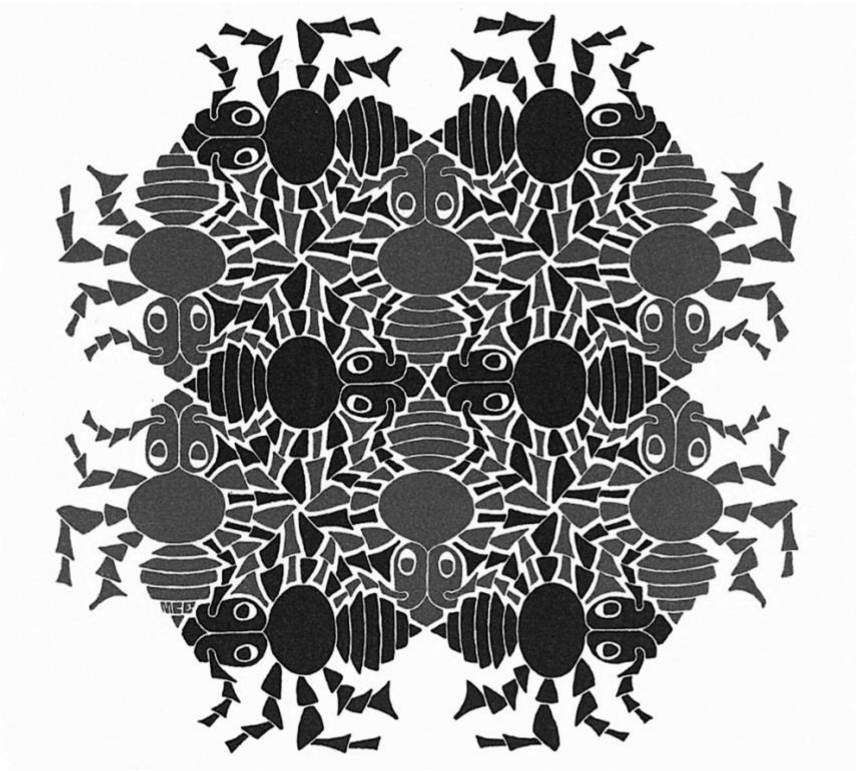
The Evolution of Cooperation and Division of Labour in Insects



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A thesis submitted for the degree of
Doctor of Philosophy

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. This work has not been submitted for any degree or professional qualification except as specified.

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Cover art: M. C. Escher.

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Publications and Contributions

Chapter 2: Social behaviour in insects

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Chapter 3: Larger colony sizes favour the evolution of worker size variation in ants

The work described in this chapter contributed to a publication in the journal *Nature Ecology & Evolution:* Louis Bell-Roberts, Juliet F. R. Turner, Gijsbert D. A. Werner., Philip A. Downing, Laura Ross, and Stuart A. West. Larger colony sizes favoured the evolution of more worker castes in ants. *Nature Ecology & Evolution* **8**, 1959–1971 (2024). https://doi.org/10.1038/s41559-024-02512-7

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Chapter 4: Larger colony sizes favour greater dimorphism between queen and workers in ants

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Chapter 5: No evidence that mode of group formation predicts helper mating in Hymenoptera

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Thesis Abstract

Why have some insect lineages evolved highly cooperative societies with specialised castes, while others remain solitary or only facultatively social?

In this thesis, I explore this question by creating and analysing large-scale comparative datasets across hundreds of insect species, focusing on the evolution of cooperation and division of labour. I begin by outlining key concepts in the study of cooperation (Chapter 1) and reviewing the diversity of insect social systems (Chapter 2), highlighting the need for more precise, lineage-specific definitions of social traits to better understand their evolutionary dynamics.

In Chapter 3, I investigate morphological variation among workers in 152 ant species, testing whether life history traits can predict the degree of worker size variation – a proxy for task specialisation and division of labour. In Chapter 4, I expand the dataset to 546 ant species, examining reproductive division of labour by assessing queen-worker size dimorphism and worker sterility. I test whether life history can explain variation in reproductive specialisation, and I reconstruct the ancestral ant to estimate how often these forms of specialisation evolved independently.

Finally, in Chapter 5, I broaden the scope to 582 species of ants, bees, and wasps, testing whether the way groups are formed affects the evolution of cooperation and sterility. Together, these studies provide new insights into the conditions that favour the emergence of reproductive division of labour and major evolutionary transitions.

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Chapter 1: An introduction to cooperation

Cooperation is essential for the evolution of complex life. This is true across taxonomic groups and across levels of biological organisation: Cooperation exists between genes in a genome, between cells in a multicellular organism, and between organisms in cooperative groups or superorganismal colonies (Boomsma, 2023; Bourke, 2011; Szathmáry and Maynard Smith, 1995; West *et al.,* 2015). A trait is cooperative if it provides a benefit to another individual and has evolved at least partially because of that benefit (West *et al.,* 2015; West *et al.,* 2007). Theoretical explanations for the evolution of cooperation can be classed into two categories: direct fitness benefits, or indirect fitness benefits (West *et al.,* 2007; Hamilton, 1964; Brown and Brown, 1981; Grafen, 1984, Taylor, 1996; Lehmann and Keller, 2006; West *et al.,* 2006). Direct benefits can explain mutually beneficial cooperation (+/+), while indirect benefits can explain altruistic cooperation where the action comes with a cost to the actor (-/+) (West *et al.,* 2007; Sachs *et al.,* 2004). It is possible for one action to have both direct and indirect fitness benefits.

Direct fitness. In cases where cooperation evolves for direct fitness benefits, individuals may have aligned interests. For example, in cooperatively breeding species, group size may increase chances of survival and so individuals may be selected to help rear offspring that are not their own if this increases group size and ultimately increases their own fitness (Kokko *et al.,* 2001). Some behaviours that appear altruistic (-/+) may actually be mutually beneficial (+/+) if the benefits for the actor only become apparent in the longer term (Clutton-Brock, 2002; Griffin and West, 2002). For example, Vampire Bats often regurgitate blood to feed roost-mates who failed to obtain a meal. In the short term, this may seem altruistic, but in the longer term, the bats remember past donors and preferentially help those who have helped them before. By ‘giving blood’, they are ensuring that they can rely on a donation if they ever failed to find their own meal in future (Wilkinson, 1984).

Cooperation for direct fitness benefits may also evolve through enforcement – punishing non-cooperators (cheaters) and rewarding cooperators (Trivers, 1971; Frank, 2003). Punishment can take the form of sanctions and policing, and rewards for cooperation can take the form of reciprocal altruism and reputation-based reciprocity. Punishment as a mechanism for enforcing cooperation can be found between animals (including humans), plants, and bacteria

(Trivers, 1985; Clutton-Brock & Parker, 1995; Frank, 2003; Sachs *et al.*, 2004; Foster & Wenseleers, 2006; Kiers & van der Heijden, 2006; Ratnieks *et al.*, 2006; West *et al.*, 2006; Reeve, 1992). For example, in *Polistes* paper wasp colonies, dominant females punish subordinate workers who attempt to lay eggs by eating their eggs and physically attacking them, ensuring that workers cooperate by contributing to colony tasks rather than focusing on personal reproduction (Saigo and Tsuchida, 2004).

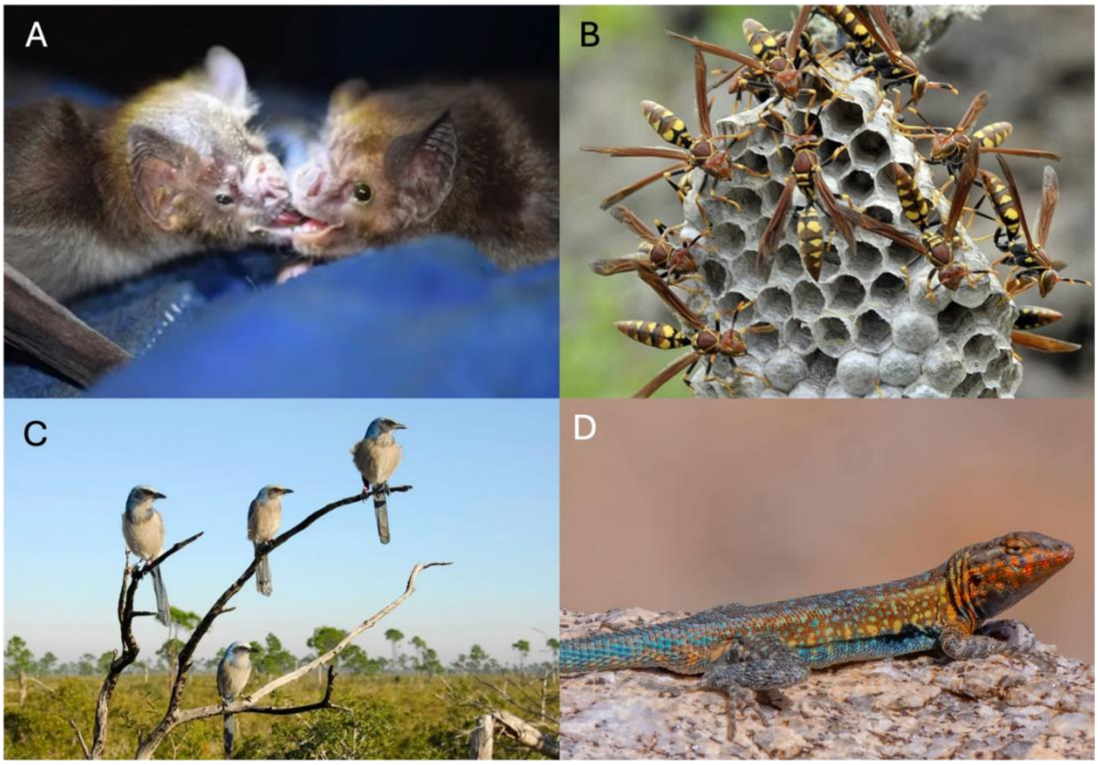

Figure 1. Cooperation in the nature. (A) Vampire bats regurgitate blood meals to roost-mates in exchange for future reciprocation. (Image: Luis Lecuona/ USDA International Services), **(B)** *Polistes* paper wasps enforce cooperation through violent policing. (Image: Frank Bungartz, CDF), **(C)** Florida Scrub Jays live in large family groups where individuals cooperate to help rear their younger siblings, thereby gaining indirect fitness benefits (Image: Reed Bowman), **(D)** Some males of Side-blotched lizards *Uta stansburiana* cooperate to protect their territories, identifying other potential cooperators using phenotypic markers (Image: uzun on iNaturalist).

