproductive division of labour that is present in eusocial insects. Across ants, species vary in the level of specialisation between the queen and worker castes. For example, in some species, such as *Kalathomyrmex emeryi*, workers resemble queens in terms of their size and morphology, and the reproductive capacity of queens is relatively low (Ferguson-Gow *et al.*, 2014). In other species, such as the leafcutter ant genus *Atta*, there is a stark contrast in morphology between queens and workers. Queens can be many times larger than the smallest workers, capable of producing millions of workers throughout their lifetime, along with thousands of new reproductive gynes and drones each year (Den Boer *et al.*, 2009). Variation also exists across species in terms of the level of reproductive potential of workers. Many species of ants are able to produce male-destined eggs (Khila and Abouheif, 2010), despite being incapable of reproducing sexually. However, total sterility has also evolved in some species, such as *Monomorium pharaonis* (Renard, Gueydan and Aron, 2022). An important

test would be to investigate whether similar factors that promote the evolution of greater non-reproductive division of labour, such as larger colony sizes, also favour the evolution of greater specialisation in reproductive division of labour. This could be assessed by examining either queen-worker dimorphism or the levels of reproductive potential within the worker caste.

Testing for genomic signatures of kin selection

In Chapter 4, I found mixed support for the hypothesis that signatures of kin selection can be found in genomes of social insects. In species with multiply mating queens: (i) consistent with theory, genes with worker-biased expression harboured greater levels of sequence polymorphism at non-synonymous sites in comparison to genes with queen-biased expression; and (ii) contradicting theoretical predictions, we found no evidence that genes with worker-biased expression harboured greater levels of divergence at non-synonymous sites in comparison to genes with queen-biased expression. Overall, the evidence for signatures of kin selection being present in the genomes of social insects is inconclusive.

My ability to detect an effect in this analysis may have been limited by the relatively small number of species for which the necessary genomic and transcriptomic data was available. However, future studies will benefit from the increasing availability of genomic data in social insects, for example due to the efforts of international collaborations such as the Global Ant Genomics Alliance (GAGA consortium) (Boomsma *et al.*, 2017). This collaboration is currently trying to obtain high-quality genomic sequence data for at least one representative from every ant genus. Such initiatives will enhance future studies by providing the data required to improve statistical power.

As the first phylogenetic comparative analysis looking for signatures of kin selection at the genomic level, this study provides a template that could be replicated for analyses performed in other taxa. For example, a recent study tested whether genes for cooperative traits, such as those induced by the quorum sensing signalling system, displayed signatures of kin selection in the genome of *Pseudomonas aeruginosa* (Belcher *et al.*, 2022). While strong support for the hypothesis was found, a comparative analysis is still required. Kin selection theory predicts that, across species, levels of polymorphism and divergence in genes with indirect fitness effects will be elevated compared to genes with direct fitness effects. This trend is predicted to occur as a negative function of relatedness (Linksvayer and Wade, 2009). Therefore, multiple species are required to test this prediction.

The challenge for studies testing this hypothesis in bacteria will be obtaining estimates of relatedness across enough species to conduct a comparative analysis. Furthermore, these estimates will need to be obtained from species where the necessary genomic data is also available, narrowing down the potential number of species that could be used.

A potential solution to this problem has been identified by a recent study, which introduced a method for calculating estimates of relatedness for bacterial populations by inferring relatedness from estimates of nucleotide diversity (Simonet and McNally, 2021). However, this method can only provide a lower-bound estimate of relatedness since it doesn't account for population structure, which can impact the level of relatedness. Given the difficulties involved in observing cooperative behaviours in natural populations of bacteria, looking for genomic signatures of kin selection will help to clarify the extent of social interactions within bacterial life.

Concluding remarks

At their conception, phylogenetic comparative methods were a monumental step forward for the study of adaptation. They enabled researchers to disentangle specific biological effects under study from patterns generated by shared ancestry between lineages (Felsenstein, 1985; Grafen, 1989; Harvey and Pagel, 1991). However, recent methods have improved significantly, allowing us to move beyond simple tests of whether two traits are correlated while controlling for the phylogenetic relationships between species. Instead, we can now test causal hypotheses by investigating the evolutionary order of traits and unravel causal networks involving multiple correlated traits (Garamszegi, 2014). The techniques that permit these types of analyses include phylogenetic path analysis, ancestral state reconstructions and transition rate models. The use of phylogenetic comparative analyses is a unifying theme across all chapters of this thesis, leveraging the power of these cutting-edge comparative methods to answer evolutionary questions.

Challenges for the future of comparative studies mainly relate to collecting biological data, building phylogenies, or synthesising the considerable amount of data that already exists. For example, large amounts of life history data are available for thousands of species of ants, although many still lack substantial information. However, much of the required data for my analyses needed to be collected from individual studies from the literature. Although methodological limitations still exist for comparative studies, particularly how data must be categorised to suit certain types of analyses, these limitations are rarely the limiting factor. In contrast, collecting and standardising biological data in a way that can be used in comparative studies is a major challenge.

Fortunately, the future of comparative research in the study of ants appears promising as issues related to data scarcity, standardisation, and synthesis are improving. Recent releases of large-scale databases (Guénard *et al.*, 2017; Parr *et al.*, 2017; La Richelière *et al.*, 2022), with more anticipated, along with the ongoing collection of additional life history data, are contributing to this positive outlook. Furthermore, studies on the phylogenetic relationships between ant species have also succeeded in producing phylogenies that are robust to at least the genus level (Economo *et al.*, 2018; Nelsen, Ree and Moreau, 2018). Hopefully, the synthesis of a large amount of ant trait data presented in this thesis will serve as a springboard for future studies exploring the evolution of other ant life history traits.

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Supplementary Material

This section contains the supplementary material for Chapters 3-4. Owing to the extensive nature of the supplementary material for Chapter 2, the Extended Data Figures of Chapter 2 are located on pages 71-82 and the Supplementary Tables can be found online (for further details see below). The Supplementary Material contains information referenced in the text such as supplementary methods, figures, tables representing the results of analyses, tables of data used for each analysis, and the list of references for the data. Links to each of these sections are provided in the following table of contents.

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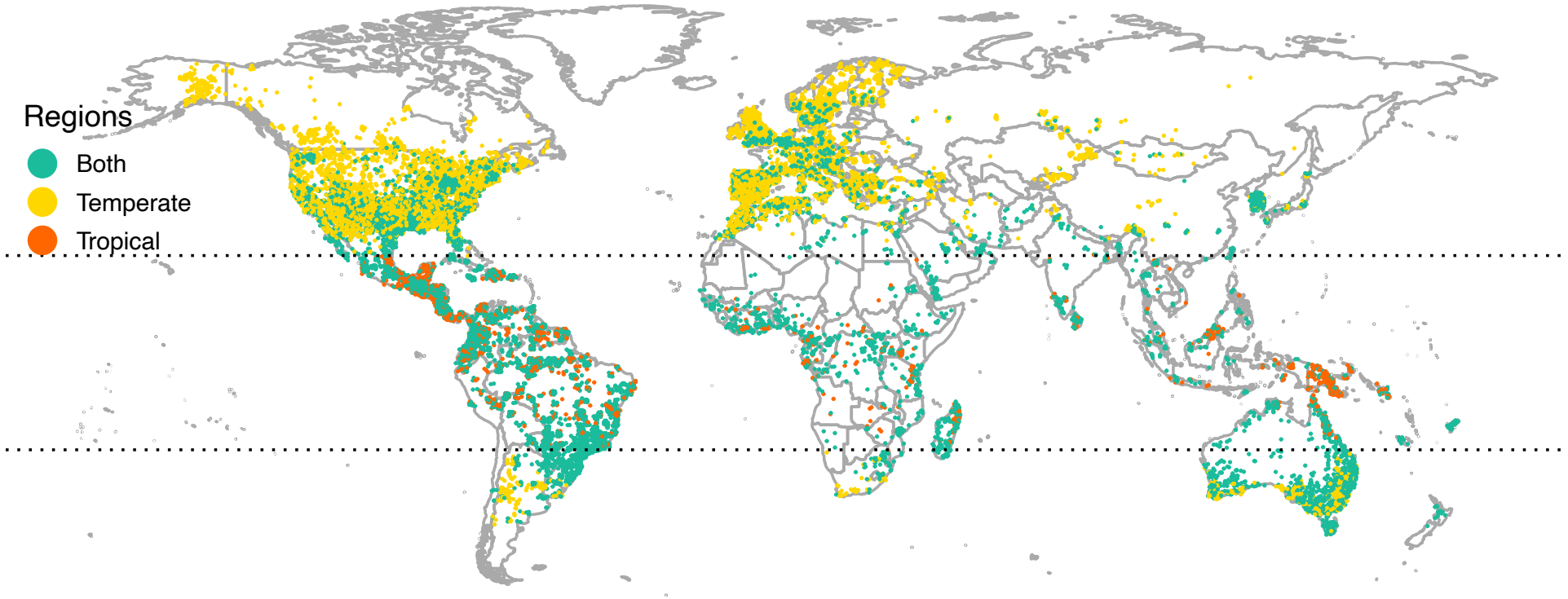
Supplementary Material for Chapter 2

All Supplementary Tables and their associated list of references can be found online at either: https://ora.ox.ac.uk/objects/uuid:453278a3-61b9-4144-b08a-98d9379d5ce8

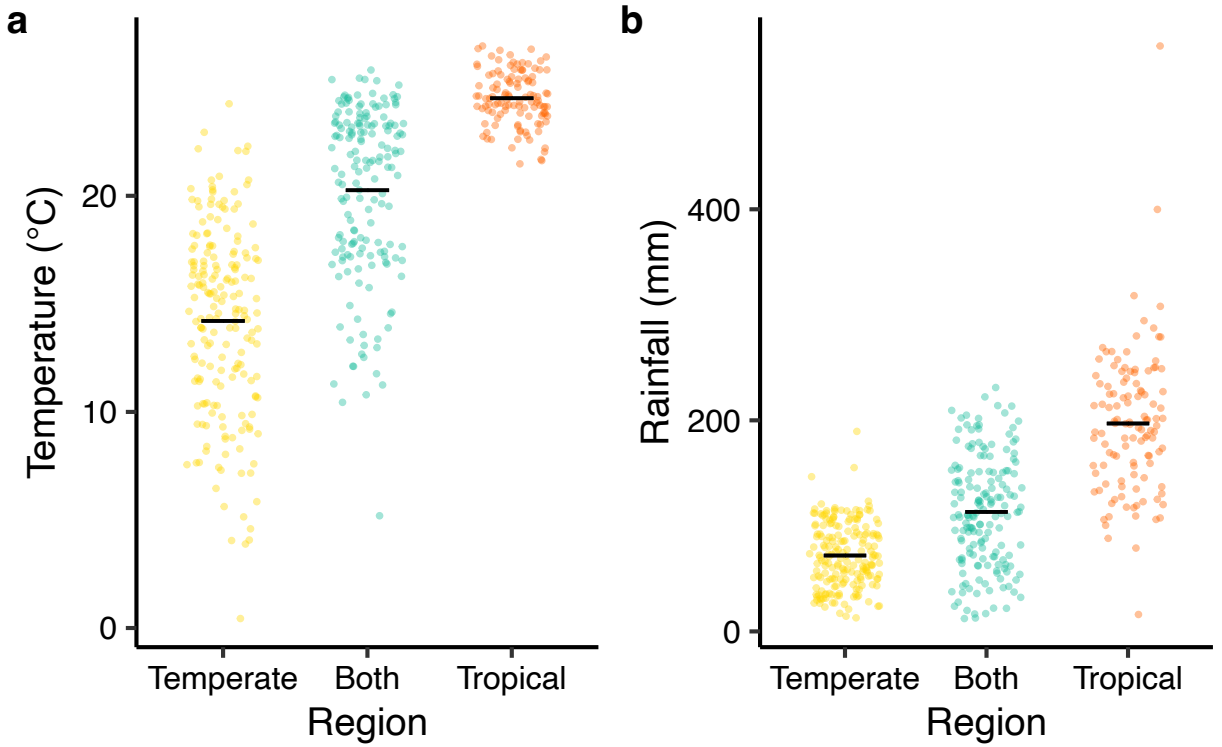
or

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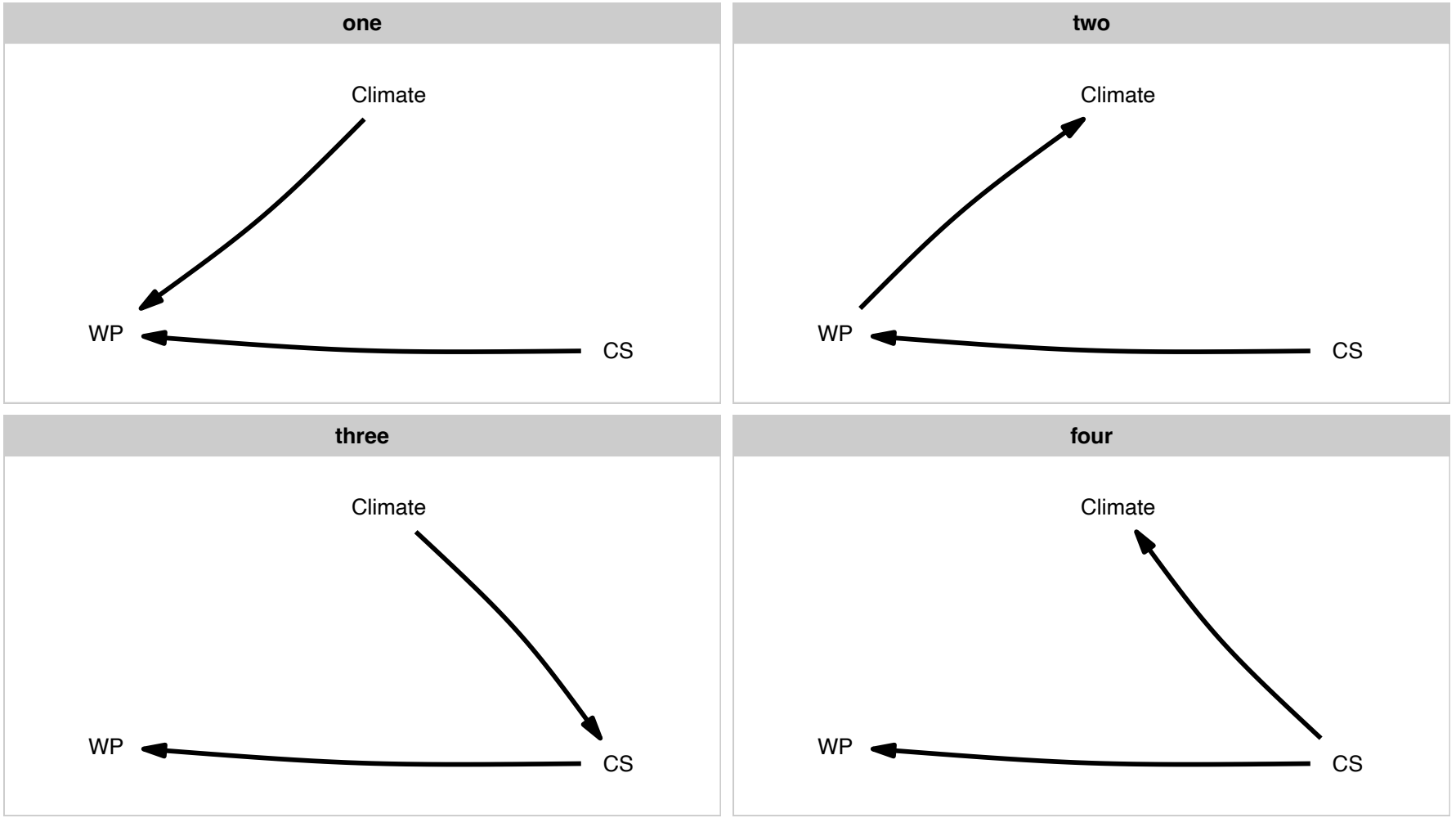
Supplementary Material for Chapter 3



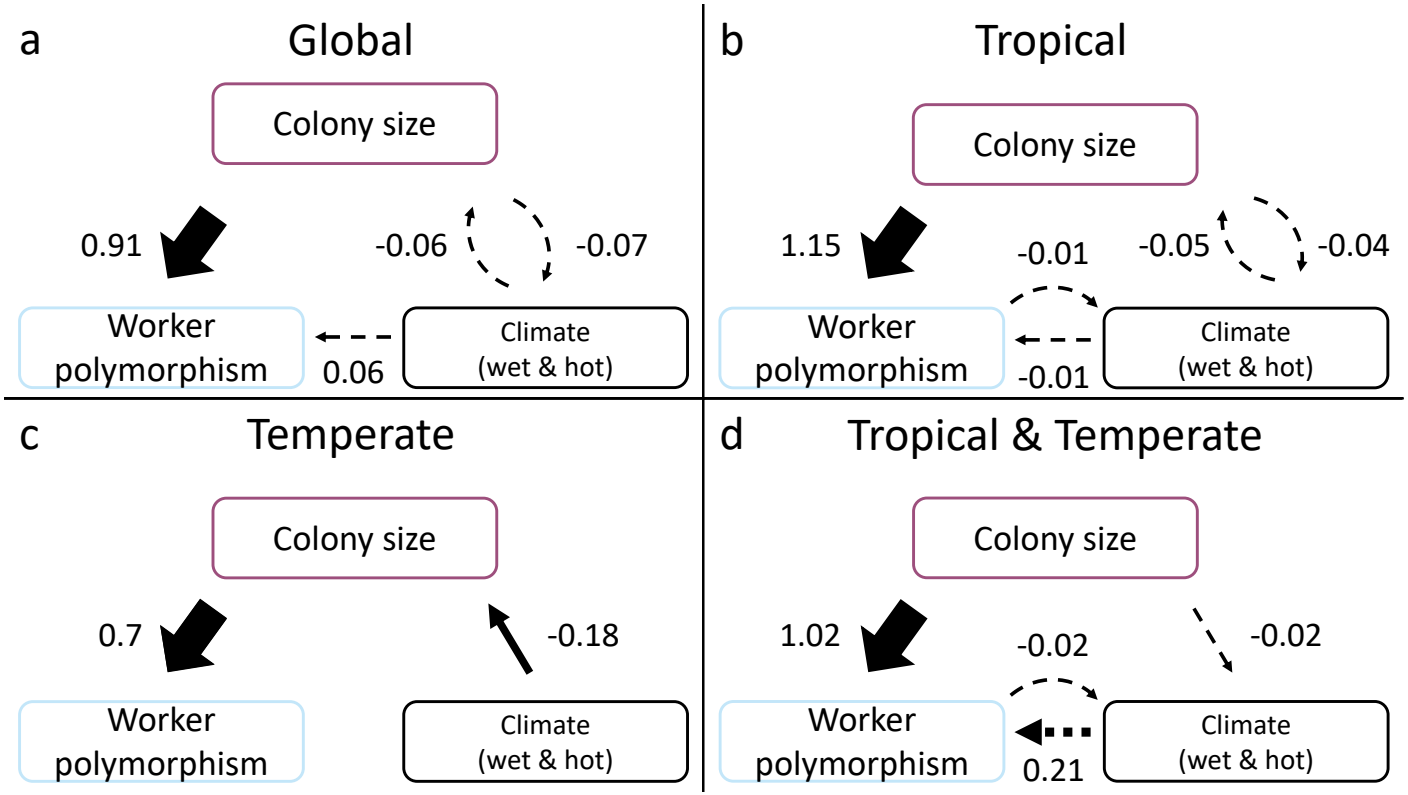
Supplementary Fig. 1. Global map displaying the geographic distributions of species categorised as occurring only in tropical regions (orange), only in temperate regions (yellow) or species that occur in both tropical and temperate regions (green). Dotted lines delineate tropical (central region) and temperate regions (top/bottom regions) and represent the Tropic of Cancer and Capricorn. Sample sizes: $n_{species\ tropical} = 116$; $n_{species\ temperate} = 190$; $n_{species\ both} = 168$.



Supplementary Fig. 2. Variation in temperature and rainfall across geographic regions. Black lines represent mean values for each region. Dots represent species averages. Sample sizes: $n_{species\ tropical} = 116$; $n_{species\ temperate} = 190$; $n_{species\ tropical\ \&\ temperate} = 168$.

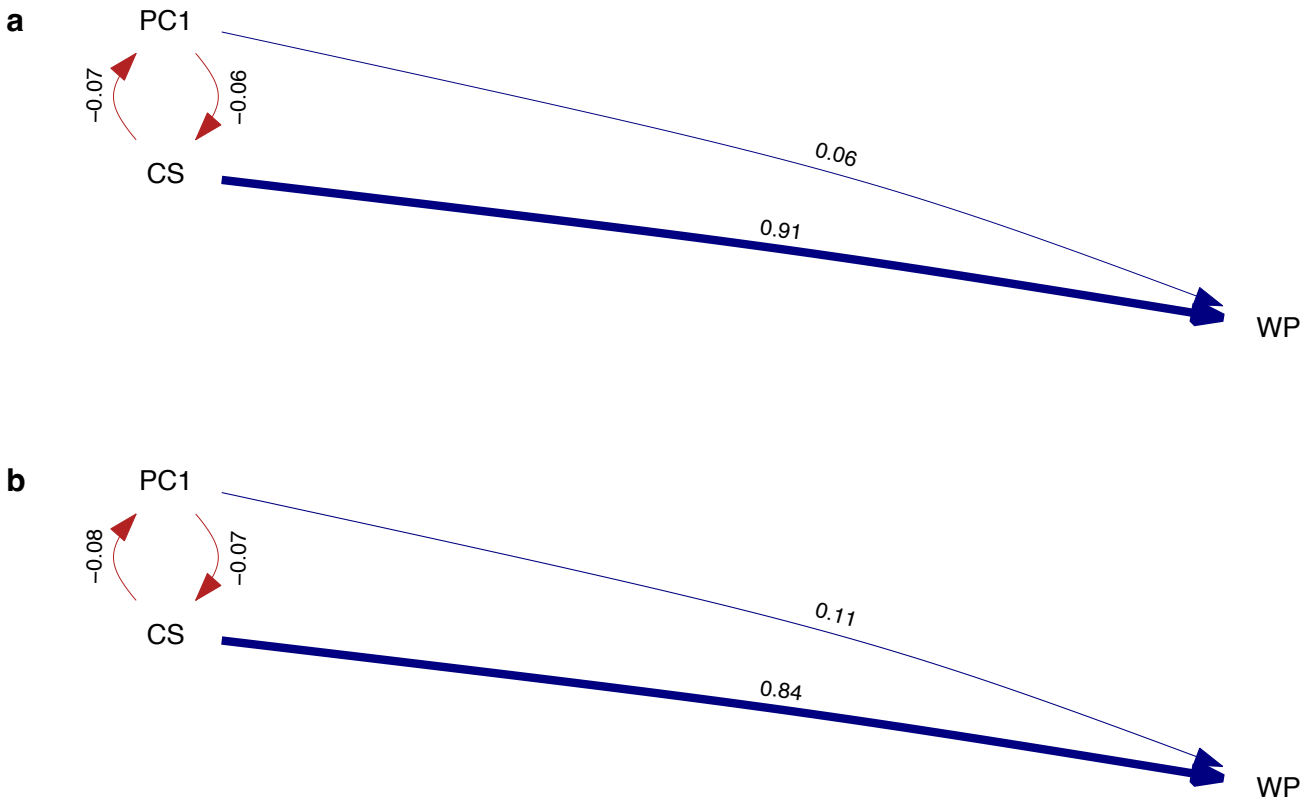


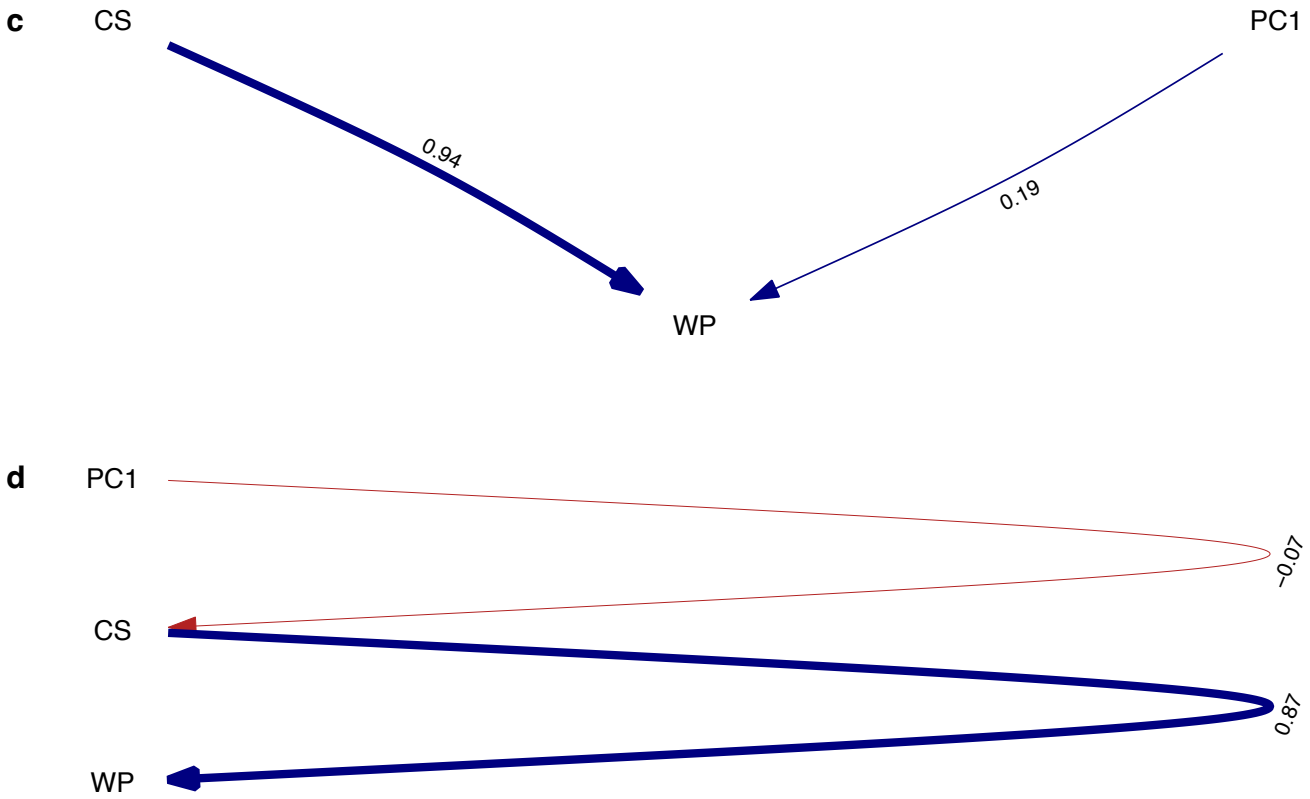
Supplementary Fig. 3. The full set of potential causal models tested in the phylogenetic path analyses illustrated using directed acyclic graphs (DAGs). Climate represents PC1 or PC2 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.



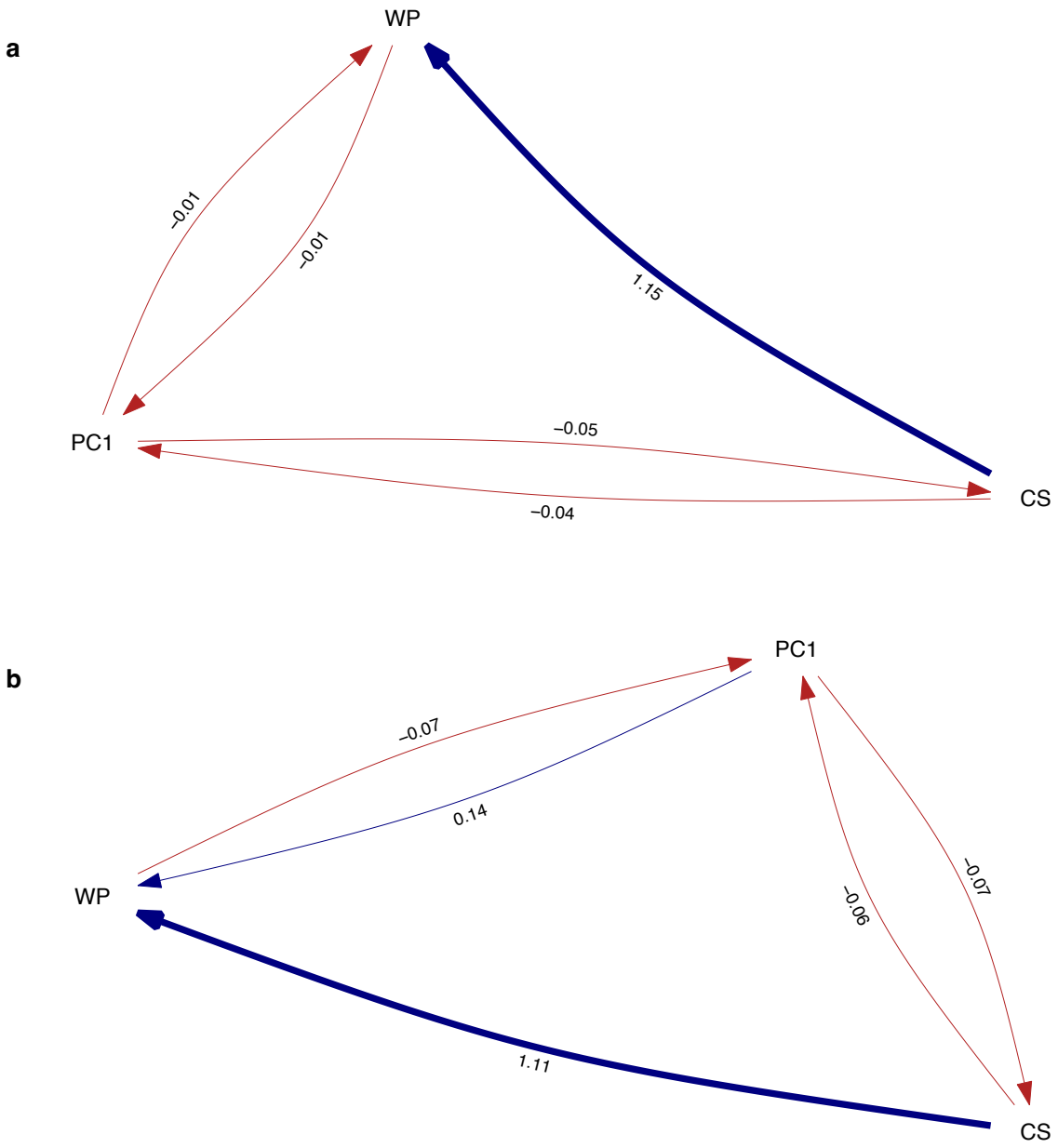
Supplementary Fig. 4. Average model from phylogenetic path analysis across different regional scales. Climate is modelled using PC1, where increasing values for this trait indicate hotter and wetter climates, while decreasing values indicate colder and drier climates. In species that occur exclusively in temperate regions, drier and colder climates favour the evolution of larger colony sizes. We found no further significant causal relationships between climate and either of the social complexity traits at any other regional scale. We analysed our species across four different geographical scales: (a) globally; (b) with distributions exclusive to tropical regions; (c) with distributions exclusive to temperate regions; and (d) with distributions that overlap both tropical and temperate regions. Solid arrows indicate the presence and direction of significant relationships between variables, with values representing the standardised average path

coefficients. Path coefficients describe the strength and direction of the relationship between two variables in terms of their correlated variance. Larger coefficients are denoted by a heavier line. Dashed arrows represent non-significant relationships between variables. We display the results from the analysis of one maximum clade credibility (MCC) consensus tree. However, we repeated the analyses on three additional MCC trees and alternative results can sometimes be obtained (Supplementary Figs. 5-8 and Supplementary Tables 2-3). Worker polymorphism is modelled as a discrete binary variable (monomorphic/polymorphic) while colony size and climate are modelled as continuous variables.

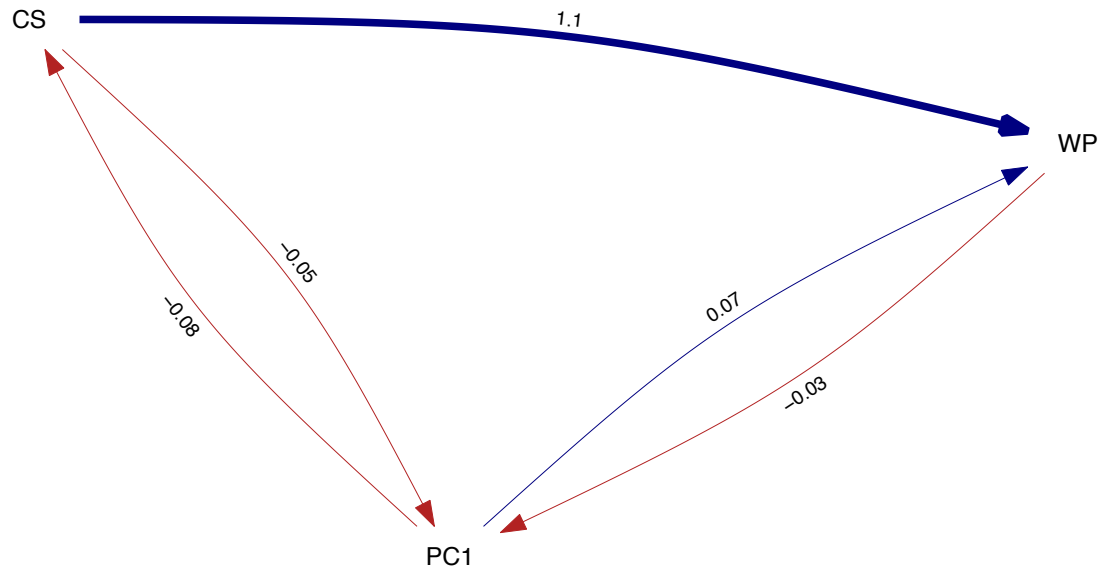

a
PC1
−0.07
−0.06
CS
0.06
0.91
WP
b
PC1
−0.08
−0.07
CS
0.11
0.84
WP



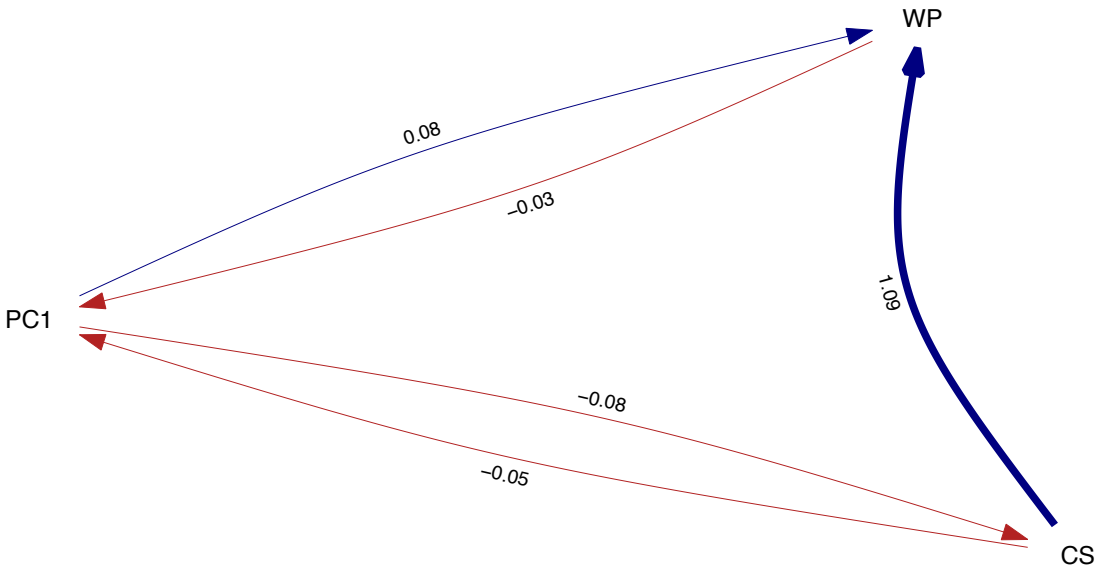
Supplementary Fig. 5. Average of the best-performing models analysing species globally. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. Climate represents PC1 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.