Indirect fitness. For cooperation to evolve for indirect fitness benefits, it must be directed towards individuals who also carry the cooperative gene (Hamilton, 1964; 1970; 1975). Carriers are likely to be close relatives, in which case cooperative individuals must either be able to either discriminate between relatives and non-relatives (kin discrimination) or live in a situation of limited dispersal (population viscosity) where they are more likely to be surrounded by close relatives who will gain the benefits of their cooperation (Hamilton, 1964). Inclusive fitness (kin selection) theory describes how indirect fitness benefits can arise through individuals helping relatives to reproduce (Maynard Smith, 1964). Examples of kin-selected cooperation are abundant in nature, including in: superorganismal insect colonies where sterile workers (daughters) cooperate to rear the offspring of the queen (their mother); in multicellular slime mould fruiting bodies where 'stalk' cells sacrifice their reproductive success to improve the dispersal ability of other 'spore' cells; and in birds and mammals where individuals are more likely to emit an alarm call or help with rearing offspring if surrounded by close kin (Hughes *et al.,* 2008; Boomsma, 2009; Alpedrinha *et al.,* 2013; Hamilton, 1964; Kay *et al.,* 2019; Griffin and West, 2003; Hatchwell *et al.,* 2014).

Indirect fitness can also involve enforcement, such as the policing behaviours seen in many social insects. Workers may try to 'cheat' by laying their own eggs instead of contributing to colony tasks and caring for the queen's offspring. Other workers in the colony act as a police force and destroy those eggs, ensuring their own indirect fitness gains through the survival of the queen's eggs – their sisters (Ratnieks, 1988; Ratnieks and Helanterä, 2009).

Greenbeards. It is theoretically also possible for a cooperative gene to be shared by nonrelatives – a 'greenbeard' gene. In this situation, the greenbeard gene may be the only gene that two or more interacting individuals share. This mechanism would require a single gene or a number of tightly linked genes that encode for both the cooperative behaviour and also the distinctive phenotypic marker that individuals can use to identify each other (such as a green beard) (Gardner and West, 2010; Hamilton, 1964; Dawkins, 1976; Jansen and van Baalen, 2006). There has been debate over whether a greenbeard gene for cooperation could exist in practice as it would be very difficult for a single gene or set of linked genes to produce the required complex phenotypic effects. Additionally, the strategy could be invaded by 'falsebeards' who display the identifying phenotypic marker without also performing the cooperative behaviour (West and Gardner, 2010; Dawkins, 1976). However, genes for cooperation which act as greenbeards have subsequently been found in slime moulds, yeast, lizards, and fire ants (West and Gardner, 2010; Keller and Ross, 1998; Queller *et al.,* 2003; Sinervo and Clobert, 2003).

Division of labour. Cooperation often involves division of labour, where different tasks are shared between individuals who specialize to their assigned roles (West and Cooper, 2016). Division of labour is a shared feature of complex life across all levels of biological organisation – genes perform different functions within a cell, cells perform different functions with an organism, and organisms perform different functions within a group or society (Oster and Wilson, 1978; West *et al.,* 2015). The specialisation of individuals to different roles through division of labour can also lead to irreversible mutual dependence which characterises major transitions in individuality (Boomsma, 2023; West *et al.,* 2015; Bourke, 2011). Following major transitions in individuality, totipotency is lost, meaning that individuals can no longer reproduce independently (West *et al.,* 2015). As a result, the level of conflict among individuals is reduced, and the collective can be viewed as a unified, fitness-maximizing entity. For instance, in multicellular animals, the cells function together as a single organism. Similarly, collectives of organisms can cooperate at a higher level, forming a new entity— a 'superorganism'.

However, not all examples of division of labour are in groups that have undergone a major transition. Division of labour can be seen in mammals such as meerkats where some individuals act as guards while others act as foragers (Clutton-Brock *et al.,* 1999). Though theories have predicted that division of labour will evolve when there are enough efficiency benefits to specialisation and when the interests of individuals align, such as when they are related, relatively little is known about why division of labour only evolves in some lineages and not in others (Cooper and West, 2018; Michod, 2007; West *et al.,* 2015). The comparative approach allows us to recognise similarities between independent transitions to higher levels of division of labour, shedding light on the conditions that facilitate major transitions in individuality and foster the emergence of more complex life forms. In the following chapters of this thesis I undertake a review of insect social behaviour, followed by several different comparative research projects in which I investigate the conditions that led to the evolution of cooperation and division of labour in insects.

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Chapter 2: Division of Labour in Insects: A Trait-Based Perspective

Abstract

The social behaviour exhibited by insects represents some of the most spectacular examples of cooperation in the natural world. The colonies of some species comprise tens of millions of sterile helpers dedicating their entire lives to foraging for, protecting, and rearing the offspring of a single reproductive queen. However, not all insect societies are so large and complex – some are smaller, less conspicuous, but demonstrate fascinating cooperative traits of their own. While research has traditionally focused on so-called "eusocial" species, many taxa that fall outside conventional definitions have been overlooked. Recent discoveries of novel social behaviours challenge established classifications and highlight the limitations of current frameworks. Social classifications are often applied inconsistently, reflecting disagreement among researchers about which traits are essential. This conceptual ambiguity impedes comparative analyses and obscures evolutionary patterns. Although it is widely accepted that sociality has evolved multiple times in insects, the number of independent origins remains uncertain, largely due to incomplete trait mapping and the ambiguity of social terminology. In this review, we identify conceptual and empirical gaps in the study of insect social evolution. We advocate for more precise descriptions of social traits and propose future research directions to clarify the evolutionary trajectories and selective pressures underlying the diverse social systems found in insects.

Defining social behaviours

Efforts to categorise and define social behaviour have been ongoing for many decades, described in 2005 as a "prevailing terminological free-for-all" by Costa & Fitzgerald. To this day, a consensus on how to define sociality still has not been reached and the language used to classify social groups is diverse and inconsistent (Crespi & Yanega, 1995; Boomsma and Gawne, 2018; West et al., 2007). This confusion is reflected in the conflict over how to think of sociality conceptually.

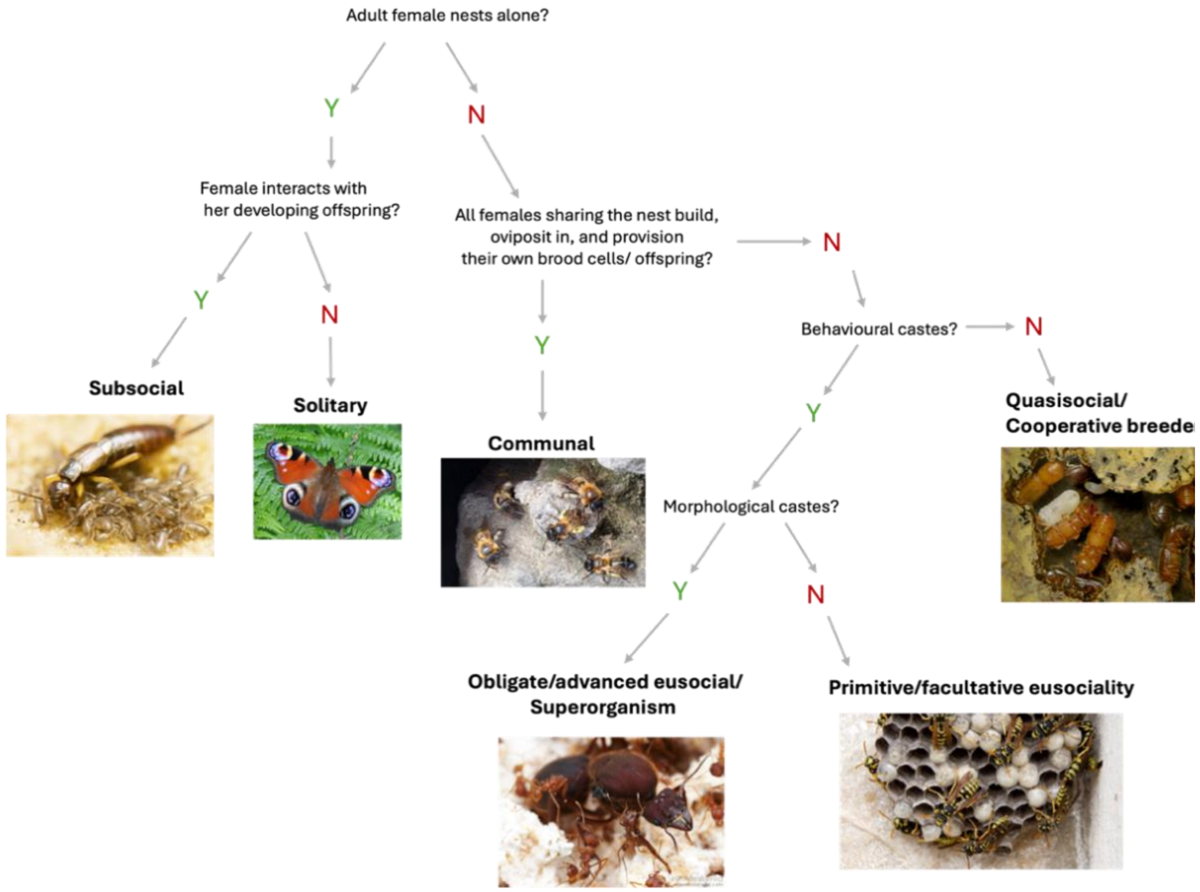

Figure 1. A key to diagnosing the social classification of an insect. Here we provide a quick guide to the commonly used social classifications of insects.