a
WP
PC1
CS
−0.01
−0.01
1.15
−0.05
−0.04
b
PC1
WP
CS
−0.07
0.14
−0.06
−0.07
1.11

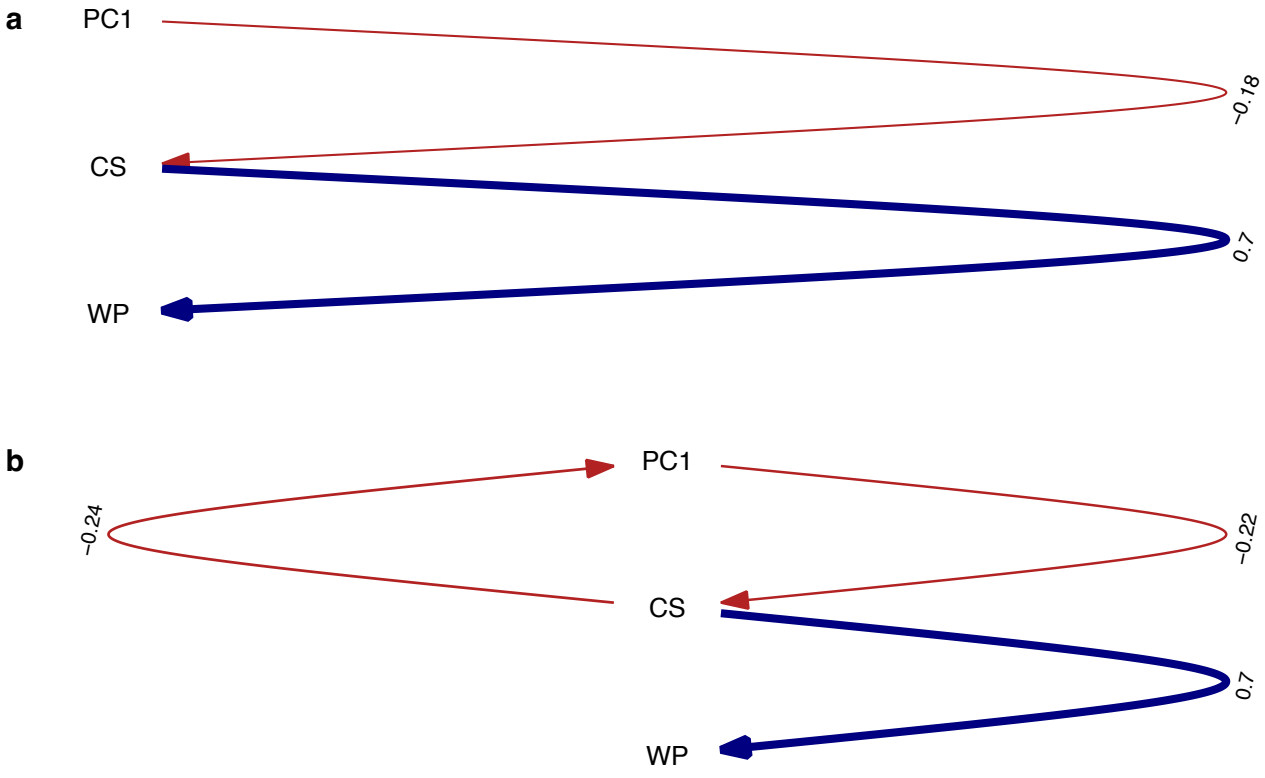
c

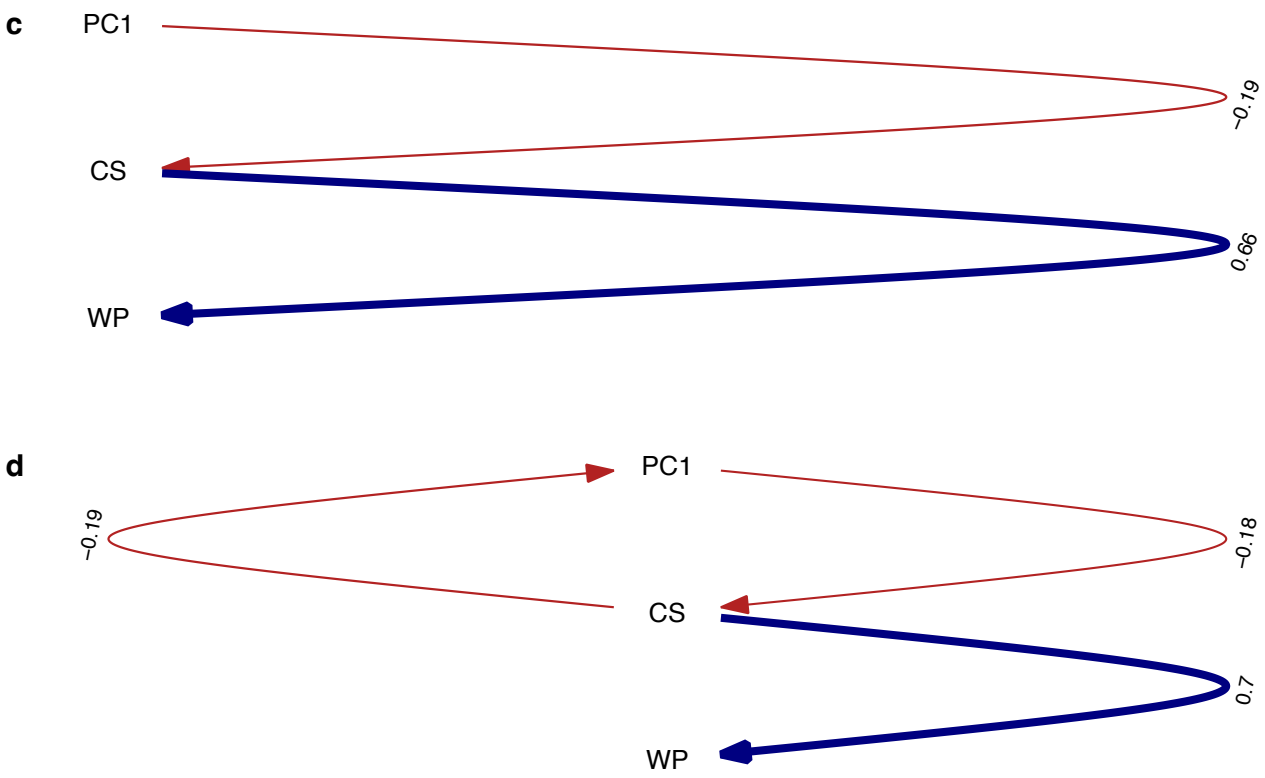


d



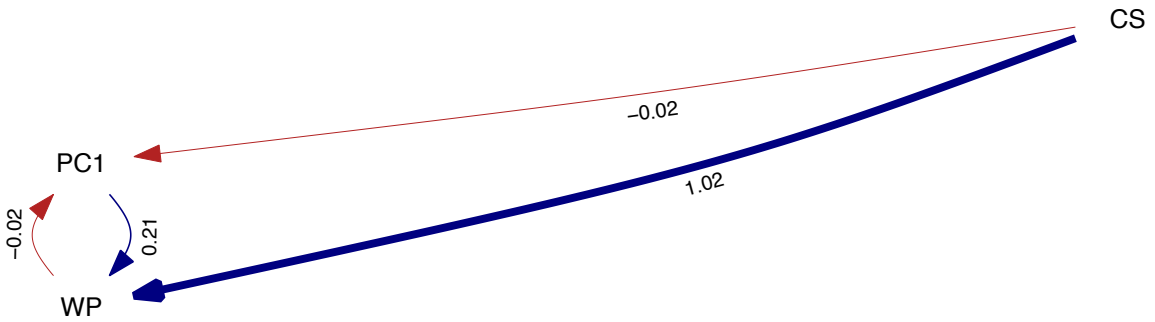
Supplementary Fig. 6. Average of the best-performing models analysing species with exclusively tropical distributions. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. Climate represents PC1 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.


a
PC1
CS
WP
−0.18
0.7
b
PC1
CS
WP
−0.24
−0.22
0.7

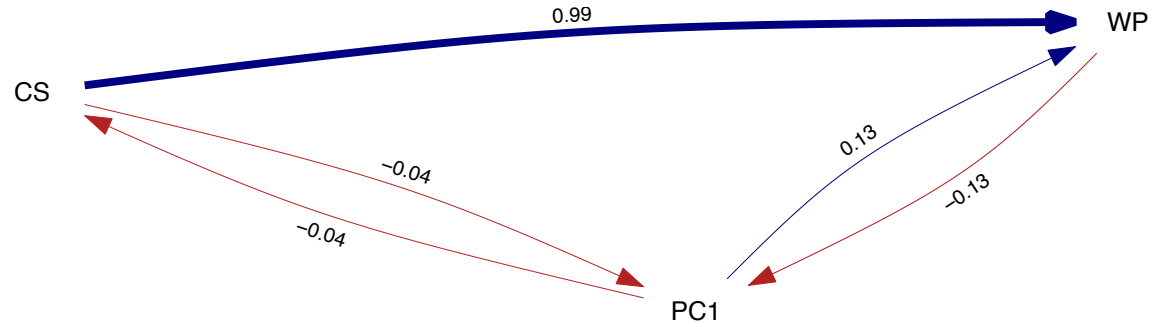


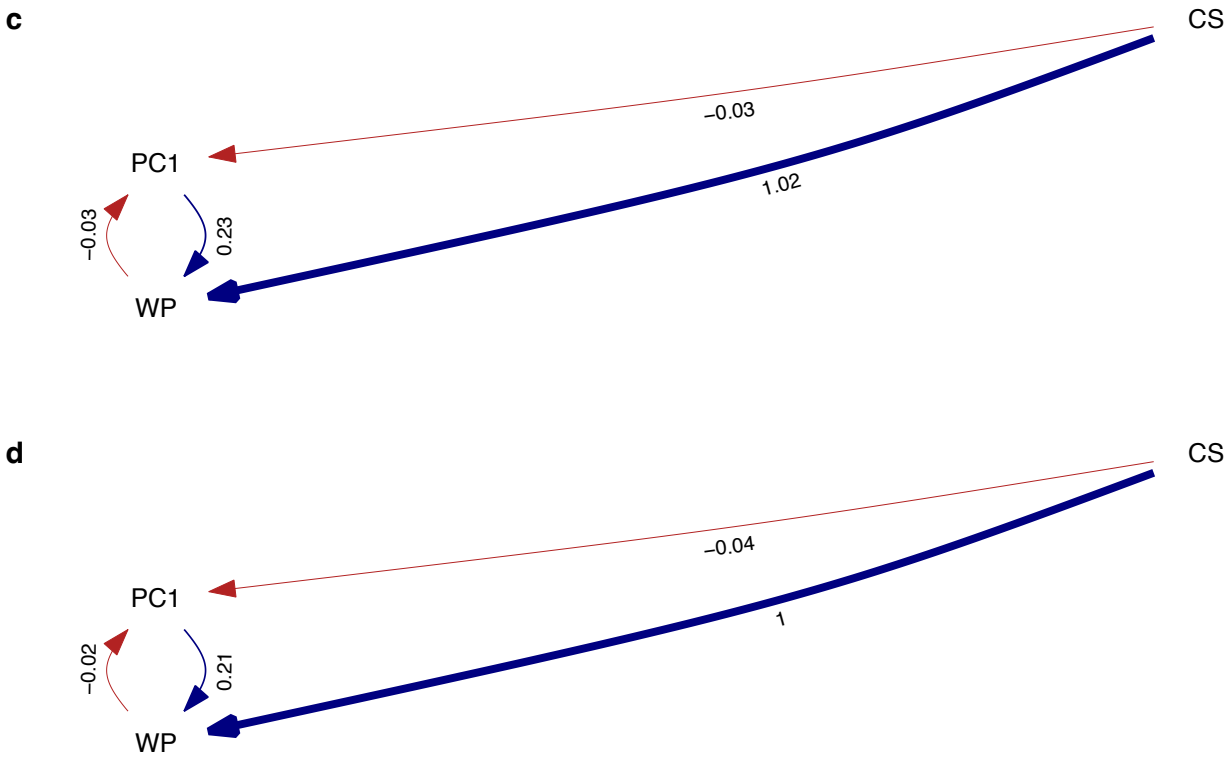
Supplementary Fig. 7. Average of the best-performing models analysing species with exclusively temperate distributions. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. Climate represents PC1 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.

a

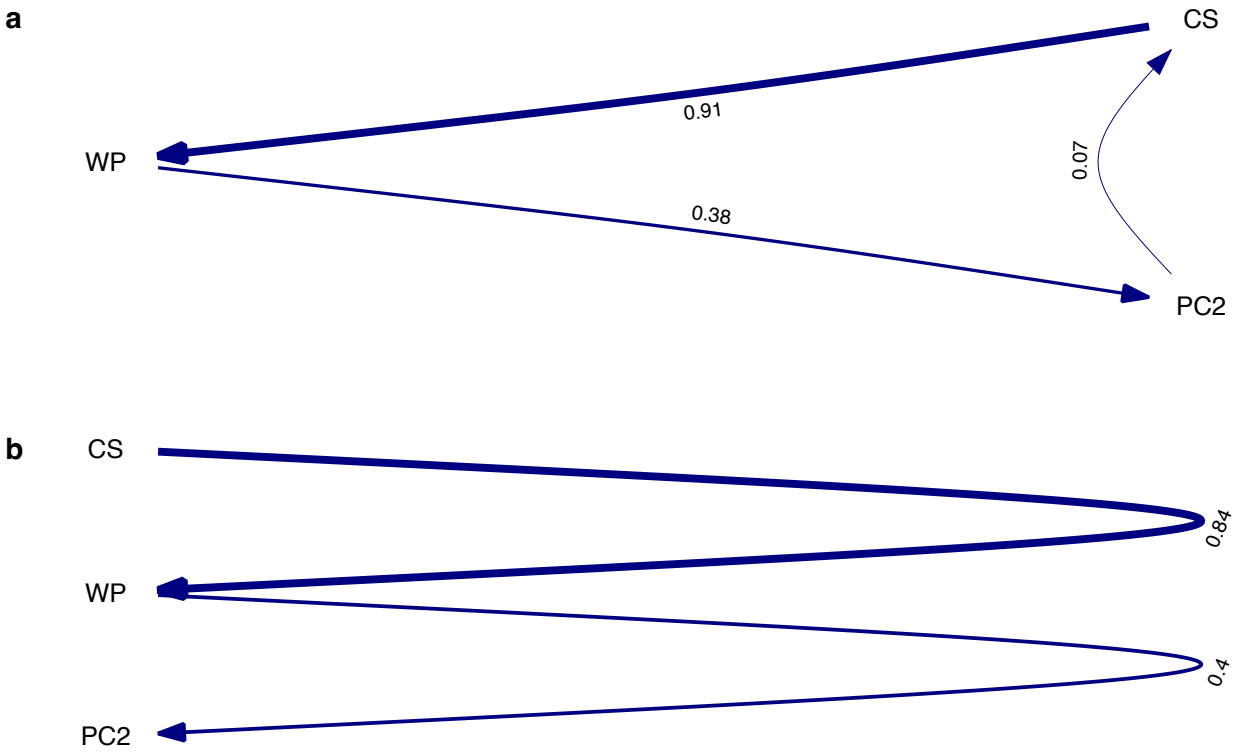


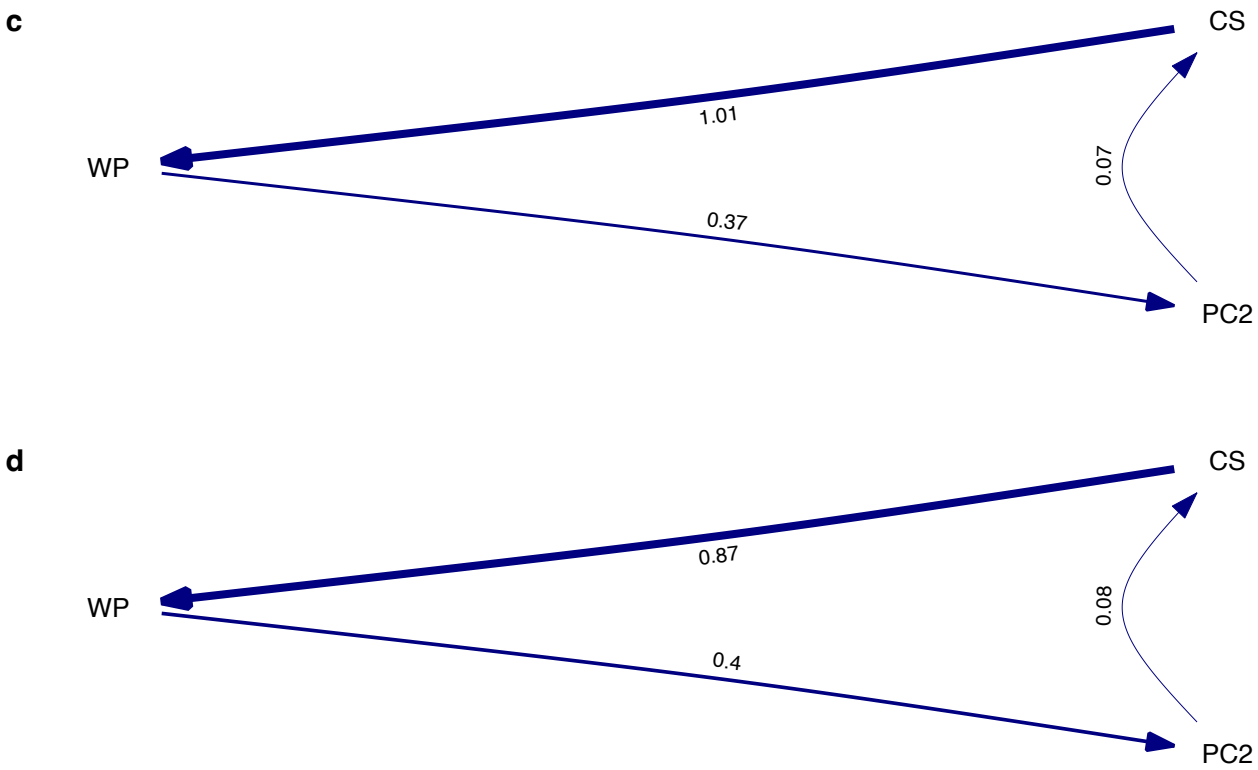
b



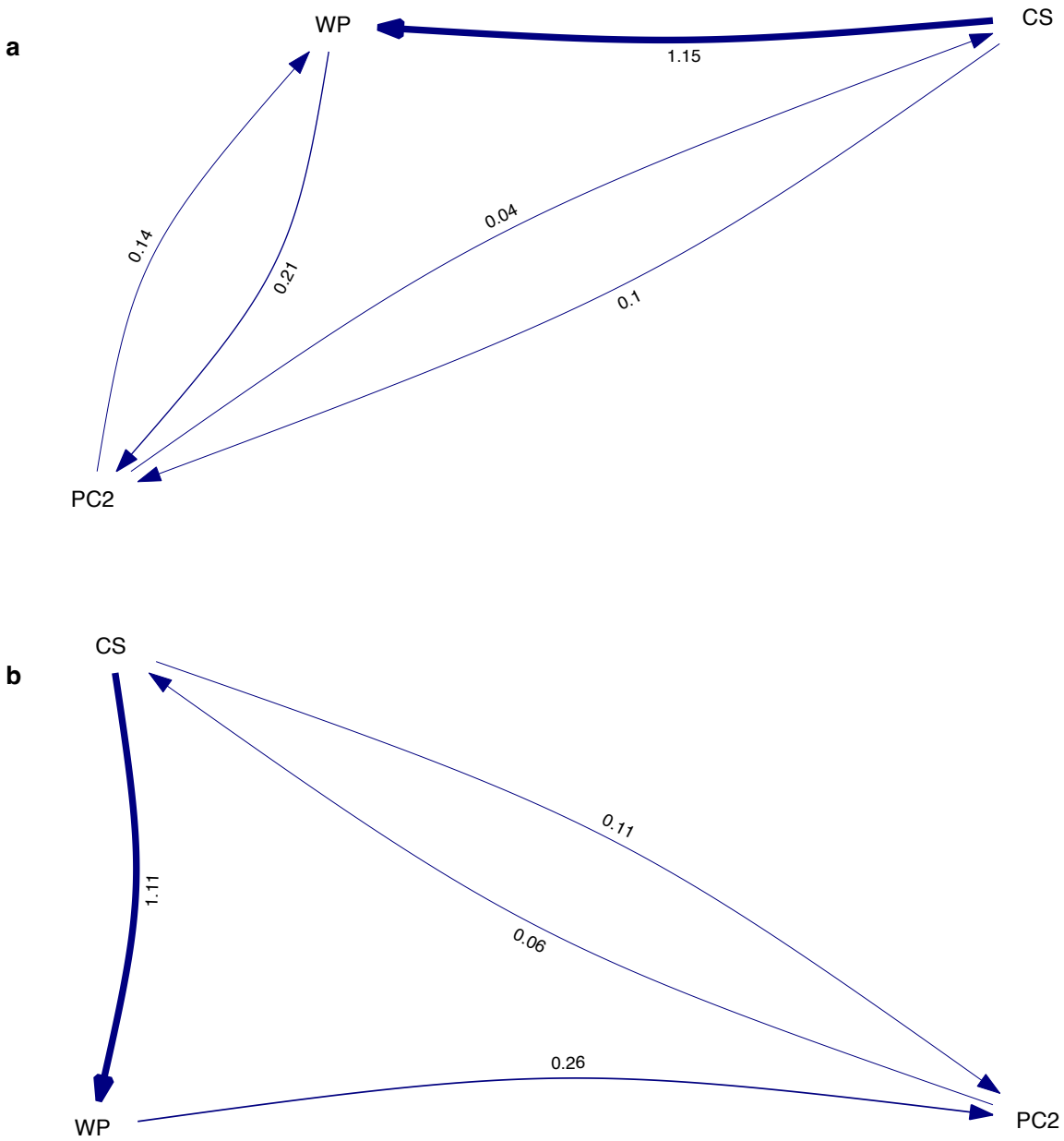


Supplementary Fig. 8. Average of the best-performing models analysing species with distributions that overlap both tropical and temperate regions. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. Climate represents PC1 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.