Social behaviour, in its broadest sense, can be used to refer to a wide variety of interactions between individuals that have fitness consequences for both the actor and another individual (West et al., 2007; West et al., 2015; Boomsma, 2018). However, the best-known social insects include “eusocial” species such as ants or vespine wasps. “Eusociality” is sometimes used interchangeably with the broader term sociality, but is traditionally characterised by cooperative brood care, reproductive division of labour, and overlap of generations (Figure 1; Wilson, 1971; Michener, 1974). The term was coined to describe ground-nesting halictid bees whose nest foundresses recruit daughters as helpers. It did not refer to social insects in general, and particularly not those with physically differentiated castes (Batra, 1966; Boomsma, 2018). In the 1970s, the definition of eusociality was broadened to include both lineages with temporal helper phenotypes and those with permanent castes (Wilson, 1971, 1975, 1985). The broadening of the eusociality criteria has subsequently been described as a loss of precision and loss of distinction between species that have or haven't undergone major transitions in individuality (Boomsma, 2018).

In contrast, the superorganismality framework was proposed to describe species with physical queen-worker caste differentiation (morphological reproductive division of labour), analogous to obligate germ-soma specialisation, where helper sterility is a proposed criterion of a major evolutionary transition (Wheeler, 1911; Bernadou et al., 2021). By this definition, superorganismal insect colonies represent a distinct level of organisation above metazoan multicellularity (Boomsma & Gawne, 2017; Smith & Szathmáry, 1995; Boomsma, 2018). Confusingly, authors have used the terms eusocial and superorganismal interchangeably (see review by Boomsma, 2018). In addition, species may be grouped together as either "eusocial" or "superorganismal" because of completely different sets of traits. This is because of a lack of consensus about what is key in the criteria: Is it the presence of helpers, such as temporal caste differentiation in Indian paper wasps, with older females acting as egg-layers and younger ones as foragers? (*Ropalida sp.;* Sinha et al., 1993; Ross, 1991). Is it morphological specialisation, such as the defensive nymphs of some aphids, which have larger bodies, thicker cuticles, and enlarged horns? (Aoki, 1977; Bourke, 2011) Maybe it is the presence of non-reproductive individuals, such as in one species of beetle that has helpers with lifetime unmatedness, but no pre-imaginable caste differentiation or morphological specialisation. (*Austroplatypus incompertus*; Boomsma, 2023; Smith et al., 2018; Kent & Simpson, 1992). All of these example species have been referred to as "eusocial" or "superorganismal" yet have very different sets of traits.

Terms and phrases like "quasisociality" (Michener, 1969, 1974) and "cooperative breeding" (Crespi & Yanega, 1995) are also ambiguous and inconsistently used. Some authors have used these terms to refer to species in which group members exhibit cooperation in provisioning and construction of brood cells within a communal nest where all females lay eggs (distinct from only 'communal' nests where each individual constructs and provisions their own brood cells) (e.g. Crespi & Yanega, 1995; Michener, 1969, 1974). Others have used "cooperative breeding' in reference to species with communal nesting, cooperation in provisioning and construction of brood, *and* reproductive division of labour – essentially, a synonym of "primitive eusociality" (e.g. Griffin & West; Grinsted & Field, 2017).

In many descriptions of social insects, a ladder-like way of viewing their state is evident, with the assumption being that solitary species or simpler social groups represent the ancestors of species with fixed morphological castes and complex social arrangements. This way of thinking is apparent in classifications such as “primitive” or “advanced” eusociality. Describing any extant species as "primitive" is problematic because all living species have undergone an equally long evolutionary history, and being extant means a species is adapted to its current environment, not simply a relic of the past. The term "primitive" also implies a particular evolutionary trajectory, suggesting that "primitive" states are the intermediate stage on the way to a more “advanced” social form. This assumption is not supported by current evidence. For example, some insect societies with physical reproductive division of labour, such as honeybees, appear to have evolved directly from solitary ancestors, rather than via facultatively social stages (da Silva, 2021; Linksvayer & Johnson, 2019; Rehan & Toth, 2015; Boomsma, 2023). In this context, behavioural or facultative forms of division of labour are better understood as alternative, equally derived states.

Regardless, many social species do not fit neatly into concepts of *primitive* or *advanced* sociality. For example, swarm-founding paper wasps could be classified as “primitively eusocial” because they do not have morphological castes and helpers may one day take over as the dominant reproductive, but they could also be considered to be “advanced eusocial” because of their dependence on workers to establish new nests and because of caste-specific differences in some species (da Silva, 2021). The same theme continues with termites, which are sometimes divided into “Higher” and “Lower” termites based on the perceived advancement of their social traits. However, Mastotermitidae is the most basal termite family yet its single representative species has many characteristics usually associated with more “advanced” sociality, such as large colony sizes, diverged developmental pathways, and a true worker caste (*Mastotermes darwiensis,* Thorne *et al.,* 2000). Once again, this demonstrates the problem with trying to place modern species on some kind of evolutionary hierarchy. Even trying to rank species on a scale of least to most socially complex is more complicated than it might initially seem: As others have highlighted, the answer is that it depends on which traits you look at, and the selection of traits presumed to indicate complexity don’t always correlate (Peled et al., 2025; Revely et al., 2024).

Despite the terminological confusions, traits such as the loss of reproductive totipotency and the development of group or colony level adaptations have major implications for our understanding of biological complexity and organisation in general (Cooper & West, 2018). It is therefore crucial to clearly identify which traits are present when describing social species. Both eusociality and superorganismality involve distinct forms of division of labour – whether reproductive or non-reproductive, behavioural or morphological – which themselves are measurable and biologically meaningful. By adopting a trait-based approach that focuses on the presence or absence of specific traits, rather than applying social group classifications that are inconsistently defined across the literature, we can enable more robust comparative analyses of social organisation across taxa.

Figure 2. Obligately colonial insects and their nests. While many insects live in groups for only part of their lives, others are obligately colonial, meaning that they are unable to survive or reproduce without their colony. Examples include: **(A)** Termites (photos from Shutterstock and iStock), **(B)** Stingless bees (photo from nativebeehives.com), **(C)** *Atta* leaf-cutter ants (photos by Michael J O'Brien and Alex Wild), **(D)** *Vespula* wasps (photos from Shutterstock and Superstock). Colonial insects are of enormous ecological and economic significance; capable of building huge nest structures and acting as ecosystem engineers by substantially altering their environment. Colonial insects have been the subject of much social research because of the clues they can give about cooperation more broadly, such as cooperation and specialisation among cells in multicellular organisms.

The aim of this review is to clearly identify which social traits are present in insect lineages, summarising the independent origins of social traits to highlight where comparative analyses may be possible, and draw attention to lesser-known lineages of social insect that have been neglected in research to date. We will focus specifically on the sociality of stable, cooperative groups, excluding species that are generally solitary but may occasionally form transient aggregations. Examples of transient insect groups that we will not discuss are those that aggregate in shelters but do not have cooperative behaviours beyond that (e.g. cockroaches in a rock crevice), insects that swarm but don't cooperate beyond coordinated movement (e.g. locust swarms), nymphs or larvae that stay together for protection after hatching but soon disperse (e.g. processionary caterpillars), insects that exhibit parental care where offspring remain on their natal nest but there is no prolonged cooperation between adults (e.g. many Hemipterans), migratory flocks of insects (e.g. monarch butterflies), and any species that come together to mate but exhibit no further social behaviours (e.g. mayflies, midges, fireflies).

In the following sections we will give an overview of key social traits across insects and detail the similarities across taxa as well as the unique ways that similar attributes may present. The traits we have selected include nest sharing, reproductive division of labour (behavioural), reproductive division of labour (morphological), non-reproductive division of labour (behavioural), and non-reproductive division of labour (morphological). Group formation and division of labour are of broad interest in the study of cooperation as they are necessary steps in the evolution of more complex life (West et al., 2014).

Communal nesting

Nest sharing is important in the study of social evolution because it acts as an enclosed, defendable base, often keeping family groups close together, and potentially selecting for the cooperative adaptations, such as the evolution of a non-reproductive defensive caste (soldier). Communal nesting, defined as the cohabitation of multiple reproductive females without clear division of labour (Figure 1; Appendix Table 2), is widespread across insects such as bees, wasps, thrips, aphids, beetles, and webspinners. While communal nesting ranges from obligate to facultative, and kin-based to non-kin associations, individuals in these systems typically maintain their own brood care with no further task specialisation. Though sometimes proposed as a potential precursor to more complex cooperation, communal nesting often persists as a stable, low-cost strategy (da Silva, 2021).

Most bee species are solitary, but among those usually considered to live and breed alone, you may find several individuals (or sometimes hundreds) co-habiting in a single nest burrow, seeming to live together in relative harmony. The orchid bees are a tribe of strikingly colourful metallic bees whose males are known for making perfumes from orchid flowers. They will nest in communes facultatively, depending on whether offspring choose to disperse or remain at their natal nest (Cameron & Ramírez, 2001; Santos & Garófalo, 1994; Eltz, 1999; Eltz *et al.,* 2003). In general, orchid bees exhibit a range of social states both within and between species – solitary-nesting, communal-nesting, and group-living with division of labour between reproductive and non-reproductive individuals – providing good opportunities for comparative analyses. Communal associations also occur in sweat bees (Halictidae), carpenter bees (Xylocopinae), mining bees (Andrenidae), plasterer bees (Colletidae), longhorn bees (Eucerini), and allodapine bees (Allodapini) (Figure 3 A; Michener, 1974; Córdoba-Aguilar et al., 2018; Iwata, 1964; Anzenberger, 1977; Nielsen, 1902; Sakagami & Zucchi, 1978; Paxton et al., 1999; Giovanetti et al., 1999; Spessa et al., 2000; da Silva et al., 2015).

Among wasps, communal nesting has been reported in several vespid subfamilies, (Matthews & Matthews, 2004; Ross, 1991; Krombein, 1978; Zucchi et al., 1976; Yoshikawa, 1969; West-Eberhard, 1978; Wcislo et al., 1988). In *Montezumia dimidiata*, marked females were observed returning to their maternal nests to reproduce, although it remains unclear whether they nested with their mothers (subsociality) or sisters (semisociality) (Ross, 1991). Communal nesting is also known in some Sphecid and Crabronid wasps, although the nature of nest sharing – whether females share brood cells as well as the nest ("quasisociality") or provision separately (communal nesting) – is uncertain (Ross, 1991). Spider wasps (Pompillidae: Pepsinae) have also been documented to form communal nests: *Auplopus semialatus* forms a group with up to eight females, jointly building and maintaining a single nest (Wcislo *et al.,* 1988).

Communal nesting is also fairly common in thrips, where unrelated females may form multi-foundress groups (Figure 3 C; Morris & Schwarz, 2002; Bono & Crespi, 2006; Gilbert & Simpson, 2013; Abbot & Chapman, 2017). Similarly, some aphid species form communal galls inhabited by multiple foundresses or clones, sometimes involving mixed-species assemblages (Figure 3 B; Miller, 1998, 2005; Akimonto, 1989; Abbot & Chapman, 2017). In Ambrosia beetles, such as *Ambrosiophilus* spp., daughters may stay in their natal nest and breed communally alongside their mother (Kasson et al., 2016). Webspinners (order Embioptera) show remarkable variation in social organisation – from solitary to obligate communal nesting – and can form groups of hundreds of individuals (Figure 3 D; Bradoo, 1967; Edgerly-Rooks, 1986, 1987; Edgerly, 1997, 2018, 2022; Hoell et al., 1998; Kusnezov, 1904; Miller et al., 2012). Relatedness within these groups differs; some species are parthenogenetic, with females producing viable eggs without fertilisation (Hoell *et al.,* 1998). Group formation may occur either through offspring staying with their mother or non-relatives joining together, as seen in facultatively communal species like *A. urichi* (Edgerly, 2022, 2018, 1987). Despite their fascinating behaviours, Webspinners remain one of the least studied insect groups.

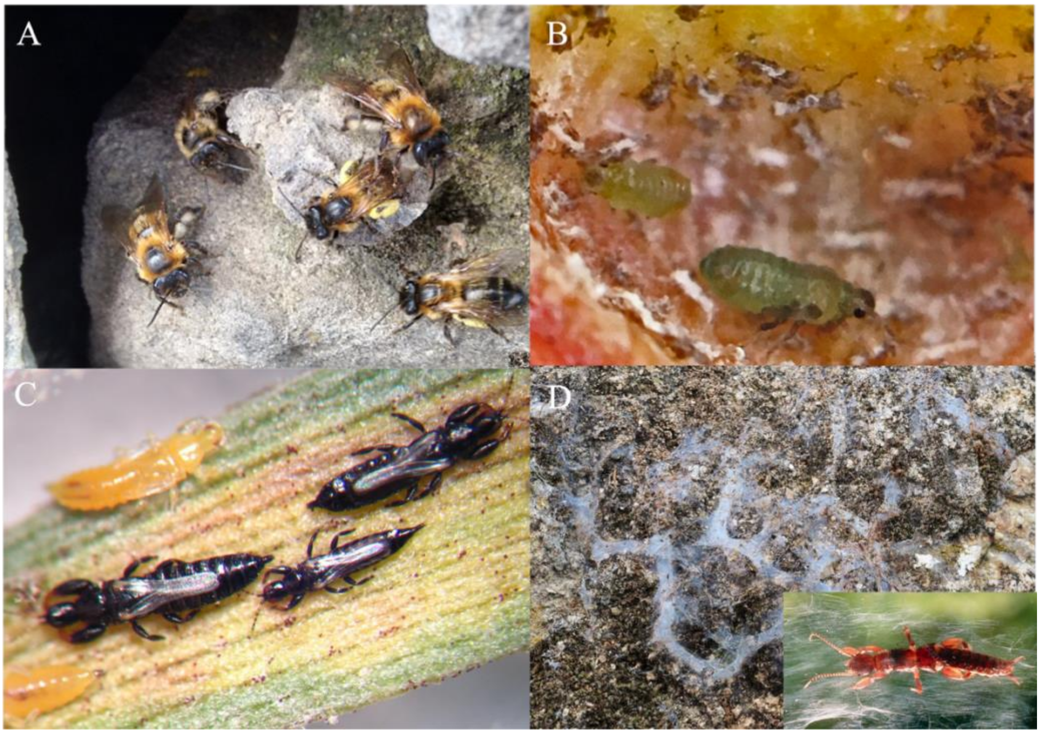

Figure 3. Communal nesting insects. Some insects share a nest with other related or unrelated adults but do not exhibit cooperative brood care or division of labour. **(A)** The Chocolate Mining Bee, *Andrena scotica*, is usually solitary, but occasionally hundreds of females can be found sharing a nest. Photo by Athayde Tonhasca. **(B)** In the aphid *Tamalia coweni,* multiple foundresses may live together communally inside a leaf gall. Photo by Damon Tighe. **(C)** Communal nesting is fairly common in thrips (order: Thysanoptera), with some groups formed by multiple foundresses, as in the genus *Dunatothrips.* Photo by James Gilbert. **(D)** Webspinners live in large silk nests, with tunnels connecting the domiciles of sometimes hundreds of individuals. Photos by Gordon Ramel and George Bernard.

The ecological diversity of communal nesting across taxa offers rich opportunities to examine the conditions under which cooperative living evolves. However, communal systems often remain poorly understood, especially in species with cryptic nesting habits. Even in well-studied groups like bees, it is difficult to determine whether nestmates interact cooperatively, provision brood separately, or engage in any division of tasks, for the practical reason that it is difficult to observe interactions in natural nests. These uncertainties highlight the need for more detailed behavioural studies in the field and lab.

Reproductive Division of Labour

Division of labour is the specialisation of individuals to different tasks – each performs only a subset of what is necessary for survival and group maintenance, and each is dependent on the other fulfilling their role (Cooper & West, 2018). Even reproduction is a task that can be outsourced, with some individuals specialised to breeding and others specialised to non-reproductive helper roles. Reproductive division of labour may be behavioural or morphological. In cases of behavioural division of labour with no clear morphological castes, roles are often facultative and changeable, with individuals acting as a helper for part of their life but then becoming a reproductive later. Behavioural differentiation into reproductive and non-reproductive roles is found in wasps, ants, bees, beetles, thrips, and aphids. These species have been described as “primitively eusocial” or “facultatively eusocial”, though these labels are also applied based on other characteristics such as colony size or longevity.

Reproductive division of labour: behavioural differentiation

Behavioural differentiation into breeder and helper roles is widespread in insects – found across bees, wasps, ants, beetles, thrips, and aphids.

Among the bees, examples of behavioural differentiation can be found in many ordinarily solitary lineages, such as orchid bees, sweat bees, longhorn bees, and carpenter bees. In some cases, there are subtle differences in size between the dominant reproductive and her helper (Figure 4 B; Appendix Table 3). There may also be differences in reproductive output that do not reflect physical differences in reproductive potential: In orchid bees, such as those in the genus *Euglossa*, there is division of labour between mothers and daughters where daughters lay eggs but are policed by their mother who destroy any eggs other than her own (Pech et al., 2008). In these arrangements, daughters retain full reproductive potential and their ‘sterility’ is behavioural rather than physical.

Behavioural reproductive division is especially well-studied in wasps, such as the paper wasp *Polistes dominula,* where dominant breeders are assisted by subordinate helpers. In many species, helpers may later inherit the nest or transition to reproduction themselves.

Behavioural reproductive divisions can be found in many wasp lineages, including paper wasps (Polistinae), *Microstigmus* wasps, hover wasps (Stenogastrinae), square-headed wasps (Crabronidae) and potter wasps (Eumeninae) (Figure 4 A; Field & Cant, 2007; Ross, 1991; Leadbeater et al., 2011; Field & Leadbeater, 2016; Sinha et al., 1993; Bonifacii & Field, 2023; Asís, 2003; Melo & Evans, 1993; Melo & Campos, 1993; Ross & Matthews, 1989; Archer, 2012; Krombein, 1976; Matthews & Naumann, 1988; Krombein, 1978).

In ants, most species show strong morphological caste systems, but a small number of genera have females with full reproductive capacity. These "gamergate" systems are regulated by dominance interactions, not morphology, and are believed to be a secondary loss of the queen caste (Figure 4 D; Ross, 1991; Hölldobler & Wilson, 1990).

Some beetles form social groups via the subsocial route, with daughters assisting their mothers in brood care or fungus-farming before dispersing to reproduce themselves (Figure 4 C; Biedermann, 2020; Peer & Taborsky, 2007). These helpers eventually disperse and can produce their own offspring (Boomsma, 2009; Boomsma, 2023; Wilson, 1971; Michener, 1974). In one species, helpers have lifetime unmatedness (functional sterility), though without distinct morphological castes (Boomsma, 2023; Smith et al., 2018; Kent & Simpson, 1992). Despite increasing interest, social beetles remain understudied due to cryptic nesting behaviours (Kirkendall et al., 2015; Saunders & Knoke, 1967; French & Roeper, 1972; Roeper et al., 1980; Mizuno & Kajimura, 2002; Biedermann et al., 2009; Lake Maner et al., 2013).

In thrips, sociality often centres on gall defence, where 'soldiers' (if fixed in their role) or 'defenders' (if they are later able to disperse and reproduce) exhibit self-sacrificial behaviours to protect kin (Crespi, 1996; Crespi & Abbot, 1999; Chapman et al., 2008; Abbot & Chapman, 2017). Aphids also produce soldier morphs – typically early-instar nymphs – that protect the group. These may be either morphologically specialised (e.g. Hormaphidinae) or not (e.g. Eriosomatinae). Non-specialised defenders are monomorphic and totipotent, and also perform cleaning behaviours (Benton & Foster, 1992).