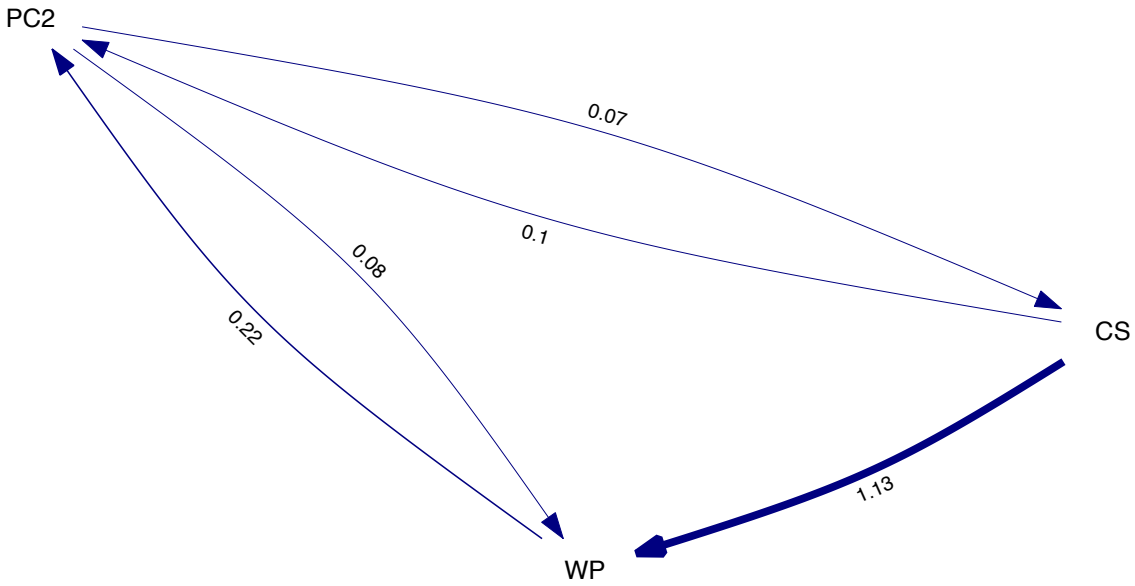

a
CS
0.91
WP
0.07
0.38
PC2
b
CS
0.84
WP
0.4
PC2



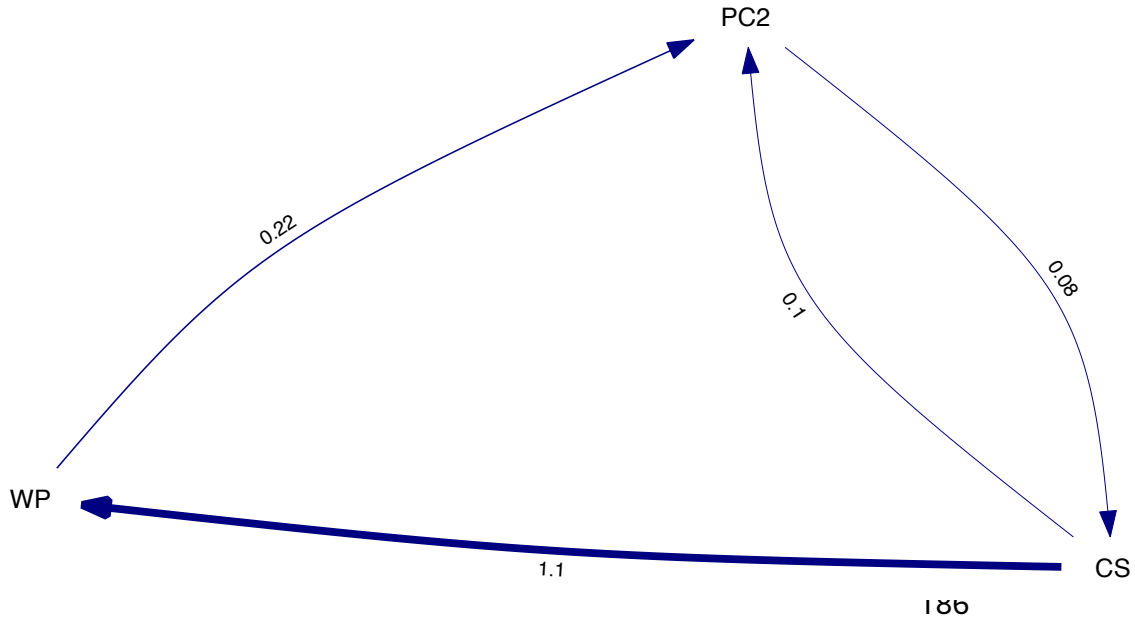
Supplementary Fig. 9. Average of the best-performing models analysing species globally. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. Climate represents PC2 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.


a
WP
CS
1.15
0.14
0.21
0.04
0.1
PC2
b
CS
0.11
1.11
0.06
0.26
WP
PC2

c

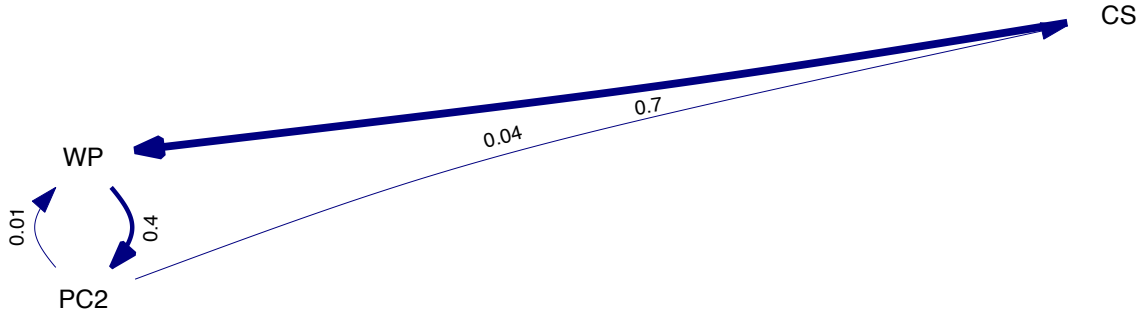


d

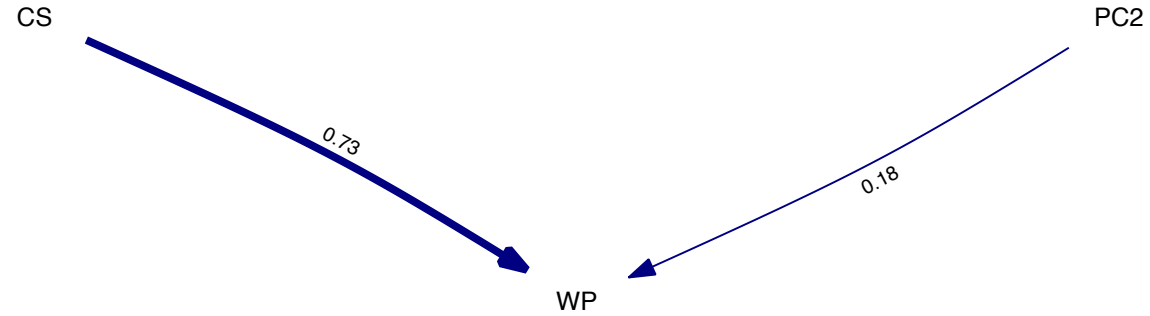


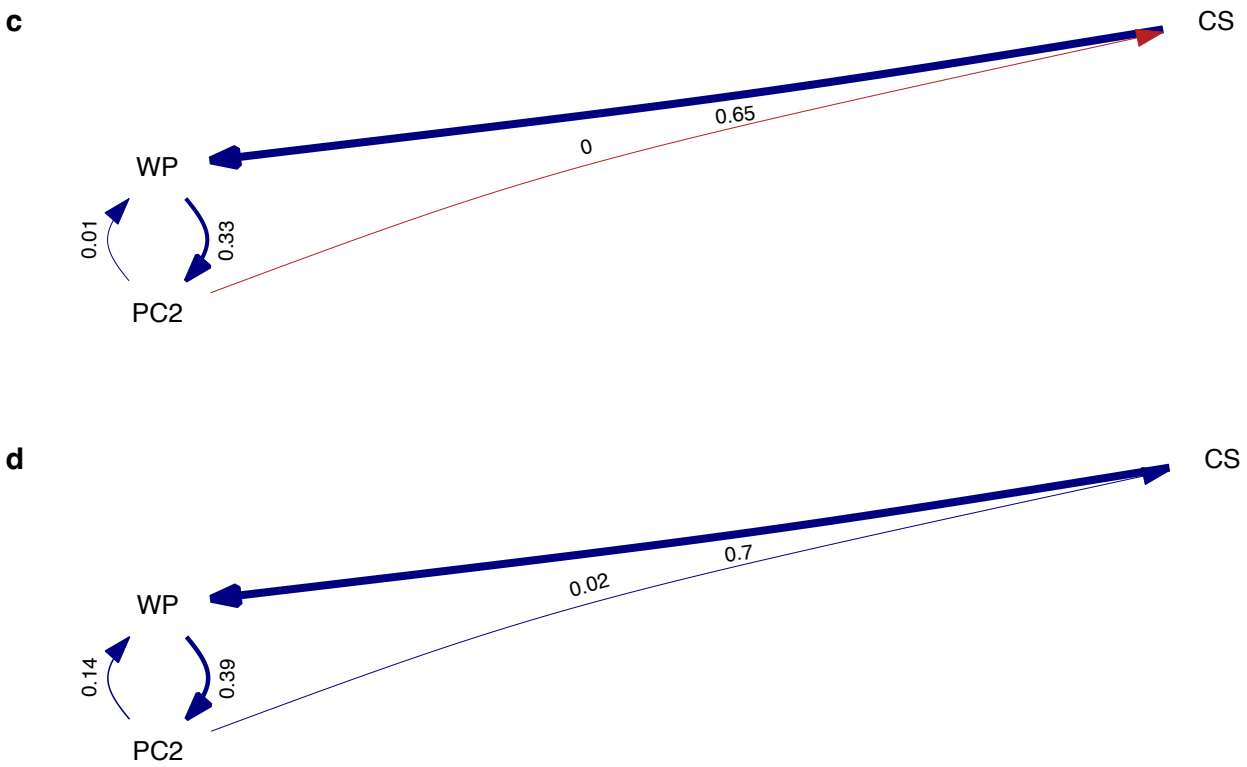
Supplementary Fig. 10. Average of the best-performing models analysing species with exclusively tropical distributions. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. Climate represents PC2 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.

a

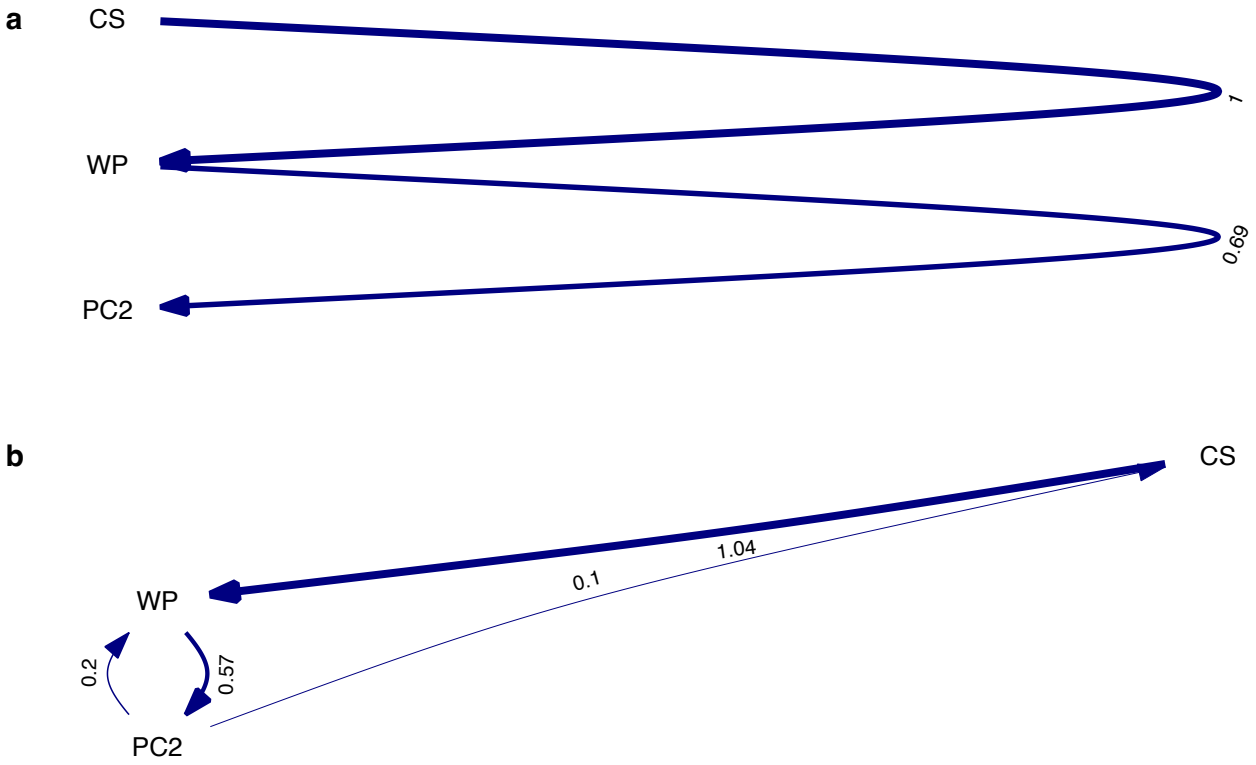


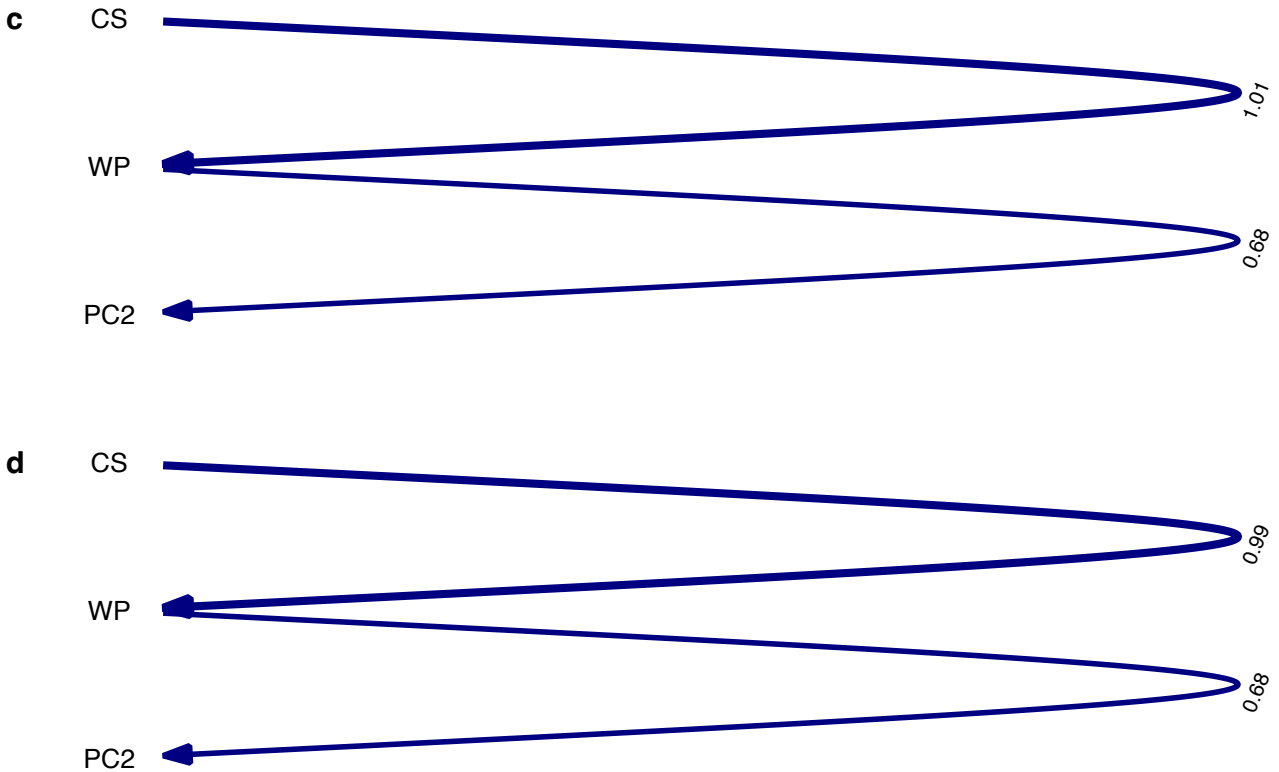
b





Supplementary Fig. 11. Average of the best-performing models analysing species with exclusively temperate distributions. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. Climate represents PC2 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.


a
CS
WP
PC2
1
0.69
b
CS
WP
PC2
1.04
0.1
0.2
0.57



Supplementary Fig. 12. Average of the best-performing models analysing species with distributions that overlap both tropical and temperate regions. Analysis repeated over four different MCC ant trees from Economo et al., 2018: a) NC uniform stem b) NC uniform crown c) FBD stem d) FBD crown. Values represent standardised average coefficients. Climate represents PC2 from our principal component analysis which captures variation in temperature and rainfall. Polymorphism is modelled as a discrete binary variable. WP = worker polymorphism and CS = colony size.

Supplementary Table 1. VIF scores across different regional scales. To assess collinearity among our climatic variables, we (i) calculated variance inflation factors (VIF) and (ii) inspected scatter plots for all possible pairwise combinations of these variables. A common rule of thumb is to consider VIF scores >10 as being a sign of severe multicollinearity (Kutner et al., 1983). Therefore, the results confirmed that collinearity did not pose an issue in our analysis, as all VIFs were less than 3.

Neter, J., Wasserman, W. & Kutner, M. H., Applied Linear Regression Models, Homewood, IL: Richard D. Irwin, Inc. 1983.

Regional scale	Temperature	Rainfall	Diurnal temperature range
Global	1.64	2	1.38
Tropical	1.37	1.08	1.32
Temperate	1.23	1.39	1.44
Both	1.51	2.82	2.3

Supplementary Table 2. Model selection summary table for phylogenetic path analysis analysing the relationship between colony size, worker polymorphism and climate (PC1). Analysis repeated over four different MCC ant trees from Economo et al., 2018. k = number of conditional independencies tested; q = number of parameters estimated in the path model; C = Fisher's C statistic; p = P-value of the Fisher's C statistic obtained through the d-sep test; CICc = C statistic Information Criterion with correction for small sample sizes; ΔCICc = difference between the CIC of the best model and subsequent models; ω = CICc weights that represent the probability of each path model given the data and the set of models being compared.