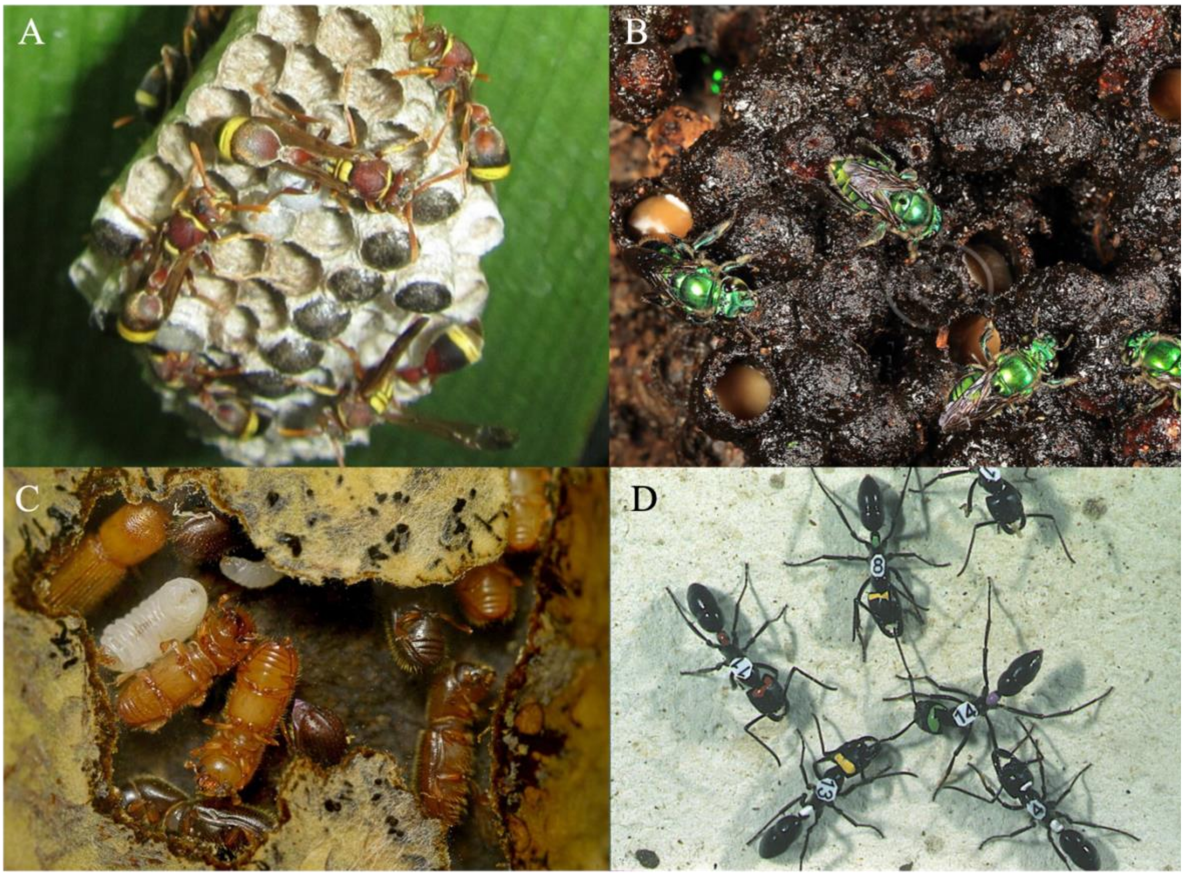

Figure 4. Behavioural division of labour. (A) In the paper wasp genus *Ropalidia*, adult females show temporal caste differentiation, with older females acting as egg-layers and younger ones as foragers. Photo by Matthew Connors. **(B)** In the orchid bee *Euglossa imperialis,* 3 or 4 females build brood cells next to each other and a dominant female (possibly the mother) may control the nest. Photo by Eric Tourneret. **(C)** In the Pinhole Borer beetle *Xyleborinus saxeseni,* females may delay dispersal to help with tasks such as brood care. Photo by Antonio Gugliuzzo. **(D)** Reproductive females compete aggressively for reproductive dominance in the queenless gamergate societies of the ant *Dinoponera quadriceps.* Photo by Thibaud Monnin.

Reproduction division of labour: morphological differentiation

Reproductive division of labour can involve morphological specialisation as well as behavioural. In some species, this divide can be extreme: An ant queen can be more than 300 times larger than her workers, and reproductive capacity varies on a similar scale – some queens can produce millions of offspring in her lifetime, while her workers completely lack any reproductive capacity (Figure 4 E, F; Appendix Table 4).

The evolution of obligate reproductive division of labour represents a critical threshold in terms of a major evolutionary transition in individuality: An oversized queen may be so full of eggs that she can barely move and relies completely on helpers to feed and guard her. Similarly, sterile workers will never be able to have offspring of their own and so can never gain direct fitness benefits. They instead depend entirely on the reproductive success of their kin to gain indirect fitness. Honeybees, bumblebees, stingless bees, and ants all have obligate coloniality, where queens and workers live and reproduce together as a single cohesive entity, analogous to germline and somatic cells in an organism (a superorganism; Grüter, 2020; Cardinal & Danforth, 2011; Romiguier et al., 2016; Hölldobler & Wilson, 1990). However, not all reproductive dimorphism is this extreme. Some insects have much subtler distinctions between dominant reproductives and their helpers (Figure 5 A, C, D).

In bees, caste differentiation is often determined during larval development. Queens and workers can differ dramatically in size, ovariole number, or egg-laying ability, although some species show only subtle differences. Queens of the Western Honeybee can lay up to 2,000 eggs per day, while workers of the same species are usually sterile (despite this, the queens are only slightly larger in body size; Figure 5 D) (Falk, 2016; Linksvayer et al., 2011; Wang et al., 2014). Honeybee caste is determined by larval diet – larvae fed "royal jelly" become queens. If the queen dies, some workers can activate their ovaries and lay male eggs. These workers also take on roles like foraging and maintaining the colony, showing some behavioural flexibility (Peso et al., 2016; Boomsma, 2009). In contrast, *Melipona* stingless bee queens and workers are reared on identical diets and are similar in size but differ in reproductive output. This results in an overproduction of queens, which later must be eliminated by nestmate workers – a scenario known as a Tragedy of the Commons where individual selfishness leads to an outcome that is detrimental to all (Grüter, 2020; Oliveira *et al.,* 2022).

Bumblebees also have caste determined during larval development, and queens are usually much larger than workers, with non-overlapping size ranges (Michener, 1974). However, bumblebee colonies are relatively small, and workers retain their ability to reproduce, so they are not usually considered "advanced eusocial" like honeybees (Ge et al., 2023). Only queens can mate with males, though many workers activate their ovaries and try to produce haploid male offspring, but few of their offspring survive in colonies with a queen (Holland & Bloch, 2020; Owen & Plowright, 1982; Bloch et al., 1996; Bloch & Hefetz, 1999; Brown et al., 2003; Takahashi et al., 2008). Evidence of morphological castes and reproductive specialisation have been found in nests of allodapine (tribe: Allodapini) bee species such as *Exoneura teidentata,* where a bimodal distribution of female size was identified, suggesting two distinct size morphs. The larger morphs have greater ovarian activity and higher mating frequency (Figure 5 B; Houston, 1977). They also found evidence that the larger, more-reproductive morph was more sedentary, as all females they identified outside of the nests were of the smaller morph. Subtle size differences between dominant reproductives and their helpers have been described in carpenter bees (Shell & Rehan, 2022).

In wasps, all known members of the Vespinae (yellowjackets and hornets) have morphological castes, with queens and workers differing in size and ovariole number (Hunt, 2007; Ross, 1991). However, no wasp species has completely sterile workers lacking spermathecae (Bell & Sumner, 2013). In some paper wasps, such as *Belonogaster grisea*, subtly dimorphic females differ in mating frequency and foraging behaviour (Pardi & Marino Piccilo, 1970, 1981).

Termites show particularly extreme caste differentiation. Most species in the Termitidae (sometimes referred to as the "Higher" termites) have irreversible developmental pathways leading to fixed castes, including highly fecund queens (Boomsma, 2023; Inward et al., 2007; Bourguignon et al., 2014; Engel et al., 2016; Evangelista et al., 2019). Some species have extreme reproductive dimorphism – in the subfamily Macrotermitinae, queens can lay up to 20,000 eggs per day (Grasse, 1949). Worker reproductive potential varies substantially across termite species – some workers have full reproductive potential, some have functional sterility, and others have fully sterile workers (Abe, 1987; Korb, 2019). Termites within the families Archotermopsidae, Stolotermitidae, Kalotermitidae, Stylotermitidae, and Rhinotermitidae retain the capacity to differentiate into reproductives, alates (primary founding reproductives), or defenders, even into their late instars.

Sociality in aphids (and thrips) was predicted before it was discovered: In 1972, Hamilton wrote about the need to explain the lack of social behaviour in male-haploid and clonal groups and subsequently, aphid and thrip sociality was found to take the form of family groups with specialised defensive morphs (Aoki, 1977; Crespi, 1992). Around 60 species of aphid have been identified as having some kind of specialised defensive nymph, which may be sterile or totipotent depending on the subfamily. Some even possess venom (Kutsukake et al., 2004; Benton & Foster, 1992). These helpers may also perform non-defensive roles, such as housekeeping or gall repair. The gall-forming aphid *Quadrartus yoshinomiyai* has defensive grandmothers (post-reproductive females with extended lifespans similar to menopause in humans) that altruistically defend the nest (Uematsu et al., 2010).

In thrips, all species with non-dispersing soldiers belong to the genus *Kladothrips*, with other species showing unusual roles such as "medic" castes. No thrip species has obligately sterile helpers (Crespi et al., 1992, 1997; Turnbull et al., 2012; Abbot & Chapman, 2017).

Figure 5. Morphological differentiation into reproductive and less reproductive castes. (A) Queen and worker of the stingless bee *Trigonisca mepecheu.* Photo from

Grüter, 2020. **(B)** Morphs of the allodapine bee *Exoneura tridentata,* where the larger females have greater ovarian activity. Photo from Grüter (2020), adapted from Engel *et al.,* 2019. **(C)** *Melipona* bees have the power of self-determination and as a consequence, one in five of them choose to be a queen. This overproduction of queens is resolved when they are eliminated by nestmates workers. Photo by Denise Alves. **(D)** Honeybee queens are fed on "royal jelly" and can lay up to 2,000 per day, though are only slightly larger than the workers. Photo from www.harryshoney.co.uk. **(E)** A *Dorylus* army ant queens can be more than 300 x larger than her worker daughter. Photo by Daniel Kronaeur. **(F)** Termites can also show extreme size dimorphism between queens and workers. Photo by Kurt Treftz.

Nymphal and larval castes

In some insects, morphological specialisation may exist only within the nymphal stage and in some cases these nymphs eventually disperse to mate, making their specialisation to helper roles only a temporary state. We discuss these cases here, distinguishing between them and species with lifelong helper specialisation, with the key distinction being whether helpers can eventually become reproductive. Nymphal castes exist in some hemimetabolous species, where young insects are more mobile and better able to contribute to colony tasks (termites, aphids). However, some holometabolous insects, such as beetles and ants, are also known to have division of labour between their larvae and adults.

Although some termites do have specialised helpers with obligate sterility, others have workers that are completely fertile. Fertile workers may act only temporarily as workers in their first few instars and then mature to either become replacement reproductives or leave the nest to establish new colonies (Boomsma, 2023; Krishna *et al.,* 2013). These are sometimes described as "false workers" or "pseudergates" and are found within Termposidae, Kalotermitidae, and Rhinotermitidae (Korb, 2016; Revely *et al.,* 2024).

In the aphid subfamily Eriosomatinae, nymphs aggressively defend the group and may have larger bodies, thicker cuticles, and enlarged horns compared to other nymphs in the group (Aoki, 1977; Bourke, 2011). These nymphs retain totipotency and will later disperse to reproduce and so are not truly sterile (Abbot & Chapman, 2017). In contrast, other aphid species have nymphs that never reach reproductive age and therefore may be considered true soldiers.

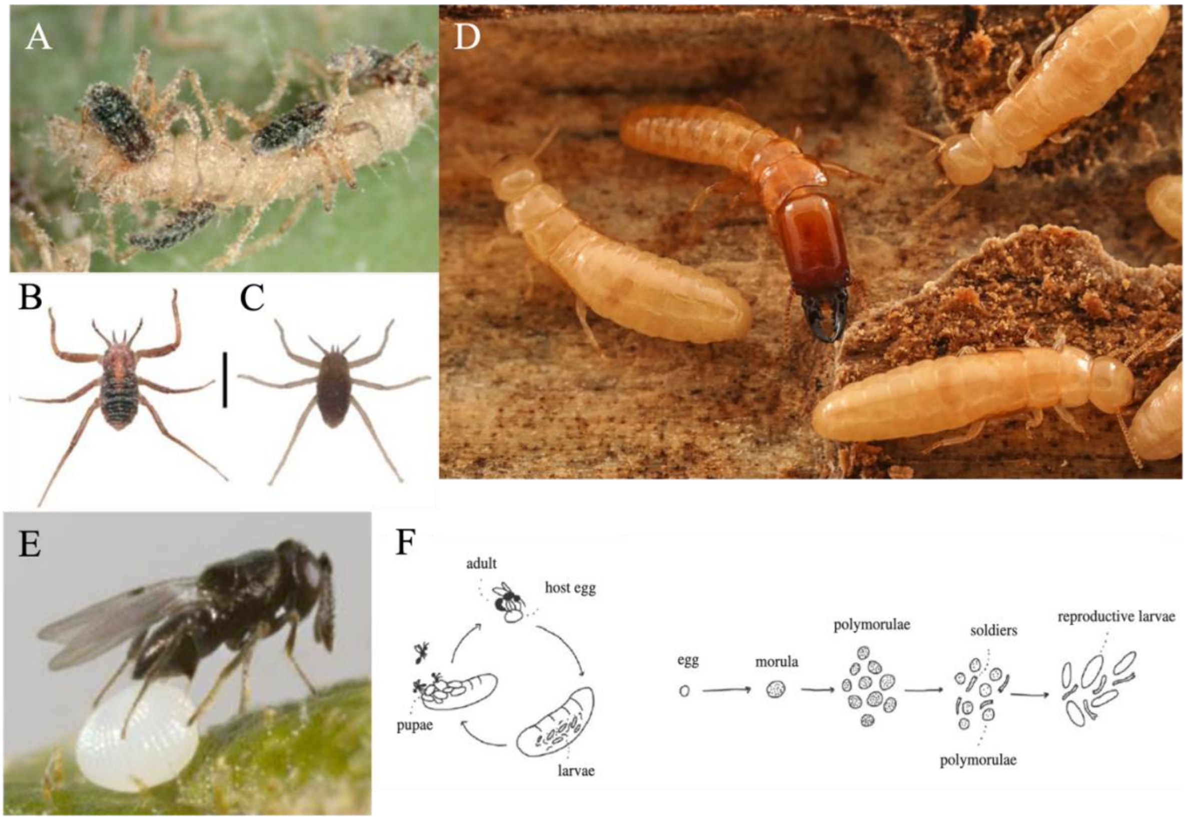

Figure 6. Nymphal and larval castes. Some insects have morphs, or castes, that are only present in their juvenile stage. In some species, the helper morphs never develop into adulthood and so are sterile, but in other species the opposite is true, and so they may not be considered true castes. **(A)** At least sixty species of aphid have nymphs specialised for defence; some of these later disperse to become reproductives, while others never disperse and may be considered sterile 'true soldiers'. In *Pseudoregma bambucicola,* there are two types of first instar nymphs: **(B)** Sterile soldier, and **(C)** a normal nymph. Soldiers are easily recognized by their thick armour, sharp horns, and enlarged forelegs. Photos from Shibao *et al.* (2009). **(D)** In some termites, such as *Kelotermes flavicollis,* colony tasks are performed by nymphs that may be temporarily specialised to roles such as defence but can later disperse and reproduce. Photo by Jan Šobotník**.** **(E)** The wasps *Copidosoma floridanum* lays its egg inside the egg of another, larger insect. Photo by Michael Strand. **(F)** The eggs *of C. floridanum* have a unique biology where multiple embryos develop from one egg, some of which become sterile soldiers protecting the future reproductives while still inside their host. Diagram taken from Whyte (2021).

Some species of ambrosia beetles, such as *Xyleborinus saxesenii,* have division of labour between larvae and adults; larvae care for brood and maintain their nest but may later disperse (Biedermann *et al.,* 2013).

In ants, division of labour can occur between larvae and adults, particularly in the handling and processing of food. Adults typically consume liquid resources such as nectar or honeydew and provide solid foods (such as protein-rich insect prey) to the larvae that are better able to process them. After digestion, the larvae may then return the nutrients to the adult workers via trophallaxis, effectively acting as living digestive organs for the colony (Dussutour & Simpson, 2009).

Larval castes can also be found in the parasitic wasp family Encyrtidae. Their lifecycle begins when a female oviposits into the eggs of their host species, laying one or two of her own eggs per host (Figure 6 E). Multiple larvae develop from each parasitic egg, giving the wasps their common name – Polyembryonic Wasps. This unusual biology creates a unique situation for the developing larvae: Clonally related, and in a defendable home (the host – arguably analogous to a nest), these larvae have evolved division of labour between sterile soldiers and reproductives (Figure 6 F; Cruz, 1981; Giron et al., 2007). The sterile soldiers kill competitors, but also siblings of the opposite sex because asymmetric relatedness between male and female siblings in these haplodiploid insects leads to sex ratio conflict (Gardner et al., 2006; Grbic et al., 1992; Giron et al., 2004).

The Polyembryonic wasps, although possessing completely sterile helpers, would not fit easily into the traditional eusocial classification because they don't have overlapping generations; considered by some to be an essential part of the criteria (Whyte, 2021; Wilson, 1971; Oster & Wilson, 1978). However, it is contested that the requirement for overlapping generations was based on the observation that most of the complex social Hymenopteran groups have subsocial group formation (mother-offspring associations) and that this is just one mode of achieving high relatedness, while polyembryony is another (Whyte, 2021; Downing *et al.,* 2017).

Non-reproductive division of labour

Aside from the differentiation of nestmates into reproductive and helper roles, some insects exhibit differentiation into multiple non-reproductive roles within their groups, such as foraging, guarding, nursing, or brood care. Well-known examples of non-reproductive division of labour include the multiple worker sub-castes of ants or termites, but other insect groups also exhibit specialisation among their helpers, including age-based task allocation in honeybee workers, body size-correlated behavioural differences in bumblebees, and specialised defender morphs in stingless bees, thrips, and aphids. In this section we cover the varied forms of non-reproductive division of labour within the insect helper castes.