Phylogeny	model	k	q	C	p	CICc	ΔCICc	l	ω	Region
NC uniform stem	three	1	5	1.76	0.41	11.89	0	1	0.52	Global
NC uniform stem	four	1	5	3.62	0.16	13.75	1.86	0.39	0.21	Global
NC uniform stem	one	1	5	3.75	0.15	13.88	1.99	0.37	0.19	Global
NC uniform stem	two	1	5	5.62	0.06	15.74	3.86	0.15	0.08	Global
NC uniform crown	three	1	5	3.03	0.22	13.16	0	1	0.47	Global
NC uniform crown	four	1	5	4.4	0.11	14.53	1.37	0.5	0.24	Global
NC uniform crown	one	1	5	4.59	0.1	14.71	1.55	0.46	0.22	Global
NC uniform crown	two	1	5	6.87	0.03	17	3.84	0.15	0.07	Global
FBD stem	one	1	5	2.41	0.3	12.53	0	1	0.58	Global
FBD stem	two	1	5	4.65	0.1	14.78	2.24	0.33	0.19	Global
FBD stem	four	1	5	5.44	0.07	15.56	3.03	0.22	0.13	Global
FBD stem	three	1	5	5.91	0.05	16.03	3.5	0.17	0.1	Global
FBD crown	three	1	5	1.6	0.45	11.73	0	1	0.7	Global
FBD crown	one	1	5	4.19	0.12	14.31	2.58	0.27	0.19	Global

FBD crown	four	1	5	6.47	0.04	16.6	4.87	0.09	0.06	Global
FBD crown	two	1	5	7.31	0.03	17.44	5.71	0.06	0.04	Global
NC uniform stem	three	1	5	0.09	0.96	10.63	0	1	0.32	Tropical
NC uniform stem	four	1	5	0.38	0.83	10.92	0.29	0.86	0.27	Tropical
NC uniform stem	one	1	5	0.9	0.64	11.45	0.81	0.67	0.21	Tropical
NC uniform stem	two	1	5	1	0.61	11.54	0.91	0.63	0.2	Tropical
NC uniform crown	four	1	5	0	1	10.55	0	1	0.36	Tropical
NC uniform crown	three	1	5	0.82	0.66	11.36	0.81	0.67	0.24	Tropical
NC uniform crown	two	1	5	1.1	0.58	11.65	1.1	0.58	0.21	Tropical
NC uniform crown	one	1	5	1.35	0.51	11.89	1.34	0.51	0.19	Tropical
FBD stem	four	1	5	0.25	0.88	10.79	0	1	0.31	Tropical
FBD stem	three	1	5	0.48	0.79	11.02	0.23	0.89	0.28	Tropical
FBD stem	two	1	5	1.03	0.6	11.58	0.78	0.68	0.21	Tropical
FBD stem	one	1	5	1.04	0.59	11.59	0.79	0.67	0.21	Tropical
FBD crown	four	1	5	0.29	0.87	10.83	0	1	0.31	Tropical
FBD crown	three	1	5	0.61	0.74	11.15	0.32	0.85	0.26	Tropical
FBD crown	one	1	5	1	0.61	11.54	0.71	0.7	0.22	Tropical
FBD crown	two	1	5	1.02	0.6	11.57	0.74	0.69	0.21	Tropical
NC uniform stem	three	1	5	3.31	0.19	13.64	0	1	0.71	Temperate
NC uniform stem	four	1	5	5.42	0.07	15.75	2.11	0.35	0.25	Temperate
NC uniform stem	one	1	5	9.18	0.01	19.51	5.87	0.05	0.04	Temperate

NC uniform stem	two	1	5	12.23	0	22.56	8.92	0.01	0.01	Temperate
NC uniform crown	three	1	5	3.64	0.16	13.96	0	1	0.59	Temperate
NC uniform crown	four	1	5	4.39	0.11	14.72	0.75	0.69	0.4	Temperate
NC uniform crown	one	1	5	12.17	0	22.49	8.53	0.01	0.01	Temperate
NC uniform crown	two	1	5	14.75	0	25.08	11.11	0	0	Temperate
FBD stem	three	1	5	3.1	0.21	13.43	0	1	0.71	Temperate
FBD stem	four	1	5	5.17	0.08	15.5	2.07	0.36	0.25	Temperate
FBD stem	one	1	5	9.22	0.01	19.55	6.12	0.05	0.03	Temperate
FBD stem	two	1	5	12.14	0	22.47	9.04	0.01	0.01	Temperate
FBD crown	three	1	5	3.91	0.14	14.24	0	1	0.66	Temperate
FBD crown	four	1	5	5.74	0.06	16.06	1.83	0.4	0.27	Temperate
FBD crown	one	1	5	8.72	0.01	19.05	4.81	0.09	0.06	Temperate
FBD crown	two	1	5	12.03	0	22.35	8.12	0.02	0.01	Temperate
NC uniform stem	four	1	5	0.03	0.98	10.4	0	1	0.36	Both
NC uniform stem	two	1	5	0.43	0.81	10.8	0.39	0.82	0.3	Both
NC uniform stem	one	1	5	0.5	0.78	10.87	0.47	0.79	0.29	Both
NC uniform stem	three	1	5	3.75	0.15	14.12	3.72	0.16	0.06	Both
NC uniform crown	two	1	5	0.18	0.91	10.56	0	1	0.37	Both
NC uniform crown	one	1	5	0.9	0.64	11.27	0.71	0.7	0.26	Both
NC uniform crown	four	1	5	1.33	0.51	11.7	1.15	0.56	0.21	Both
NC uniform crown	three	1	5	1.78	0.41	12.16	1.6	0.45	0.17	Both

FBD stem	four	1	5	0.06	0.97	10.44	0	1	0.36	Both
FBD stem	two	1	5	0.48	0.79	10.85	0.42	0.81	0.3	Both
FBD stem	one	1	5	0.58	0.75	10.95	0.52	0.77	0.28	Both
FBD stem	three	1	5	3.74	0.15	14.11	3.68	0.16	0.06	Both
FBD crown	four	1	5	0.2	0.91	10.57	0	1	0.39	Both
FBD crown	two	1	5	1	0.61	11.37	0.81	0.67	0.26	Both
FBD crown	one	1	5	1.01	0.6	11.38	0.82	0.66	0.26	Both
FBD crown	three	1	5	3.37	0.19	13.74	3.17	0.2	0.08	Both

Supplementary Table 3. Coefficients for each of the averaged models from phylogenetic path analysis analysing the relationship between colony size, worker polymorphism and climate (PC1). Analysis repeated over four different MCC ant trees from Economo et al., 2018. SE = standard error, CI = confidence interval, WP = worker polymorphism, CS = colony size and Climate = PC1. Polymorphism is modelled as a discrete binary variable.

Causal relationship	Path coefficient	SE	Lower 95 CI	Upper 95 CI	Tree	Region
CS_affects_WP	0.91	0.14	0.64	1.17	NCuniform_stem	Global
Climate_affects_WP	0.06	0.1	-0.15	0.26	NCuniform_stem	Global
WP_affects_Climate	0	0	0	0	NCuniform_stem	Global
CS_affects_Climate	-0.07	0.05	-0.16	0.02	NCuniform_stem	Global
Climate_affects_CS	-0.06	0.05	-0.15	0.02	NCuniform_stem	Global
CS_affects_WP	0.84	0.12	0.61	1.08	NCuniform_crown	Global
Climate_affects_WP	0.11	0.09	-0.07	0.28	NCuniform_crown	Global
WP_affects_Climate	0	0	0	0	NCuniform_crown	Global
CS_affects_Climate	-0.08	0.05	-0.17	0.01	NCuniform_crown	Global
Climate_affects_CS	-0.07	0.04	-0.16	0.01	NCuniform_crown	Global
CS_affects_WP	0.94	0.13	0.69	1.19	FBD_stem	Global
Climate_affects_WP	0.19	0.09	0.01	0.37	FBD_stem	Global
WP_affects_Climate	0	0	0	0	FBD_stem	Global
CS_affects_Climate	0	0	0	0	FBD_stem	Global
Climate_affects_CS	0	0	0	0	FBD_stem	Global
CS_affects_WP	0.87	0.13	0.62	1.12	FBD_crown	Global

Climate_affects_WP	0	0	0	0	FBD_crown	Global
WP_affects_Climate	0	0	0	0	FBD_crown	Global
CS_affects_Climate	0	0	0	0	FBD_crown	Global
Climate_affects_CS	-0.07	0.05	-0.16	0.02	FBD_crown	Global
CS_affects_WP	1.15	0.28	0.6	1.69	NCuniform_stem	Tropical
Climate_affects_WP	-0.01	0.24	-0.48	0.46	NCuniform_stem	Tropical
WP_affects_Climate	-0.01	0.21	-0.42	0.4	NCuniform_stem	Tropical
CS_affects_Climate	-0.04	0.09	-0.23	0.14	NCuniform_stem	Tropical
Climate_affects_CS	-0.05	0.06	-0.17	0.06	NCuniform_stem	Tropical
CS_affects_WP	1.11	0.27	0.58	1.64	NCuniform_crown	Tropical
Climate_affects_WP	0.14	0.22	-0.29	0.57	NCuniform_crown	Tropical
WP_affects_Climate	-0.07	0.21	-0.48	0.34	NCuniform_crown	Tropical
CS_affects_Climate	-0.06	0.09	-0.24	0.12	NCuniform_crown	Tropical
Climate_affects_CS	-0.07	0.06	-0.19	0.05	NCuniform_crown	Tropical
CS_affects_WP	1.1	0.28	0.55	1.65	FBD_stem	Tropical
Climate_affects_WP	0.07	0.19	-0.31	0.45	FBD_stem	Tropical
WP_affects_Climate	-0.03	0.21	-0.44	0.38	FBD_stem	Tropical
CS_affects_Climate	-0.05	0.09	-0.23	0.13	FBD_stem	Tropical
Climate_affects_CS	-0.08	0.06	-0.2	0.05	FBD_stem	Tropical
CS_affects_WP	1.09	0.27	0.56	1.62	FBD_crown	Tropical
Climate_affects_WP	0.08	0.22	-0.35	0.51	FBD_crown	Tropical

WP_affects_Climate	-0.03	0.21	-0.43	0.38	FBD_crown	Tropical
CS_affects_Climate	-0.05	0.09	-0.23	0.14	FBD_crown	Tropical
Climate_affects_CS	-0.08	0.06	-0.2	0.04	FBD_crown	Tropical
CS_affects_WP	0.7	0.18	0.35	1.05	NCuniform_stem	Temperate
Climate_affects_WP	0	0	0	0	NCuniform_stem	Temperate
WP_affects_Climate	0	0	0	0	NCuniform_stem	Temperate
CS_affects_Climate	0	0	0	0	NCuniform_stem	Temperate
Climate_affects_CS	-0.18	0.07	-0.31	-0.05	NCuniform_stem	Temperate
CS_affects_WP	0.7	0.18	0.35	1.06	NCuniform_crown	Temperate
Climate_affects_WP	0	0	0	0	NCuniform_crown	Temperate
WP_affects_Climate	0	0	0	0	NCuniform_crown	Temperate
CS_affects_Climate	-0.24	0.08	-0.39	-0.09	NCuniform_crown	Temperate
Climate_affects_CS	-0.22	0.06	-0.34	-0.09	NCuniform_crown	Temperate
CS_affects_WP	0.66	0.16	0.33	0.98	FBD_stem	Temperate
Climate_affects_WP	0	0	0	0	FBD_stem	Temperate
WP_affects_Climate	0	0	0	0	FBD_stem	Temperate
CS_affects_Climate	0	0	0	0	FBD_stem	Temperate
Climate_affects_CS	-0.19	0.06	-0.31	-0.06	FBD_stem	Temperate
CS_affects_WP	0.7	0.17	0.36	1.04	FBD_crown	Temperate
Climate_affects_WP	0	0	0	0	FBD_crown	Temperate
WP_affects_Climate	0	0	0	0	FBD_crown	Temperate

CS_affects_Climate	-0.19	0.08	-0.34	-0.04	FBD_crown	Temperate
Climate_affects_CS	-0.18	0.06	-0.3	-0.06	FBD_crown	Temperate
CS_affects_WP	1.02	0.22	0.58	1.46	NCuniform_stem	Both
Climate_affects_WP	0.21	0.16	-0.1	0.52	NCuniform_stem	Both
WP_affects_Climate	-0.02	0.17	-0.36	0.31	NCuniform_stem	Both
CS_affects_Climate	-0.02	0.08	-0.19	0.14	NCuniform_stem	Both
Climate_affects_CS	0	0	0	0	NCuniform_stem	Both
CS_affects_WP	0.99	0.22	0.56	1.42	NCuniform_crown	Both
Climate_affects_WP	0.13	0.16	-0.18	0.44	NCuniform_crown	Both
WP_affects_Climate	-0.13	0.17	-0.47	0.2	NCuniform_crown	Both
CS_affects_Climate	-0.04	0.08	-0.19	0.12	NCuniform_crown	Both
Climate_affects_CS	-0.04	0.07	-0.18	0.11	NCuniform_crown	Both
CS_affects_WP	1.02	0.22	0.58	1.46	FBD_stem	Both
Climate_affects_WP	0.23	0.16	-0.08	0.54	FBD_stem	Both
WP_affects_Climate	-0.03	0.17	-0.37	0.31	FBD_stem	Both
CS_affects_Climate	-0.03	0.08	-0.18	0.13	FBD_stem	Both
Climate_affects_CS	0	0	0	0	FBD_stem	Both
CS_affects_WP	1	0.22	0.56	1.43	FBD_crown	Both
Climate_affects_WP	0.21	0.16	-0.1	0.52	FBD_crown	Both
WP_affects_Climate	-0.02	0.17	-0.36	0.32	FBD_crown	Both
CS_affects_Climate	-0.04	0.08	-0.2	0.11	FBD_crown	Both

Climate_affects_CS	0	0	0	0	FBD_crown	Both	

Supplementary Table 4. Model selection summary table for phylogenetic path analysis analysing the relationship between colony size, worker polymorphism and climate (PC2). Analysis repeated over four different MCC ant trees from Economo et al., 2018. k = number of conditional independencies tested; q = number of parameters estimated in the path model; C = Fisher's C statistic; p = P-value of the Fisher's C statistic obtained through the d-sep test; CICc = C statistic Information Criterion with correction for small sample sizes; ΔCICc = difference between the CIC of the best model and subsequent models; ω = CICc weights that represent the probability of each path model given the data and the set of models being compared.

phylogeny	model	k	q	C	p	CICc	ΔCICc	l	ω	Region
NC uniform stem	three	1	5	2.33	0.31	12.46	0	1	0.49	Global
NC uniform stem	two	1	5	2.47	0.29	12.6	0.14	0.93	0.46	Global
NC uniform stem	one	1	5	7.99	0.02	18.12	5.66	0.06	0.03	Global
NC uniform stem	four	1	5	8.92	0.01	19.05	6.59	0.04	0.02	Global
NC uniform crown	two	1	5	0.84	0.66	10.97	0	1	0.91	Global
NC uniform crown	one	1	5	5.77	0.06	15.9	4.93	0.09	0.08	Global
NC uniform crown	three	1	5	11.48	0	21.6	10.64	0	0	Global

NC uniform crown	four	1	5	12.4	0	22.53	11.56	0	0	Global
FBD stem	two	1	5	2.73	0.26	12.86	0	1	0.49	Global
FBD stem	three	1	5	2.84	0.24	12.97	0.11	0.95	0.46	Global
FBD stem	one	1	5	8.31	0.02	18.44	5.58	0.06	0.03	Global
FBD stem	four	1	5	8.52	0.01	18.65	5.8	0.06	0.03	Global
FBD crown	three	1	5	2.26	0.32	12.38	0	1	0.53	Global
FBD crown	two	1	5	2.65	0.27	12.78	0.39	0.82	0.43	Global
FBD crown	one	1	5	8.62	0.01	18.74	6.36	0.04	0.02	Global
FBD crown	four	1	5	9.42	0.01	19.54	7.16	0.03	0.01	Global
NC uniform stem	three	1	5	0.95	0.62	11.49	0	1	0.31	Tropical
NC uniform stem	four	1	5	1.07	0.58	11.62	0.13	0.94	0.29	Tropical
NC uniform stem	two	1	5	1.27	0.53	11.82	0.33	0.85	0.26	Tropical
NC uniform stem	one	1	5	2.52	0.28	13.06	1.57	0.46	0.14	Tropical
NC uniform crown	three	1	5	0.17	0.92	10.72	0	1	0.43	Tropical

NC uniform crown	two	1	5	1.3	0.52	11.84	1.13	0.57	0.24	Tropical
NC uniform crown	four	1	5	1.55	0.46	12.09	1.37	0.5	0.22	Tropical
NC uniform crown	one	1	5	2.92	0.23	13.47	2.75	0.25	0.11	Tropical
FBD stem	three	1	5	0.68	0.71	11.23	0	1	0.34	Tropical
FBD stem	four	1	5	1.17	0.56	11.72	0.49	0.78	0.27	Tropical
FBD stem	two	1	5	1.28	0.53	11.83	0.6	0.74	0.26	Tropical
FBD stem	one	1	5	2.61	0.27	13.15	1.92	0.38	0.13	Tropical
FBD crown	three	1	5	0.43	0.81	10.98	0	1	0.37	Tropical
FBD crown	four	1	5	1.14	0.56	11.69	0.71	0.7	0.26	Tropical
FBD crown	two	1	5	1.28	0.53	11.83	0.85	0.65	0.24	Tropical
FBD crown	one	1	5	2.58	0.28	13.12	2.15	0.34	0.13	Tropical
NC uniform stem	two	1	5	0.38	0.83	10.7	0	1	0.38	Temperate
NC uniform stem	three	1	5	0.42	0.81	10.74	0.04	0.98	0.37	Temperate
NC uniform stem	one	1	5	1.26	0.53	11.59	0.89	0.64	0.24	Temperate

NC uniform stem	four	1	5	7.64	0.02	17.97	7.27	0.03	0.01	Temperate
NC uniform crown	one	1	5	0.65	0.72	10.98	0	1	0.6	Temperate
NC uniform crown	two	1	5	2.75	0.25	13.08	2.1	0.35	0.21	Temperate
NC uniform crown	three	1	5	3.14	0.21	13.46	2.48	0.29	0.17	Temperate
NC uniform crown	four	1	5	7.8	0.02	18.12	7.14	0.03	0.02	Temperate
FBD stem	one	1	5	0.63	0.73	10.96	0	1	0.39	Temperate
FBD stem	two	1	5	0.67	0.72	10.99	0.04	0.98	0.38	Temperate
FBD stem	three	1	5	2.01	0.37	12.34	1.38	0.5	0.2	Temperate
FBD stem	four	1	5	6.02	0.05	16.34	5.39	0.07	0.03	Temperate
FBD crown	two	1	5	0.67	0.72	10.99	0	1	0.43	Temperate
FBD crown	one	1	5	0.98	0.61	11.31	0.31	0.85	0.36	Temperate
FBD crown	three	1	5	2.22	0.33	12.54	1.55	0.46	0.2	Temperate
FBD crown	four	1	5	7.55	0.02	17.87	6.88	0.03	0.01	Temperate
NC uniform stem	two	1	5	0.13	0.94	10.5	0	1	0.92	Both

NC uniform stem	one	1	5	6.03	0.05	16.4	5.9	0.05	0.05	Both
NC uniform stem	three	1	5	6.67	0.04	17.04	6.54	0.04	0.03	Both
NC uniform stem	four	1	5	14.59	0	24.96	14.46	0	0	Both
NC uniform crown	two	1	5	1.87	0.39	12.24	0	1	0.52	Both
NC uniform crown	three	1	5	3.31	0.19	13.68	1.44	0.49	0.25	Both
NC uniform crown	one	1	5	3.49	0.17	13.87	1.63	0.44	0.23	Both
NC uniform crown	four	1	5	14.52	0	24.89	12.65	0	0	Both
FBD stem	two	1	5	0.07	0.97	10.44	0	1	0.92	Both
FBD stem	one	1	5	5.92	0.05	16.3	5.85	0.05	0.05	Both
FBD stem	three	1	5	7.29	0.03	17.66	7.22	0.03	0.03	Both
FBD stem	four	1	5	14.24	0	24.61	14.17	0	0	Both
FBD crown	two	1	5	0.07	0.97	10.44	0	1	0.93	Both
FBD crown	one	1	5	5.86	0.05	16.24	5.8	0.06	0.05	Both
FBD crown	three	1	5	7.46	0.02	17.83	7.4	0.02	0.02	Both

FBD crown	four	1	5	14.17	0	24.54	14.1	0	0	Both

Supplementary Table 5. Coefficients for each of the averaged models from phylogenetic path analysis analysing the relationship between colony size, worker polymorphism and climate (PC2). Analysis repeated over four different MCC ant trees from Economo et al., 2018. SE = standard error, CI = confidence interval, WP = worker polymorphism, CS = colony size and Climate = PC2. Polymorphism is modelled as a discrete binary variable.