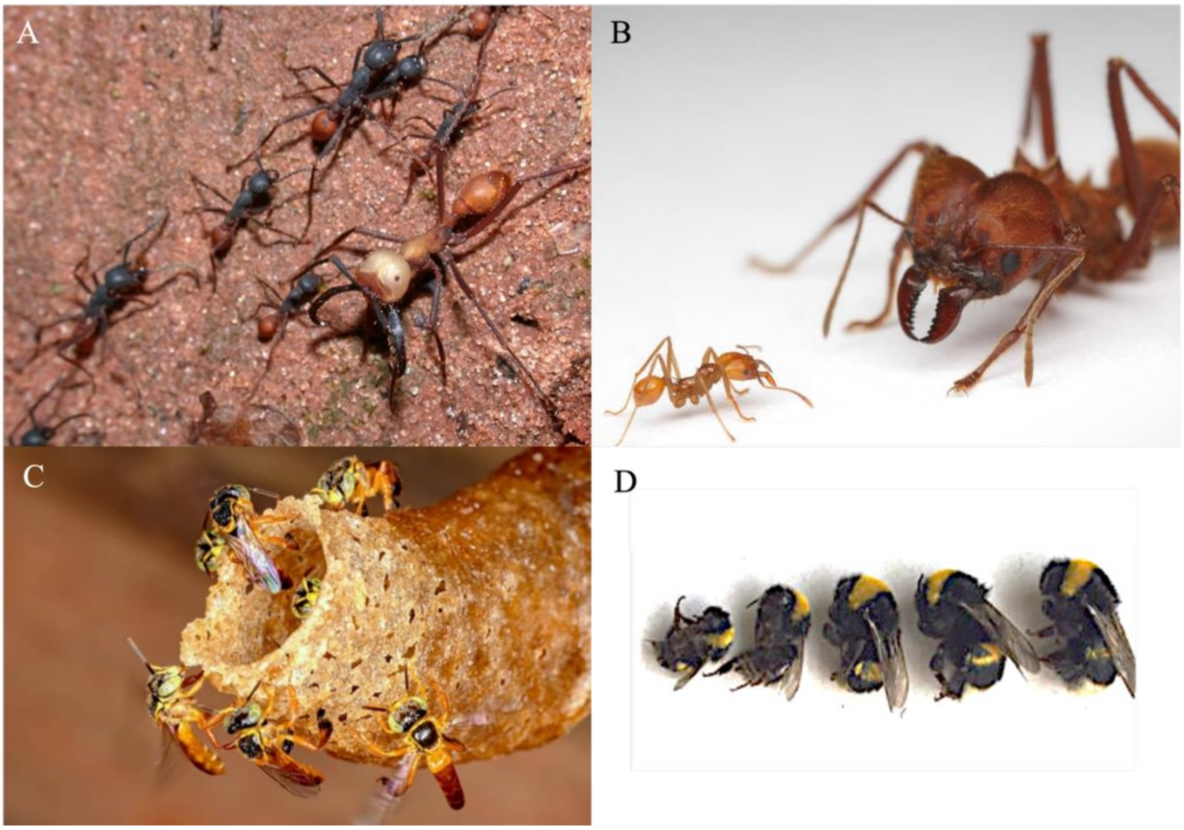

Figure 7. Division of labour within the worker caste. Some insects have non-reproductive division of labour where tasks such as defence, foraging, or nursing are divided up between specialised between nestmates. **(A)** *Eciton* army ants have extreme worker caste differentiation; here two sister workers of the same colony are shown, one specialised for defence, and the others for foraging. The smallest workers stay inside the colony and tend to the queen's eggs. Photo by Alex Wild. **(B)** *Atta* leafcutter ants also show extreme worker size differentiation. Photo by Alex Wild. **(C)** Several species of

stingless bee, such as *Tetragonisca angustula,* have specialised guards that may be 30% larger than their nestmates. Photo by Unknown. **(D)** In bumblebees, worker morphology varies on a continuous scale, with sizes corresponding to task performance. Photo by Unknown.

Non-reproductive division of labour: behavioural differentiation

In many social insects, workers don't just help – they specialise into distinct helper roles. Even without physical differences, colonies often show temporal polyethism, where workers change roles as they age. This flexible division of labour is seen across bees, ants, and termites, allowing colonies to efficiently assign tasks based on individual age, risk, and sometimes size (Appendix Table 5).

Bees such as the Western Honeybee (*Apis mellifera*) have a well-defined sequence of worker roles: cleaning → nursing → general tasks including nest-building, maintenance, nectar processing and nest-guarding → foraging (Johnson, 2009). Some stingless bees follow a similar age-based progression, while others show behavioural division of labour that appears to not be linked to their age or size (Grüter et al., 2023; Inoue et al., 1996; Mateus et al., 2019). Bumblebees also vary in task performance by size, but lack clear-cut subcastes (Goulson, 2010; Mares et al., 2005; Cumber, 1949; Holland & Bloch, 2020).

In ants, both behavioural and morphological worker differentiation is common. Leafcutter ants such as *Atta* and *Acromyrmex* combine age-based role changes with physical castes (Hölldobler & Wilson, 1980). Some ants also divide tasks spatially, such as *Leptothorax unifasciatus*, while *Temnothorax albipennis* colonies have both specialist and generalist workers (Sendova-Franks & Franks, 1995; Chittka & Muller, 2009). Even termites, famous for extreme body castes, often show age-based task allocation within morphologically similar workers. In *Reticulitermes speratus*, young workers perform care duties while older workers forage or defend the nest (Yanagihara et al., 2018).

Non-reproductive division of labour: morphological differentiation

Morphological specialisation within the helper caste varies widely across insect societies. In ants, this ranges from very subtle continuous size variation among workers, to highly differentiated sub-castes with dramatic differences in morphology and behaviour (Figure 7 A & B; Appendix Table 6). The leafcutter ant *Atta cephalotes* has huge soldiers with heads nearly 4 mm bigger than their smallest nestmates (Bell-Roberts et al., 2024; Chapter 3 of this thesis). However, the majority of ant species do not have discrete worker sub-castes: *Cardiocondyla batesii* has monomorphic workers with <0.01mm difference between the smallest and largest head size of its workers (Chapter 3 of this thesis). There is evidence that some ant species with continuous worker size variation may have behavioural differences corresponding to their different body sizes (*Formica* sp*.;* West & Purcell, 2020).

Termites also possess worker sub-castes, and in fact boast the greatest diversity of body forms from a single genome than any other known group, with up to three types of reproductives of each sex, and several sub-castes of workers or soldiers (Korb & Thorne, 2017). In *Macrotermes bellicosus*, major and minor workers differ in both form and task: majors forage, while minors tend fungus gardens and raise young (Gerber et al., 1988; Lys & Lethold, 1991). Yet, this complexity doesn't always mean increased sterility: *Trinervitermes bettonianus* workers are highly differentiated but not obligately sterile (Revely et al., 2024). Other termites have lower worker size variation, sometimes due to a secondary loss of the soldier sub-caste, as in Apicotermitinae (Revely *et al.,* 2024).

Morphological specialisation within the helper caste is rare in bees, but it does exist. In *Tetragonisca angustula* and at least 10 other stingless bees, guards are 30% larger than their nestmates, and some show bimodal size distributions indicating that these may be discrete sub-castes (Figure 7 C; Grüter et al., 2017, 2012). In some bumblebees, body size varies tenfold across workers and is correlated with behavioural differences, but this variation is continuous and does not fall into discrete sub-castes (Figure 7 D; Goulson, 2010; Holland & Bloch, 2020).

Summary and Conclusions

The study of insect social behaviour reveals a remarkable spectrum of cooperative strategies, stretching far beyond the well-characterised societies of leafcutter ants or honeybees. This review has highlighted the complexity and variety of insect social systems, especially in obscure and under-studied groups. By focusing on traits like nest sharing and division of labour in its various forms, we show that the ecological and evolutionary pathways to cooperation, and the ways that it can present, are highly diverse.

One of the most striking ways that cooperation manifests in insect societies is through specialisation into reproductive and helper roles. In some species, specialisation is extreme, like the huge queens and sterile workers in ants and termites. It can also be subtle, such as internal reproductive differences or slight size dimorphism. Specialisation can also be only behavioural, such as helper task allocation organised by factors such as age, body size, or social context. The variation in social organisation across insects raises many questions for evolutionary biologists: Why do some lineages evolve extreme caste dimorphism while others do not? Does extreme size dimorphism always evolve from subtler forms? Are reproductive and morphological specialisation tightly linked? Do some forms of division of labour facilitate the evolution of other forms?

To understand this diversity, we must treat individual traits as separate axes of variation rather than components of a pre-defined social package. A key message from our synthesis is that popular social classifications like "primitively eusocial" or “advanced eusocial” are problematic for several reasons: (i) They are often used in imprecise or inconsistent ways – either applied to species with very different traits or to mean different things by different authors. This makes it difficult to compare species or understand what these labels are actually describing. (ii) They can imply an evolutionary hierarchy or trajectory of ladder-like increases in complexity, where a solitary species is on the path to becoming a simple cooperative group, and ultimately a more complex cooperative group. These labels can give the false impression that all social insects are heading toward the same end point, when evolution is often much messier. It is possible that extreme division of labour could arise directly from a completely solitary state or that a species with complex cooperation could transition back into a solitary state or group with reduced division of labour, as has happened in the loss of ant queen caste in gamergate species, and the loss of the soldier caste in the termite subfamily Apicotermitinae (Hölldobler & Wilson; Revely et al., 2024 (iii) Social classifications can also obscure fascinating differences between cooperative adaptations across species. A mother and adult daughter Carpenter bee cooperating in building and provisioning brood cells is very different to the sterile defensive larvae of polyembryonic parasitoid wasps, yet these divergent cases might find themselves together under terms such as “incipiently social”.

To avoid these pitfalls, we advocate a trait-based approach. By clearly reporting the presence or absence of measurable features – such as reproductive skew, size dimorphism, or task allocation – we can make more meaningful comparisons across lineages and better identify the conditions that favour cooperative behaviour. Atypical or under-studied systems, including those in beetles, aphids, thrips, and polyembryonic wasps, are especially important in this respect. These groups defy assumptions about "true" sociality and demonstrate that features like task allocation or helper sterility can evolve in unexpected ways.

Looking forward, clearer reporting of trait presence or absence, standardisation of behavioural observations, and improvement of molecular phylogenies will help resolve outstanding questions about how insect sociality evolves and persists. Ultimately, refining our understanding of insect social behaviour will not only clarify the number of independent origins of cooperation, but answer broader questions about the evolution of complexity and individuality across all forms of life.

Appendix Tables

Table 1. Estimates for number of evolutionary origins of sociality in insects.

*da Silva (2021) defines "primitive eusociality" as small, short-lived colonies where morphological differences between reproductive and non- reproductive are minimal or non-existent, while "advanced eusociality" describes large and complex colonies that are long-lived and have distinct morphological castes.