Causal relationship	Path coefficient	SE	Lower 95 CI	Upper 95 CI	Tree	Region
CS_affects_WP	0.91	0.14	0.64	1.17	NCuniform_stem	Global
Climate_affects_WP	0	0	0	0	NCuniform_stem	Global
WP_affects_Climate	0.38	0.12	0.16	0.61	NCuniform_stem	Global
CS_affects_Climate	0	0	0	0	NCuniform_stem	Global
Climate_affects_CS	0.07	0.04	0	0.15	NCuniform_stem	Global
CS_affects_WP	0.84	0.12	0.61	1.08	NCuniform_crown	Global
Climate_affects_WP	0	0	0	0	NCuniform_crown	Global
WP_affects_Climate	0.4	0.11	0.18	0.62	NCuniform_crown	Global
CS_affects_Climate	0	0	0	0	NCuniform_crown	Global
Climate_affects_CS	0	0	0	0	NCuniform_crown	Global
CS_affects_WP	1.01	0.15	0.71	1.3	FBD_stem	Global
Climate_affects_WP	0	0	0	0	FBD_stem	Global
WP_affects_Climate	0.37	0.11	0.15	0.59	FBD_stem	Global
CS_affects_Climate	0	0	0	0	FBD_stem	Global
Climate_affects_CS	0.07	0.04	0	0.15	FBD_stem	Global
CS_affects_WP	0.87	0.13	0.62	1.12	FBD_crown	Global

Climate_affects_WP	0	0	0	0	FBD_crown	Global
WP_affects_Climate	0.4	0.12	0.17	0.62	FBD_crown	Global
CS_affects_Climate	0	0	0	0	FBD_crown	Global
Climate_affects_CS	0.08	0.04	0	0.15	FBD_crown	Global
CS_affects_WP	1.15	0.28	0.6	1.69	NCuniform_stem	Tropical
Climate_affects_WP	0.14	0.25	-0.36	0.64	NCuniform_stem	Tropical
WP_affects_Climate	0.21	0.21	-0.19	0.62	NCuniform_stem	Tropical
CS_affects_Climate	0.1	0.09	-0.08	0.28	NCuniform_stem	Tropical
Climate_affects_CS	0.04	0.06	-0.07	0.16	NCuniform_stem	Tropical
CS_affects_WP	1.11	0.27	0.58	1.64	NCuniform_crown	Tropical
Climate_affects_WP	0	0	0	0	NCuniform_crown	Tropical
WP_affects_Climate	0.26	0.21	-0.15	0.67	NCuniform_crown	Tropical
CS_affects_Climate	0.11	0.09	-0.07	0.29	NCuniform_crown	Tropical
Climate_affects_CS	0.06	0.06	-0.07	0.18	NCuniform_crown	Tropical
CS_affects_WP	1.13	0.27	0.59	1.67	FBD_stem	Tropical
Climate_affects_WP	0.08	0.24	-0.39	0.55	FBD_stem	Tropical
WP_affects_Climate	0.22	0.21	-0.18	0.63	FBD_stem	Tropical
CS_affects_Climate	0.1	0.09	-0.08	0.29	FBD_stem	Tropical
Climate_affects_CS	0.07	0.06	-0.05	0.19	FBD_stem	Tropical
CS_affects_WP	1.1	0.27	0.57	1.62	FBD_crown	Tropical
Climate_affects_WP	0	0	0	0	FBD_crown	Tropical

WP_affects_Climate	0.22	0.21	-0.19	0.63	FBD_crown	Tropical
CS_affects_Climate	0.1	0.09	-0.08	0.28	FBD_crown	Tropical
Climate_affects_CS	0.08	0.06	-0.05	0.2	FBD_crown	Tropical
CS_affects_WP	0.7	0.18	0.35	1.05	NCuniform_stem	Temperate
Climate_affects_WP	0.01	0.13	-0.25	0.27	NCuniform_stem	Temperate
WP_affects_Climate	0.4	0.17	0.07	0.73	NCuniform_stem	Temperate
CS_affects_Climate	0	0	0	0	NCuniform_stem	Temperate
Climate_affects_CS	0.04	0.08	-0.1	0.19	NCuniform_stem	Temperate
CS_affects_WP	0.73	0.18	0.36	1.09	NCuniform_crown	Temperate
Climate_affects_WP	0.18	0.14	-0.1	0.46	NCuniform_crown	Temperate
WP_affects_Climate	0	0	0	0	NCuniform_crown	Temperate
CS_affects_Climate	0	0	0	0	NCuniform_crown	Temperate
Climate_affects_CS	0	0	0	0	NCuniform_crown	Temperate
CS_affects_WP	0.65	0.16	0.33	0.97	FBD_stem	Temperate
Climate_affects_WP	0.01	0.13	-0.24	0.25	FBD_stem	Temperate
WP_affects_Climate	0.33	0.16	0	0.65	FBD_stem	Temperate
CS_affects_Climate	0	0	0	0	FBD_stem	Temperate
Climate_affects_CS	0	0.07	-0.14	0.14	FBD_stem	Temperate
CS_affects_WP	0.7	0.17	0.36	1.04	FBD_crown	Temperate
Climate_affects_WP	0.14	0.14	-0.14	0.41	FBD_crown	Temperate
WP_affects_Climate	0.39	0.17	0.06	0.73	FBD_crown	Temperate

CS_affects_Climate	0	0	0	0	FBD_crown	Temperate
Climate_affects_CS	0.02	0.07	-0.13	0.16	FBD_crown	Temperate
CS_affects_WP	1	0.22	0.58	1.43	NCuniform_stem	Both
Climate_affects_WP	0	0	0	0	NCuniform_stem	Both
WP_affects_Climate	0.69	0.17	0.35	1.02	NCuniform_stem	Both
CS_affects_Climate	0	0	0	0	NCuniform_stem	Both
Climate_affects_CS	0	0	0	0	NCuniform_stem	Both
CS_affects_WP	1.04	0.24	0.56	1.52	NCuniform_crown	Both
Climate_affects_WP	0.2	0.16	-0.12	0.52	NCuniform_crown	Both
WP_affects_Climate	0.57	0.16	0.26	0.88	NCuniform_crown	Both
CS_affects_Climate	0	0	0	0	NCuniform_crown	Both
Climate_affects_CS	0.1	0.06	-0.02	0.22	NCuniform_crown	Both
CS_affects_WP	1.01	0.22	0.58	1.43	FBD_stem	Both
Climate_affects_WP	0	0	0	0	FBD_stem	Both
WP_affects_Climate	0.68	0.17	0.34	1.01	FBD_stem	Both
CS_affects_Climate	0	0	0	0	FBD_stem	Both
Climate_affects_CS	0	0	0	0	FBD_stem	Both
CS_affects_WP	0.99	0.22	0.57	1.42	FBD_crown	Both
Climate_affects_WP	0	0	0	0	FBD_crown	Both
WP_affects_Climate	0.68	0.17	0.34	1.02	FBD_crown	Both
CS_affects_Climate	0	0	0	0	FBD_crown	Both

Climate_affects_CS	0	0	0	0	FBD_crown	Both	

Supplementary Table 6. Phylogenetic heritability or intraclass correlation coefficient estimates for colony size and worker polymorphism calculated using BPMMs across 400 trees. For BPMMSs with binary response variables, the residual variance cannot be identified. Therefore, when analysing worker polymorphism, the residual variance is set to 1. We estimate the amount of variation in worker polymorphism explained by shared ancestry between species calculated as the intraclass correlation coefficient (phylogenetic variance/ (phylogenetic variance + 1) + π^(2/3)) — an analogous measure of the amount of variation explained by phylogenetic history appropriate for binary traits.

Response trait	**Random effects**	**Posterior mode (CI)**	**Phylogenetic H^2/Intraclass correlation coefficient (CI)**
Colony size	Phylogenetic variance (V_A)	1.19 (0.78 to 1.84)	72.26% (58.13 to 83.88)
	Residual variance (V_R)	0.49 (0.35 to 0.64)	
Worker polymorphism	Phylogenetic variance (V_A)	12.87 (6.74 to 22.58)	77.02% (64.77 to 85.43)
	Residual variance (V_R)	1	

Supplementary Table 7. Parameter estimates for Bayesian phylogenetic mixed models (BPMMs) analysing the relationship between colony size and worker polymorphism. Values for colony size were log_{10} transformed for this analysis. Bold text indicates the presence of a significant effect. Parameters estimated separately for the analysis of global species distributions, as well as for distinct geographical regions: (i) tropical; (ii) temperate; and (iii) species with distributions that overlap both temperate and tropical regions.

	Worker polymorphism							
	Global		**Tropical**		**Temperate**		**Tropical & Temperate**	
Predictor	*estimate*	*CI*	*estimate*	*CI*	*estimate*	*CI*	*estimate*	*CI*
Intercept	**-7.44**	**(-10.63, -4.86)**	**-6**	**(-9.65, -3.41)**	**-8.32**	**(-12.51, -4.67)**	**-5.67**	**(-8.86, -3.57)**
Log_{10}(Colony size)	**1.84**	**(1.33, 2.47)**	**1.38**	**(0.74, 2.29)**	**1.84**	**(1.05, 2.9)**	**1.67**	**(1.06, 2.45)**

Supplementary Table 8. Parameter estimates for Bayesian phylogenetic mixed models (BPMMs) analysing the relationship between either PC1 or PC2 and colony size or worker polymorphism. Polymorphism is modelled as a discrete binary variable.

Values for colony size were log_{10} transformed for this analysis. Bold text indicates the presence of a significant effect. Parameters estimated separately for the analysis of global species distributions, as well as for distinct geographical regions: (i) tropical; (ii) temperate; and (iii) species with distributions that overlap both temperate and tropical regions. Analysis repeated over four different MCC ant trees from Economo et al., 2018.

	Global		**Tropical**		**Temperate**		**Tropical & Temperate**		**Response**	**MCC tree**
Predictor	*estimate*	*CI*	*estimate*	*CI*	*estimate*	*CI*	*estimate*	*CI*		
Intercept	**-2.89**	**(-5.38, -0.23)**	-1.59	(-4.18, 0.40)	**-2.88**	**(-5.42, -0.09)**	-1.28	(-3.60, 0.45)	Worker polymorphism	NCuniform stem
PC1	0.11	(-0.3, 0.49)	-0.26	(-1.64, 0.97)	0.56	(-0.57, 1.12)	-0.04	(-0.83, 0.66)	Worker polymorphism	NCuniform stem
Intercept	**-2.31**	**(-4.72, -0.09)**	-2.07	(-4.10, 0.06)	-2.17	(-5.57, 0.39)	-1.44	(-3.46, 0.61)	Worker polymorphism	NCuniform crown
PC1	-0.04	(-0.38, 0.44)	-0.32	(-1.25, 0.71)	0.16	(-0.76, 1.18)	-0.21	(-1.11, 0.46)	Worker polymorphism	NCuniform crown
Intercept	**-1.99**	**(-4.87, -0.36)**	-2.18	(-4.32, 0.06)	-1.95	(-4.87, 0.22)	-1.59	(-3.60, 0.45)	Worker polymorphism	FBD stem
PC1	0.03	(-0.29, 0.49)	-0.19	(-1.30, 0.92)	0.48	(-0.44, 1.25)	-0.3	(-0.8, 0.73)	Worker polymorphism	FBD stem
Intercept	**-2.51**	**(-4.72, -0.11)**	-1.56	(-4.36, 0.25)	-1.95	(-4.85, 0.24)	-0.85	(-3.75, 0.83)	Worker polymorphism	FBD crown
PC1	0.1	(-0.30, 0.53)	-0.17	(-1.54, 0.86)	0.22	(-0.47, 1.24)	-0.23	(-0.98, 0.65)	Worker polymorphism	FBD crown

		(-5.48,		**(-4.51, -**		**(-5.79,**		(-3.85,	Worker	
Intercept	**-2.99**	**-0.47)**	**-2.09**	**0.41)**	**-2.75**	**-0.85)**	-2.04	0.09)	polymorphism	NCuniform stem
		(0.40,		(-0.38,		**(0.22,**		**(0.94,**	Worker	
PC2	**0.99**	**1.96)**	0.64	1.56)	**1.54**	**2.69)**	**2.5**	**4.03)**	polymorphism	NCuniform stem
		(-4.78,		**(-4.66, -**		**(-5.44,**		(-3.73,	Worker	
Intercept	-2.87	0.08)	**-2.36**	**0.40)**	**-2.41**	**-0.45)**	-1.26	0.14)	polymorphism	NCuniform crown
		(0.53,		(0.48,		(-0.06,		**(0.77,**	Worker	
PC2	**1.33**	**1.92)**	0.69	2.07)	0.97	1.91)	**2.33**	**3.72)**	polymorphism	NCuniform crown
		(-4.72,		**(-4.32, -**		**(-5.11,**		(-3.84,	Worker	
Intercept	**-2.39**	**-0.23)**	**-1.88**	**0.33)**	**-2.31**	**-0.66)**	-2.12	0.06)	polymorphism	FBD stem
		(0.37,		(-0.46,		(-0.05,		**(1.16,**	Worker	
PC2	**1.13**	**1.92)**	0.41	1.59)	1.53	2.30)	**2.01**	**4.10)**	polymorphism	FBD stem
		(-4.81,		**(-4.52, -**		**(-5.18,**		(-4.16,	Worker	
Intercept	**-2.42**	**-0.30)**	**-2.62**	**0.63)**	**-2.48**	**-0.48)**	-1.98	0.02)	polymorphism	FBD crown
		(0.50,		(-0.43,		**(0.14,**		**(0.97,**	Worker	
PC2	**1.14**	**2.07)**	0.78	1.70)	**1.11**	**2.58)**	**2.08**	**3.98)**	polymorphism	FBD crown
		(1.88,		**(1.77,**		**(1.54,**		**(1.74,**		
Intercept	**2.57**	**3.24)**	**2.58**	**3.98)**	**2.19**	**2.83)**	**2.48**	**3.40)**	Colony size	NCuniform stem
		(-0.15,		(-0.42,		**(-0.37,**		(-0.21,		
PC1	-0.07	0.02)	-0.09	0.14)	**-0.24**	**-0.08)**	-0.06	0.19)	Colony size	NCuniform stem
		(1.86,		**(1.79,**		**(1.37,**		**(1.65,**		
Intercept	**2.54**	**3.33)**	**2.92**	**3.83)**	**2.18**	**2.87)**	**2.74**	**3.47)**	Colony size	NCuniform crown
		(-0.15,		(-0.37,		**(-0.47,**		(-0.27,		
PC1	-0.07	0.01)	-0.19	0.09)	**-0.29**	**-0.13)**	-0.06	0.15)	Colony size	NCuniform crown
		(1.97,		**(1.98,**		**(1.48,**		**(1.9,**		
Intercept	**2.73**	**3.25)**	**3.1**	**3.96)**	**2.25**	**2.92)**	**2.5**	**3.34)**	Colony size	FBD stem

		(-0.13,		(-0.47,		**(-0.38,**		(-0.26,	
PC1	-0.05	0.04)	-0.19	0.09)	**-0.23**	**-0.09)**	-0.06	0.16)	Colony size	FBD stem
		(1.87,		**(1.9,**		**(1.58,**		**(1.85,**	
Intercept	**2.57**	**3.15)**	**2.83**	**3.89)**	**2.35**	**2.95)**	**2.75**	**3.35)**	Colony size	FBD crown
		(-0.15,		(-0.51,		**(-0.36,**		(-0.3,	
PC1	-0.05	0.02)	-0.21	0.07)	**-0.18**	**-0.06)**	-0.08	0.15)	Colony size	FBD crown
		(1.97,		**(1.68,**		**(1.82,**		**(1.81,**	
Intercept	**2.66**	**3.26)**	**2.74**	**3.85)**	**2.34**	**3.12)**	**2.54**	**3.46)**	Colony size	NCuniform stem
		(0.01,		(-0.12,		(-0.16,		**(0.02,**	
PC2	**0.16**	**0.28)**	0.07	0.27)	0.07	0.28)	**0.4**	**0.72)**	Colony size	NCuniform stem
		(1.84,		**(1.72,**		**(1.89,**		**(1.68,**	
Intercept	**2.64**	**3.29)**	**2.44**	**3.75)**	**2.46**	**3.19)**	**2.56**	**3.45)**	Colony size	NCuniform crown
		(-0.02,		(-0.13,		(-0.24,		(-0.04,	
PC2	0.08	0.28)	0.23	0.45)	0.01	0.13)	0.28	0.60)	Colony size	NCuniform crown
		(1.96,		**(1.83,**		**(1.93,**		**(1.84,**	
Intercept	**2.47**	**3.16)**	**2.98**	**3.66)**	**2.59**	**3.32)**	**2.59**	**3.31)**	Colony size	FBD stem
		(0.00,		(-0.07,		(-0.22,		(-0.02,	
PC2	**0.13**	**0.30)**	0.18	0.40)	0.01	0.21)	0.31	0.72)	Colony size	FBD stem
		(2.03,		**(1.88,**		**(2.01,**		**(1.94,**	
Intercept	**2.69**	**3.21)**	**2.7**	**3.63)**	**2.54**	**3.28)**	**2.63**	**3.36)**	Colony size	FBD crown
		(-0.01,		(-0.08,		(-0.21,		(-0.08,	
PC2	0.13	0.29)	0.1	0.37)	0.08	0.26)	0.36	0.67)	Colony size	FBD crown

Supplementary Table 9. Factor loadings and variance explained for the phylogenetic principal component analysis (PCA).

Variable	**PC1**	**PC2**	**MCC_tree**
Average Temperature	0.87	0.5	NCuniform_stem
Average Rainfall	0.76	-0.65	NCuniform_stem
Proportion of variance	0.67	0.33	NCuniform_stem
Cumulative proportion	0.67	1	NCuniform_stem
Average Temperature	0.78	0.63	NCuniform_crown
Average Rainfall	0.94	-0.35	NCuniform_crown
Proportion of variance	0.77	0.23	NCuniform_crown
Cumulative proportion	0.77	1	NCuniform_crown
Average Temperature	0.88	0.48	FBD_stem
Average Rainfall	0.86	-0.52	FBD_stem

Proportion of variance	0.75	0.25	FBD_stem
Cumulative proportion	0.75	1	FBD_stem
Average Temperature	0.83	0.56	FBD_crown
Average Rainfall	0.77	-0.64	FBD_crown
Proportion of variance	0.64	0.36	FBD_crown
Cumulative proportion	0.64	1	FBD_crown

Supplementary Table 10. Ant life history data and their associated references can be found online at: https://github.com/LouisBell-Roberts/Ant_complexity_climate

Supplementary Table 11. Ant species excluded from analyses and their associated references can be found online at: https://github.com/LouisBell-Roberts/Ant_complexity_climate

Model settings and assessments for Bayesian phylogenetic models

We fitted BPMMs with Markov chain Monte Carlo (MCMC) estimation using the MCMCglmm package v2.34(Hadfield, 2010). Models were run for a minimum of 11,000,000 iterations, with a burn-in of 1,000,000 and thinning interval of 10,000. However, models were run for as long as necessary to obtain posterior effective sample sizes of at least 300 for all parameters. Overall, the majority of estimates had an effective sample size of at least 1,000. To ensure model convergence, we used the coda package v0.19-4 to calculate the degree of autocorrelation between successive iterations in each chain(Plummer *et al.*, 2006). We fitted each model independently two times and used Gelman and Rubin's convergence test to compare within- and between-chain variance(Gelman and Rubin, 1992; Plummer *et al.*, 2006).

We specify the prior settings used for each analysis in the Supplementary R code. For random effects, we specified an inverse-Wishart prior ($V = 1$, $\nu = 0.002$). However, when modelling worker polymorphism as a response variable, which is a binary trait, the mixing properties of the MCMC chain were poor. Therefore, we specified a χ^2 prior ($V = 1$, $\nu = 1{,}000$, $\alpha.\mu = 0$, $\alpha.V = 1$) which improved the mixing of the chain(De Villemereuil, Gimenez and Doligez, 2013). For binary response variables, it is not possible to identify the residual variance. Therefore, we fixed the residual variance at 1. In all other cases, an inverse-Wishart prior was specified for residual variances ($V = 1$, $\nu = 0.002$). For fixed effects, we used the default priors in MCMCglmm (independent normal priors with zero mean and large variance, 10^{10}). Here, V represents variance, ν stands for the degree of belief parameter, and $\alpha.\mu$ and $\alpha.V$ denote the prior mean and prior covariance matrix, respectively.

R code for analyses can be found at: https://github.com/LouisBell-Roberts/Ant_complexity_climate

Hadfield, J.D. (2010) ‘MCMC Methods for Multi-Response Generalized Linear Mixed Models: The **MCMCglmm** *R* Package’, *Journal of Statistical Software*, 33(2). Available at: https://doi.org/10.18637/jss.v033.i02.

Gelman, A. and Rubin, D.B. (1992) ‘Inference from Iterative Simulation Using Multiple Sequences’, *Statistical Science*, 7(4). Available at: https://doi.org/10.1214/ss/1177011136.

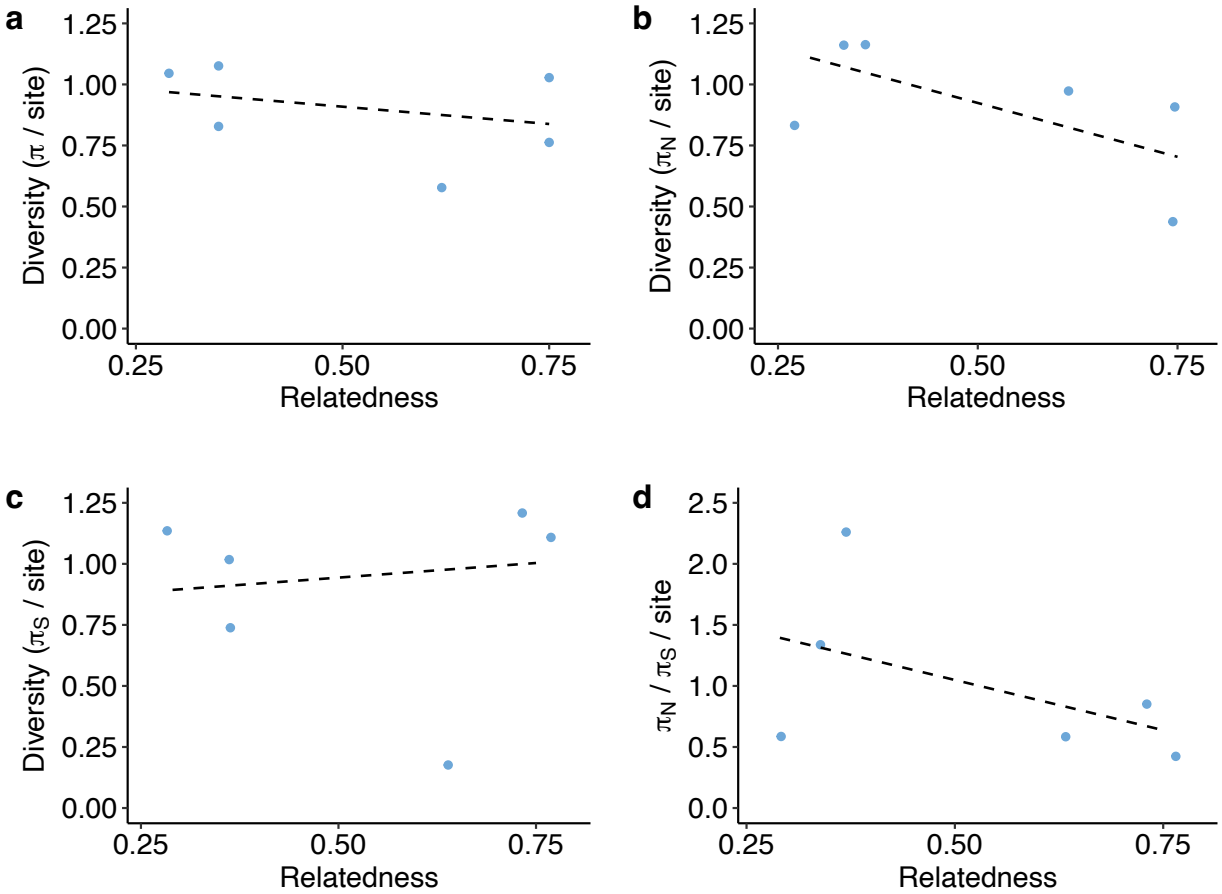
Plummer, M. *et al.* (2006) ‘CODA: convergence diagnosis and output analysis for MCMC’, *R news*, 6(1), pp. 7–11.

De Villemereuil, P., Gimenez, O. and Doligez, B. (2013) ‘Comparing parent–offspring regression with frequentist and Bayesian animal models to estimate heritability in wild populations: a simulation study for Gaussian and binary traits’, *Methods in Ecology and Evolution*. Edited by R. Freckleton, 4(3), pp. 260–275. Available at: https://doi.org/10.1111/2041-210X.12011.

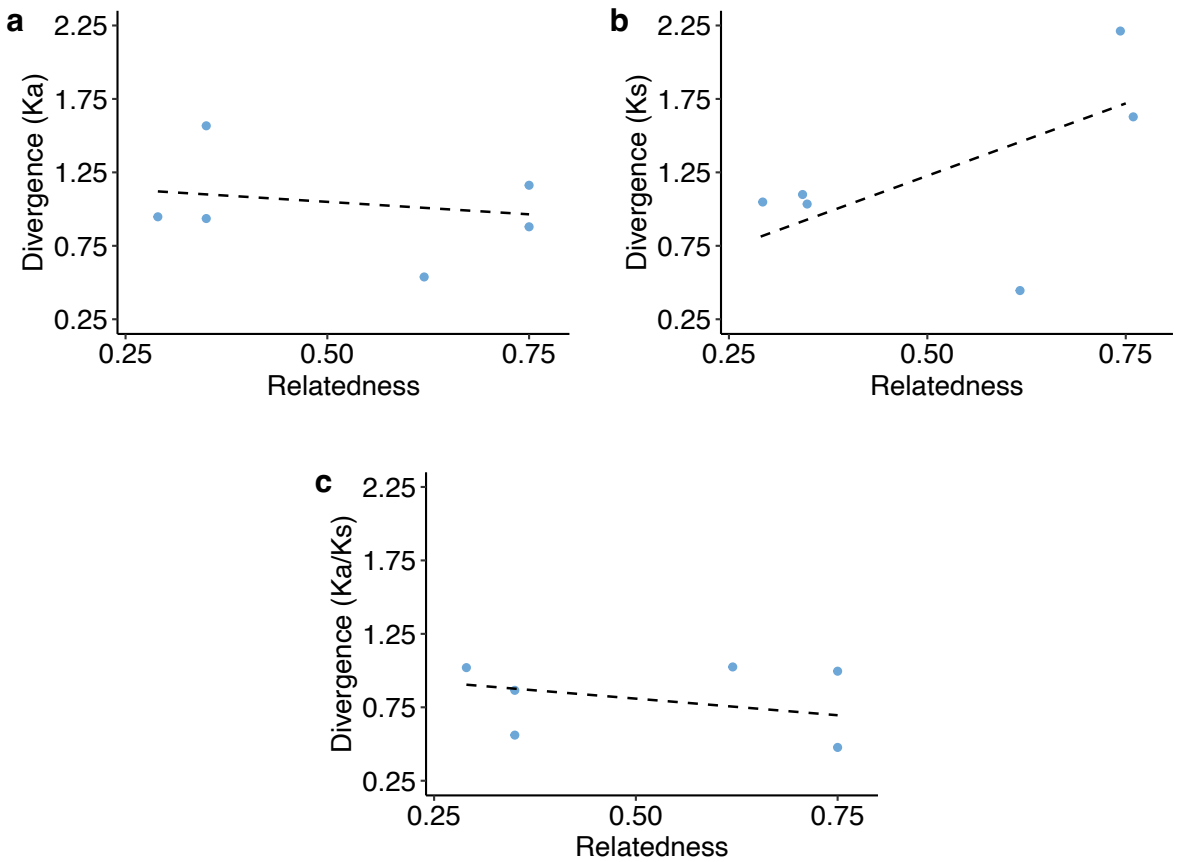
Alternative methods to quantify the level of division of labour in the worker caste in ants:

Previously, Bell-Roberts et al., 2024 quantified the level of division of labour in the worker castes of ants by counting the number of discrete worker castes for 699 ant species. Examining the 474 species used in our analysis, we found a 91% agreement in the way that species were classified between the datasets of Bell-Roberts et al., 2024 and La Richelière et al. 2022 when considering species classified by Bell-Roberts et al., 2024 as having a single worker caste as being ‘monomorphic’ and species that have more than one worker castes as being ‘polymorphic’. Furthermore, we found no phylogenetic clustering among species where the two datasets disagreed in their classification of worker polymorphism. This suggests that using either of these alternative methods to quantify division of labour is likely to produce the same results. However, we analyse division of labour as a binary variable as this method of classification offers the largest amount of data.

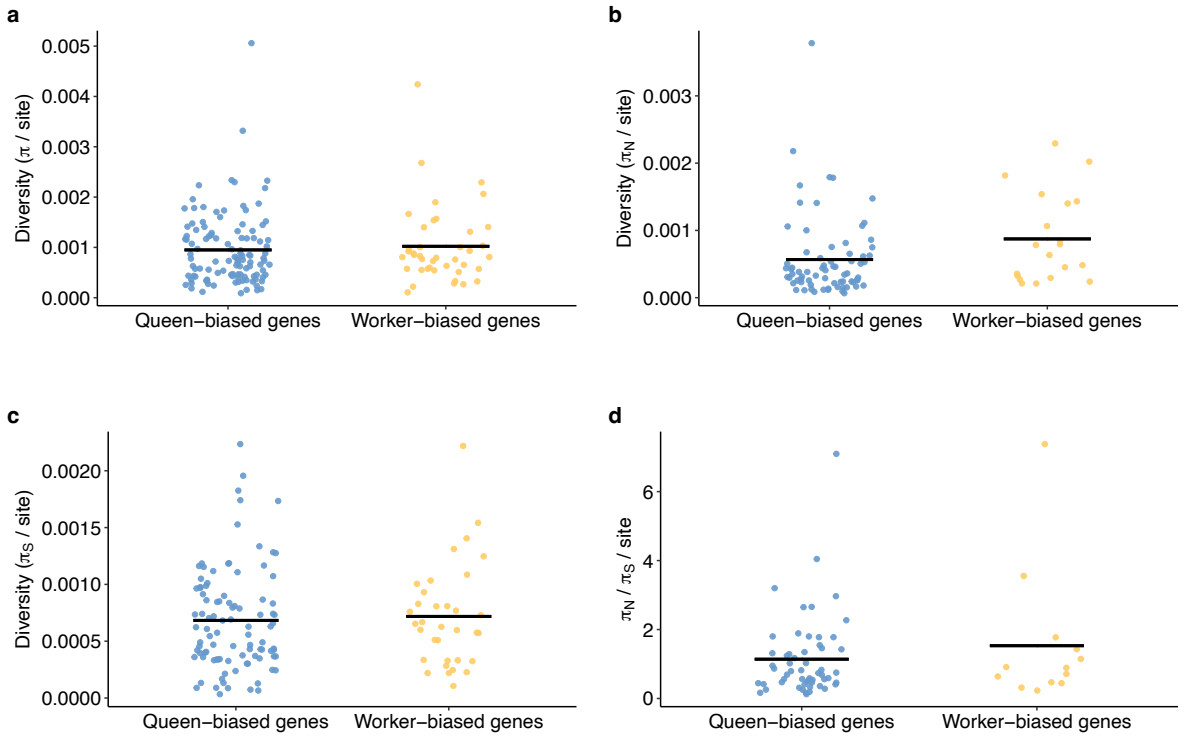
Supplementary Material for Chapter 4



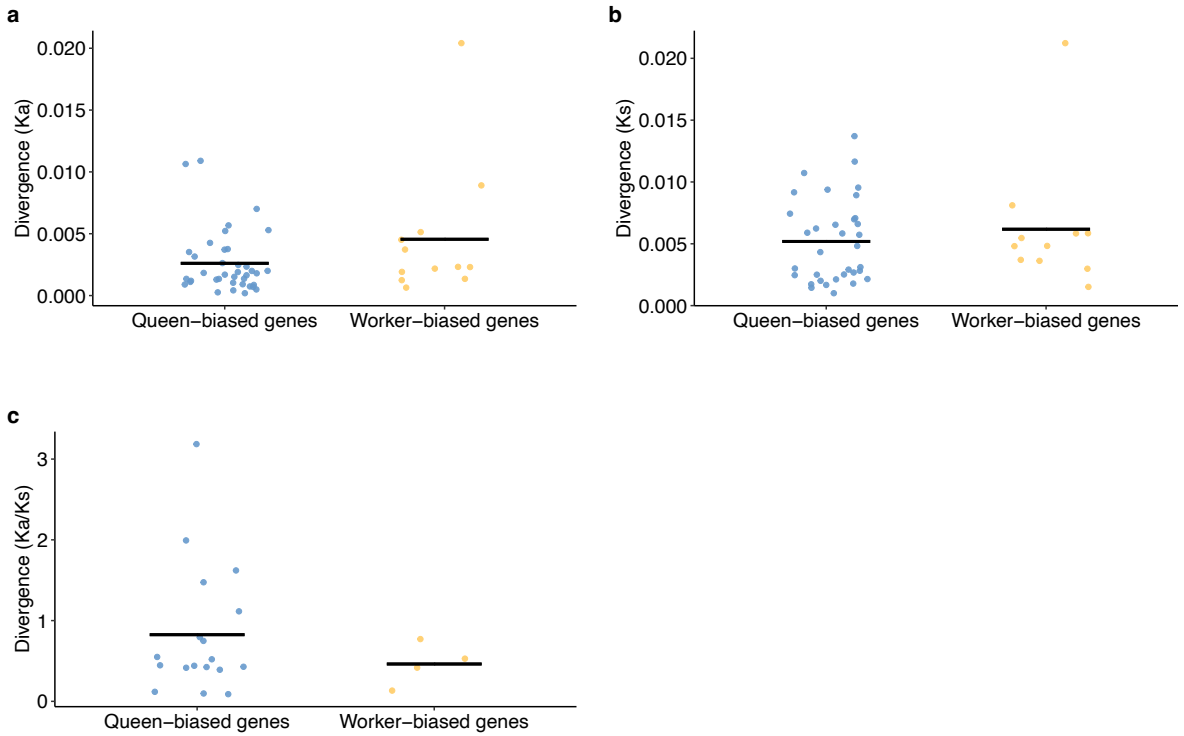
Supplementary Figure 1. Relationship between intra-colony relatedness and relative polymorphism (nucleotide diversity) in worker-biased genes compared to queen-biased genes. (b) When examining non-synonymous sites, we found that species with lower relatedness exhibit significantly higher levels of relative polymorphism. However, this relationship was not significant. Dashed lines represent non-significant relationships. Sample sizes = 6; each dot represents a species.



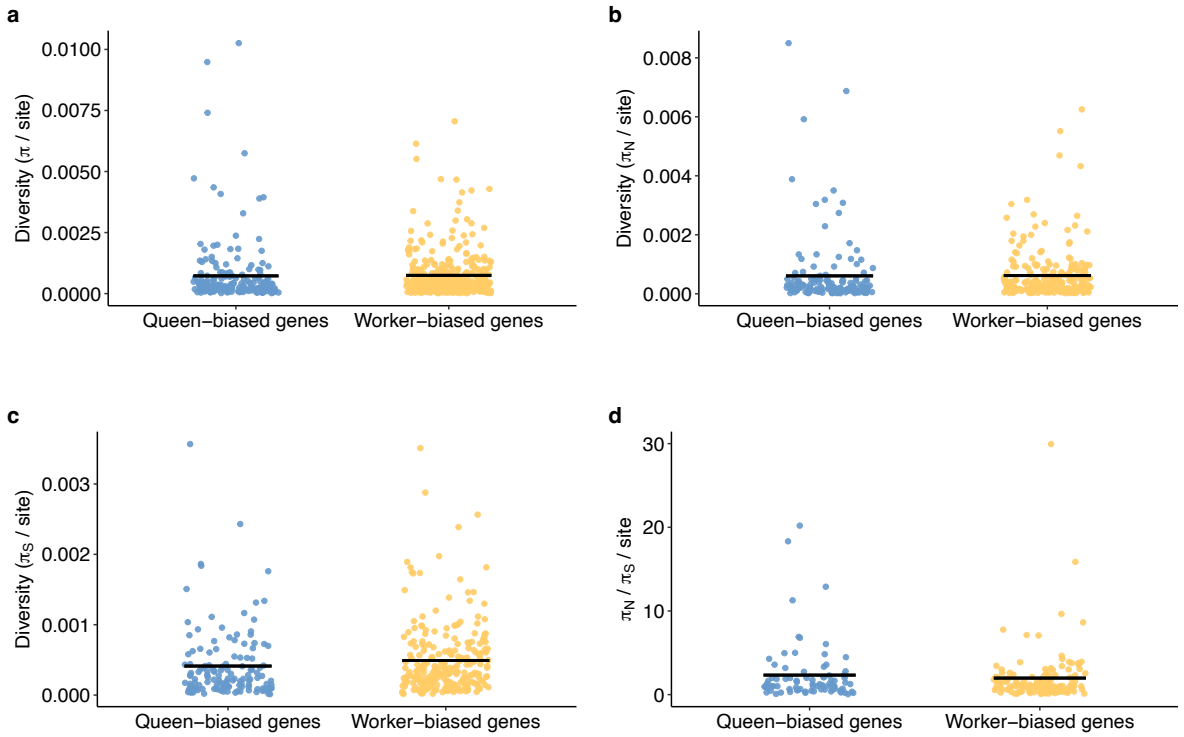
Supplementary Figure 2. Relationship between intra-colony relatedness and relative divergence in worker-biased genes compared to queen-biased genes. We found no significant relationships between relative divergence and relatedness. Dashed lines represent non-significant relationships. Sample sizes = 6; each dot represents a species.



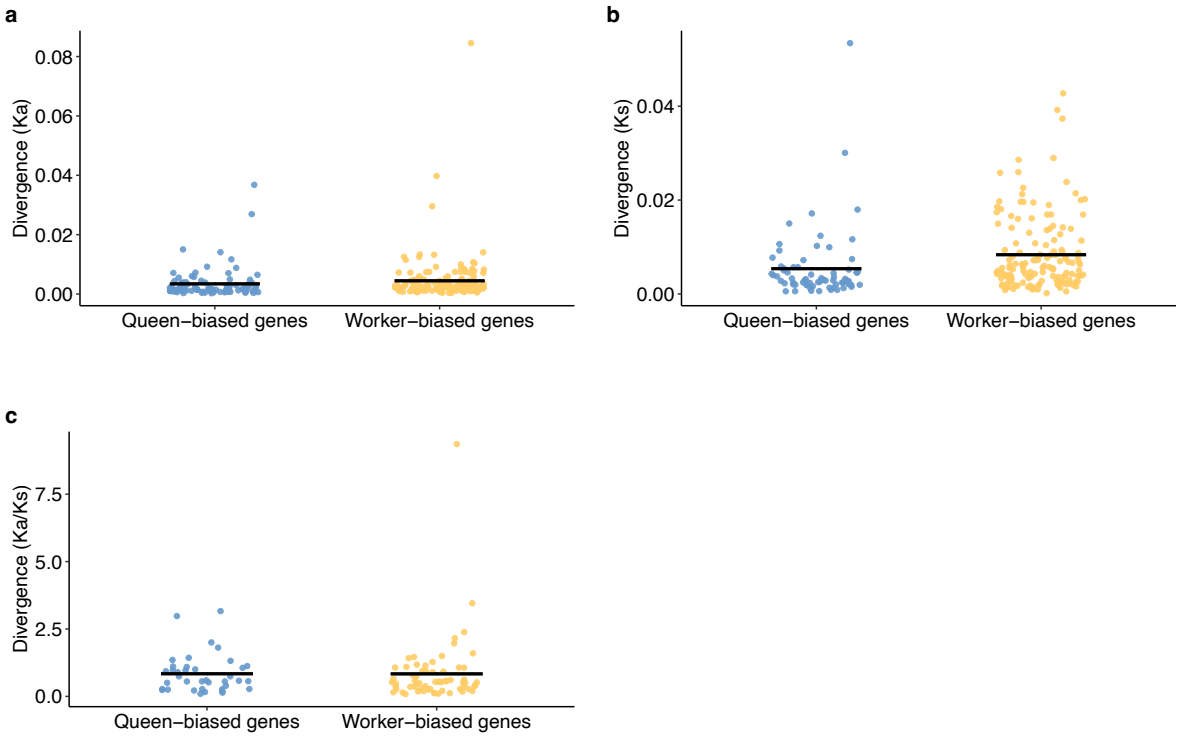
Supplementary Figure 3. ***Acromyrmex echinatior*****:** Nucleotide diversity (polymorphism) per site for genes with direct (queen-biased) and indirect (worker-biased) fitness effects. *A. echinatior* possesses queens that mate multiple times. Therefore, overall polymorphism (a), polymorphism at non-synonymous sites (b) and polymorphism ratio (d) are predicted to be greater in worker-biased genes than queen-biased genes. No difference is expected for polymorphism at synonymous sites (c). Each dot represents an estimate for a single gene and the horizontal line represents the mean.



Supplementary Figure 4. ***Acromyrmex echinatior*:** Divergence measured as rates of protein evolution for genes with direct (queen-biased) and indirect (worker-biased) fitness effects. *A. echinatior* possesses queens that mate multiple times. Therefore, divergence at non-synonymous sites (a) and divergence ratio (c) are predicted to be greater in worker-biased genes than queen-biased genes. No difference is expected for divergence at synonymous sites (b). Each dot represents an estimate for a single gene and the horizontal line represents the mean.



Supplementary Figure 5. *Camponotus floridanus*: Nucleotide diversity (polymorphism) per site for genes with direct (queen-biased) and indirect (worker-biased) fitness effects. *C. floridanus* possesses a single queen that mates once. Therefore, no significant difference is expected in the levels of overall polymorphism (a), polymorphism at non-synonymous sites (b), polymorphism at synonymous sites (c), or polymorphism ratio (d) between genes with worker-biased expression relative to genes with queen-biased expression. Each dot represents an estimate for a single gene and the horizontal line represents the mean.



Supplementary Figure 6. *Camponotus floridanus*: Divergence measured as rates of protein evolution for genes with direct (queen-biased) and indirect (worker-biased) fitness effects. *C. floridanus* possesses a single queen that mates once. Therefore, no significant difference is expected in the levels of divergence at non-synonymous sites (a), divergence at synonymous sites (b), or divergence ratio (c) between genes with worker-biased expression relative to genes with queen-biased expression. Each dot represents an estimate for a single gene and the horizontal line represents the mean.

Supplementary Table 1. Species used in whole-genome analysis.

Species	Bioproject asseccion	Number of individuals sampled	Caste	Reference	SRA	Location	Sequencing depth
Camponotus floridanus	PRJNA50203	19	Workers, males	Bonasio et al., 2010; 10.1126/science.119242	SRR059200, SRR059199, SRR059198, SRR059197, SRR059196, SRR059193, SRR059192, SRR059191, SRR059190, SRR059189, SRR059188, SRR059187, SRR059186, SRR059185, SRR059184, SRR059183, SRR059182, SRR059181, SRR059180	USA (Florida): all samples	Minimum: 20x
Monomorium pharaonis	PRJNA772784	20	Workers	Warner et al., 2017; 10.1093/molbev/msx123	SRR16493397, SRR16493398, SRR16493399, SRR16493400, SRR16493401, SRR16493402, SRR16493403, SRR16493404, SRR16493405, SRR16493406, SRR16493407, SRR16493408, SRR16493409, SRR16493410, SRR16493411, SRR16493412, SRR16493413, SRR16493414, SRR16493415, SRR16493416	USA (Florida), Ivory Coast (Lambto), USA (Texas), Taiwan, Ghana (Fete), Israel, Palau, Ghana (CRIG), Thailand, Thailand, Thailand, Poland (Warsaw), United Kingdom (London), Thailand, Malaysia (Penang), Thailand (Chiang Mai), USA (Florida), Ivory Coast (Lambto), Ghana (Fete), Cameroon	NA
Solenopsis invicta	PRJNA685290	20	Males	Stolle et al., 2022; 10.1038/s41467-022-28806-7	SRR13679678, SRR13679679, SRR13679680, SRR13679681,	Brazil (Campo Grande), Brazil (Campo Grande), Brazil (Campo Grande), Brazil	Average: 10.3x

					SRR13679691, SRR13679692, SRR13679693, SRR13679694, SRR13679695, SRR13679696, SRR13679697, SRR13679698, SRR13679700, SRR13679701, SRR13679702, SRR13679703, SRR13679704, SRR13679705, SRR13679706, SRR13679707	(Campo Grande), Brazil (Campo Grande), Brazil (Campo Grande), Brazil (Campo Grande), Argentina (San Carlos), Argentina (San Carlos), Argentina (San Carlos), Argentina (San Carlos), Brazil (Miranda), Brazil (Miranda), Brazil (Miranda), Brazil (Miranda), Brazil (Miranda), Brazil (Miranda), Brazil (Miranda)	
Formica exsecta	PRJNA393850	7	Males	Dhaygude et al., 2019; 10.1186/s12864-019-5665-6	SRR5819596, SRR5819597, SRR5819598, SRR5819599, SRR5819600, SRR5819601, SRR5819602	Finland (FuruskŠr island): all samples	Average: 47.37x
Acromyrmex echinatior	PRJNA223531	9	Queens, workers	Li et al., 2014; 10.1038/ncomms5943	SRR1015534, SRR1015530, SRR1015526, SRR1015518, SRR1015514, SRR1015510, SRR1015506, SRR1015502, SRR1015522	Panama (Gamboa): all samples	Average: 39x
Bombus terrestris	PRJEB52013	20	Workers	Barribeau et al., 2022; 10.1371/journal.pone.0277041	ERR9523411, ERR9523412, ERR9523413, ERR9523414, ERR9523415, ERR9523416, ERR9523417, ERR9523418, ERR9523419, ERR9523420, ERR9523421, ERR9523422, ERR9523423, ERR9523424,	Switzerland (North): all samples	Average: 2.5x

					ERR9523425, ERR9523426, ERR9523427, ERR9523428, ERR9523429, ERR9523430		
Apis mellifera	PRJNA516678	20	Males	Kawakami et al., 2019	SRR10055320, SRR10055321, SRR10055322, SRR10055323, SRR10055324, SRR10055325, SRR10055326, SRR10055327, SRR10055328, SRR10055329, SRR10055330, SRR10055331, SRR10055332, SRR10055333, SRR10055334, SRR10055335, SRR10055336, SRR10055337, SRR10055338, SRR10055339	Finland (Aland), Finland (Aland), South Africa, Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), South Africa, Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland), Finland (Aland)	Average: 8x
Apis cerana	PRJDB14080	20	Workers	Wakamiya et al., 2023; 10.1002/ece3.10573	DRR397653, DRR397652, DRR397651, DRR397650, DRR397649, DRR397648, DRR397647, DRR397646, DRR397645, DRR397644, DRR397643, DRR397642, DRR397641, DRR397640, DRR397639, DRR397638, DRR397637, DRR397636, DRR397635, DRR397634	Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Kanagawa), Japan (Ibaraki), Japan (Ibaraki), Japan (Ibaraki), Japan (Ibaraki), Japan (Ibaraki), Japan (Ibaraki), Japan (Ibaraki), Japan (Ibaraki)	Minimum: 29x

Supplementary Table 2. Outgroups used to calculate estimates of divergence.

Species	Bioproject asseccion	Number of individuals sampled	Caste	Reference	SRA	Location	Sequencing depth
Camponotus floridanus	PRJNA999760	1	Worker		SRR25452767	Canada (Montreal)	NA
Monomorium pharaonis	PRJEB48742	1	Worker	Romiguier et al., 2022; 10.1016/j.cub.2022.05.001	ERR8442010	Denmark	NA
Solenopsis invicta	PRJNA544804	1	Worker		SRR9127698	Korea (Gamman pier)	NA
Formica exsecta	PRJNA557080	1	Worker	Brelsford et al., 2020; 10.1016/j.cub.2019.11.032	SRR15845488	Finland	Average: 17.2x
Acromyrmex echinatior	PRJNA714946	1	Queen	Schrader et al., 2021; 10.1038/s41467-021-23178-w	SRR13980399	Panama (Gamboa)	Average: 20x
Bombus terrestris	PRJNA870415	1	Male	Larragy et al., 2023; 10.1093/gbe/evad039	SRR23684821	Ireland	Average: 16.2x
Apis mellifera	PRJNA522435	1	Female		SRR27537400	USA	NA
Apis cerana	PRJNA869845	1	Worker		SRR24658932	China (XingLong)	NA

Supplementary Table 3. Samples used for differential gene expression analysis.

Species	Bioproject asseccion	Number of queens sampled	Queen tissue	Life stage	Queen SRAs	Number of workers sampled	Worker tissue	Worker caste	Life stage	Worker SRAs	Reference
Camponotus floridanus	PRJNA327092	1	Whole body	NA	SRR3726122	2	Whole body	Major and minor	NA	SRR3726124, SRR3726123	Gospocic et al., 2017; 10.1016/j.cell.2017.07.014
Monomorium pharaonis	PRJDB3164	3	Head	NA	DRR032045, DRR032265, DRR032266	4	Head	Monomorphic workers	NA	DRR032148, DRR032150, DRR032224, DRR032226	Mikheyev & Linksvayer, 2015; 10.7554/eLife.04775
Solenopsis invicta	PRJNA472392	4	Brain	Reproductive queen, Reproductive queen, Reproductive queen, Reproductive queen	SRR7209550, SRR7209542, SRR7209541, SRR7209540	4	Brain	Minor worker	Forager, Forager, Nurse, Nurse	SRR7209532, SRR7209534, SRR7209535, SRR7209537	Chandra et al., 2018; 10.1126/science.aar5723
Formica exsecta	PRJDB6378	4	Brain	Old queen, Old queen, Emerging queen, Emerging queen	DRR118162, DRR118163, DRR118170, DRR118171	4	Brain	Monomorphic workers	Old worker, Old worker, Emerging worker, Emerging worker	DRR118166, DRR118167, DRR118174, DRR118175	Morandin et al., 2019; 10.1111/mec.15062

Acromyrmex echinatior	PRJNA427677	4	Brain	Virgin queen: all samples	SRR7772544, SRR7772545, SRR7772547, SRR7772552	4	Brain	Minor and major workers	Worker in fungus garden, Foraging worker, Foraging worker, Worker in fungus garden	SRR7772543 (minor), SRR7772546 (major), SRR7772548 (major), SRR7772550 (minor)	Qiu et al., 2018; 10.1038/s41559-018-0689-x
Bombus terrestris	PRJNA868857	3	Brain	Queen with immature ovaries, Egg-laying queen, Egg-laying queen	SRR21116033, SRR21116041, SRR21116042	3	Brain	Monomorphic workers	Inseminated worker, Inseminated worker, Inseminated worker	SRR21115987, SRR21115988, SRR21115989	Zhuang et al., 2023; 10.1038/s41467-023-41198-6
Apis mellifera	PRJNA479740	2	Head	Mature queen, Mature queen	SRR7472357, SRR7472363	2	Head	Monomorphic workers	Newly emerged worker, Newly emerged worker	SRR7472354, SRR7472361	Harris et al., 2019; 10.1186/s13072-019-0307-4
Apis cerana	PRJNA690015	2	Ovary	Egg-laying queen, Egg-laying queen	SRR13367580, SRR13367581	2	Ovary	Monomorphic workers	Mature worker, Mature worker	SRR13367578, SRR13367579	Kang et al., 2021; 10.1038/s41598-021-90635-3

Supplementary Table 4. Genomic and life history data used in the analyses for Chapter 4.

species	Polymorphism ratio (π)	Polymorphism ratio synonymous sites (π_S)	Polymorphism ratio non-synonymous sites (π_N)	Polymorphism non-synonymous sites/synonymous sites (π_N/π_S)	Divergence ratio non-synonymous sites (k_A)	Divergence ratio synonymous sites (k_S)	Divergence non-synonymous sites/synonymous sites (k_A/k_S)	Queen mating frequency	Mating frequency reference	Relatedness	Relatedness reference
Acromyrmex_echinatior	1.07579237	1.01708572	1.1702018	1.3428317	1.56699794	1.09425317	0.56053395	5.3	Sumner et al., 2004	0.35	Wenseleers & Ratnieks, 2006
Apis_mellifera	1.04552172	1.1308713	0.84896944	0.5807646	0.94753583	1.07507737	1.02007756	11.6	Tarpy et al., 2004	0.29	Wenseleers & Ratnieks, 2006
Apis_cerana	0.82805648	0.72806626	1.1695632	2.2528958	0.9356707	1.07772942	0.86543805	14.1	Tarpy et al., 2004	0.35	Wenseleers & Ratnieks, 2006
Bombus_terrestris	0.76243587	1.1067969	0.44080714	0.4377888	0.87951409	2.17438516	0.47674604	1	Schmid-Hempel & Schmid-Hempel, 2000	0.75	Wenseleers & Ratnieks, 2006
Camponotus_floridanus	1.02785413	1.20973631	0.90966583	0.8469108	1.16217854	1.61586044	0.9957935	1	Gadau et al., 1996	0.75	Wenseleers & Ratnieks, 2006
Formica_exsecta	0.5774495	0.18251923	0.96201136	0.5785752	0.53786703	0.46212441	1.02472121	1.2	Haag-Liautard et al., 2008	0.62	Wenseleers & Ratnieks, 2006
Monomorium_pharaonis	0.96390589	0.93903797	0.9937666	1.4783417	0.84762489	0.67975757	1.04144201	1	Allard et al., 2006	NA	NA
Solenopsis_invicta	0.94167964	0.92900543	0.95190984	1.0261892	0.61035664	1.05751794	0.7731363	1	Hughes et al., 2008	NA	NA

References for Supplementary Table 4

Allard, D. *et al.* (2006) 'Sperm transfer during mating in the pharaoh's ant, *Monomorium pharaonis*', *Physiological Entomology*, 31(3), pp. 294–298. Available at: https://doi.org/10.1111/j.1365-3032.2006.00519.x.

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Model settings and assessments for Bayesian phylogenetic mixed models (BPMMs)

We fitted BPMMs with Markov chain Monte Carlo (MCMC) estimation using the MCMCglmm package v2.34 (Hadfield, 2010). Models were run for 110,000 iterations, with a burn-in of 10,000 and thinning interval of 100. We ensured that parameter estimates had an effective sample size of at least 1,000. To ensure model convergence, we used the coda package v0.19-4 to calculate the degree of autocorrelation between successive iterations in each chain (Plummer *et al.*, 2006). We fitted each model independently two times and used Gelman and Rubin's convergence test to compare within-chain and between-chain variance (Gelman and Rubin, 1992; Plummer *et al.*, 2006). For random effects and residual variances, we specified an inverse-Wishart prior ($V = 1$, $\nu = 0.002$). For fixed effects, we used the default priors in MCMCglmm (independent normal priors with zero mean and large variance, 10^{10}). Here, V represents variance, ν stands for the degree of belief parameter, and $\alpha.\mu$ and $\alpha.V$ denote the prior mean and prior covariance matrix, respectively.

Hadfield, J.D. (2010) 'MCMC Methods for Multi-Response Generalized Linear Mixed Models: The **MCMCglmm** *R* Package', *Journal of Statistical Software*, 33(2). Available at: https://doi.org/10.18637/jss.v033.i02.

Gelman, A. and Rubin, D.B. (1992) 'Inference from Iterative Simulation Using Multiple Sequences', *Statistical Science*, 7(4). Available at: https://doi.org/10.1214/ss/1177011136.

Plummer, M. *et al.* (2006) 'CODA: convergence diagnosis and output analysis for MCMC', *R news*, 6(1), pp. 7–11.