Group	Origins	References
	WASPS	
Vespidae	2 origins "primitive eusociality" in Vespidae: 1 origin "primitive eusociality" in Stenogastrinae 1 origin "primitive eusociality" in Vespinae + Polistinae	da Silva, 2021; Hines et al., 2007; Piekarski et al., 2018

BEES		
Sweat bees (Halictidae)	6+ origins “primitive eusociality”: 1 – 2 origins in Halictini 1 – 4+ origins in Augochlorini No “advanced eusociality”	Halictini: 2 origins (Danforth, 2002; Brady et al., 2006; da Silva, 2021) or 1 origin (Gibbs et al., 2012). Augochlorini: 1 origin + 1 reversal (Danforth & Eickwort, 1997; Danforth, 2002; Brady et al., 2006) or 4+ origins (da Silva, 2021).
Honeybees, Bumblebees, Stingless bees, and Orchid bees (Apinae)	3 origins “eusociality”: 2 origins “primitive eusociality” (bumblebees and orchid bees) + 2 origins “advanced eusociality” (honeybees and meliponine bees)	Da Silva, 2021: “primitively eusocial” orchid bees evolved from a communal-nesting ancestors “advanced eusocial” meliponine bees evolved from “primitively eusocial” ancestors, which evolved from solitary ancestors “advanced eusocial” honeybees evolved from solitary ancestors

Carpenter bees and Longhorn bees (Xylocopinae + Eucerinae)	Up to 3 origins “primitive eusociality” in Ceratinini, Allodapini, and Xylocopini + reversals No “advanced eusociality”	3 origins (da Silva, 2021) or 1 origin (Schwarz et al., 2006)
	ANTS	
	~ 5 gains, 16 losses of worker sexual capacity in ants; 17 gains & 20 losses of complete worker sterility	Chapter 4 of this thesis
	TERMITES	
	1 origin of “true workers” in termites	Thompson et al. (2000)
	BEETLES	
	1 origin “superorganismality” in *Austroplatypus incompertus*	Boomsma (2023); Smith et al. (2018); Kent & Simpson (1992).
	APHIDS	
	6 – 9 origins of nymphal soldiers in aphids + at least 1 loss.	Stern & Foster (1996)
	THRIPS	
	1 origin + 1 loss of “true soldiers” in *Kladothrips*	Chapman et al. (2002)
	1 origin defensive morph “false soldiers” in *Koptothrips*	Crespi (1992)

Table 2. Communal nesting

Taxonomic Group	Example Species	Communal Nesting	References
BEES			
Orchid bees (Euglossini)	*Euglossa intersecta, Euglossa ignita, Eulaema meriana, Eulaema cingulata*	Facultative communal nesting; daughters may stay in natal nest or disperse	Eltz, 1999; Eltz et al., 2003; Cameron & Ramírez, 2001; Santos & Garófalo, 1994
Sweat bees (Halictidae)	*Nomia, Halictus, Pseudagapostemon, Ruizantheda, Lasioglossum, Augochloropsis*	Typically solitary; facultatively communal nesting	Michener, 1974; Córdoba-Aguilar et al., 2018
Carpenter bees (Xylocopinae)	*Xylocopa sauteri, X. nigrita*	Adult daughters cohabit maternal nest after death of mother	Iwata, 1964; Michener, 1974; Anzenberger, 1977
Mining bees (Andrenidae)	*Andrena erythronii, A. agilissima, A. carantonica*	Facultatively communal – sometimes up to 50 females sharing one nest	Michener & Rettenmeyer, 1956; Paxton et al., 1999; Giovanetti, 1999; Giovanetti et al., 1999
Longhorn and Colletid bees (Eucerinae & Colletidae)	*Eucera longicornis, Hylaeus tricolor, Amphylaeus morosus, Braunsapis puangensis*	Facultative communal nesting observed across multiple lineages of usually solitary wasps	Michener, 1974; Nielsen, 1902; Sakagami & Zucchi, 1978; Spessa et al., 2000; da Silva et al., 2015
WASPS			
Vespid wasps (Vespidae)	*Abispa australiana, Montezumia brethesi, M. dimidiata, Trimeria*	Communal nesting in several subfamilies; In *Montezumia dimidiata*, marked	Matthews & Matthews, 2004; Ross, 1991; Krombein, 1978;

	howardi, Parischnogaster striatula, Rhynchium cyanopterum	females were observed returning to their maternal nests to reproduce, although it remains unclear whether they nested with daughters (subsociality) or sisters (semisociality)	Zucchi et al., 1976; Yoshikawa, 1969; West-Eberhard, 1978
Crabronidae Sphecidae		The nature of nest sharing – whether females share brood cells as well as the nest (quasisociality) or provision separately (communal nesting) – is uncertain	Ross, 1991
Pompillidae	*Auplopus semialatus*	Documented forming communal nests, with up to eight females jointly building and maintaining a single nest	Wcislo *et al.,* 1988
		THRIPS	
Phlaeothripidae	*Dunatothrips spp.*	Multi-foundress nests, sometimes unrelated females, cooperation in domicile construction	Morris & Schwarz, 2002; Bono & Crespi, 2006; Gilbert & Simpson, 2013; Abbot & Chapman, 2017
		APHIDS	
Aphididae	*Tamalia coweni*	Multi-foundress gall sharing. Galls can sometimes be invaded by aphids of another species	Miller, 1998, 2005; Akimonto, 1989; Abbot & Chapman, 2017

		that do not produce galls of their own, resulting in mixed-species assemblages.	
		BEETLES	
Curculionidae: Ambrosia beetles	*Ambrosiophilus spp.*	Daughters remain in natal nest and breed communally alongside mother. These colonies are some of the largest ever recorded among beetles	Kasson et al., 2016
		WEBSPINNERS	
Webspinners (Embioptera)	*Oligotoma humbertiana*	solitary	Bradoo, 1967; Edgerly, 1986, 1987
	Anisembia texana, Antipluria urichi	facultatively communal	Bradoo, 1967; Edgerly, 1986, 1987
	Oligotoma saundersii, Oligotoma ceylonica	obligately communal	Bradoo, 1967; Edgerly, 1986, 1987
	Antipluria urichi	Facultatively communal; non-relatives join together	Edgerly, 2022, 2018, 1987
	General biology	Relatedness within these groups also differs; some species are parthenogenetic, with females producing viable eggs without fertilisation. The number of generations per year ranges from less than 1	Hoell *et al.,* 1998; Edgerly, 1997; Kusnezov, 1904; Bradoo, 1967

in *Embia taurica* to 4-5 in *O. ceylonica*, with more generations linked to greater social complexity.

Table 3. Reproductive division of labour: behavioural

Taxonomic Group	Example Species	Behavioural Specialisation to Reproductive and Helper Roles	References
	WASPS		
Paper wasps (Polistinae)	*Polistes dominula*	Dominance hierarchies with potentially unrelated helpers who may inherit the nest. Up to 10 females.	Field & Cant, 2007; Ross, 1991; Leadbeater et al., 2011; Field & Leadbeater, 2016
Paper wasps (Polistinae)	*Ropalidia rufoplagiata*	Temporal castes: older females lay eggs, younger ones forage	Sinha et al., 1993; Ross, 1991
Microstigmus wasps	*Microstigmus comes, M. nigrophthalmus, M. rosae*	Social nesting with reproductive division, no morphological castes	Bonifacii & Field, 2023; Asís, 2003; Melo & Evans, 1993; Melo & Campos, 1993; Ross & Matthews, 1989

	M.thripoctemus, *Anterhynchium similis*, and *A. myersi* likely also show sociality but are less studied		
Potter wasps (Eumeninae)	*Zethus miniatus*	Behavioural reproductive division, with some females acting dominantly when reproductive, while others assist in brood care.	Krombein, 1978
Crabronid wasps (Crabronidae)	*Arpactophilus spp.* and *Spilomena spp.*	Cooperative brood care among up to ten females	Matthews & Naumann, 1988; Ross, 1991
Hover wasps (Stenogastrinae)	*Eustenogaster eximia*	Dominance-based reproductive hierarchies, mother-daughter cooperation	Turillazzi in Ross, 1991; Archer, 2012; Krombein, 1976
ANTS			
Gamergate ants	*Diacamma, Dinoponera, Hagensia, Ophthalmopone, Streblognathus*	All females totipotent; dominance hierarchies and no queen caste.	
BEES			
sweat bees (Halictidae),		show behavioural	

longhorn bees (Eucerinae), and carpenter bees (Xyloponinae)		differentiation without clear morphological differences	
Orchid bees (Euglossini)	*Euglossa spp.*	Behavioural policing by mothers; daughters reproductively totipotent	Pech et al., 2008
	BEETLES		
Scolytinae Platypodinae	*Xyleborinus saxesenii, X. affinis,* *Trachyostus ghanaensis*	Helpers may be larvae or adults delaying dispersal to contribute to brood care; later reproduce	Biedermann, 2020; Peer & Taborsky, 2007; Boomsma, 2009, 2023; Wilson, 1971; Michener, 1974; Kirkendall et al., 2015
Platypodinae	*Austroplatypus incompertus*	Unmated helpers may never reproduce; lacks morphological castes	Boomsma, 2023; Smith et al., 2018; Kent & Simpson, 1992
	THRIPS		
Thrips	Various gall-forming species	Defensive castes: fixed role ‘soldiers’ or totipotent ‘defenders’	Crespi, 1996; Crespi & Abbot, 1999; Chapman et al., 2008; Abbot & Chapman, 2017

Table 4. Reproductive division of labour: morphological

Taxonomic Group	Example Species	Nature of Morphological Caste Differences	References
		BEES	
Apinae	*Apis mellifera* (honeybee)	Queens have >200 ovarioles; workers <20; queens can lay 2,000 eggs per day. Only slight size differences. Caste determined by larval diet (royal jelly). Workers can lay male eggs if queen dies.	Linksvayer et al., 2011; Wang et al., 2014; Falk, 2016; Peso et al., 2016; Boomsma, 2009
Apinae	*Bombus* spp. (bumblebees)	Queens much larger than workers; same number of ovarioles. Workers retain some reproductive potential, but only queens can mate with males.	Michener, 1974; Ge et al., 2023; Holland & Bloch, 2020; Owen & Plowright, 1982; Bloch et al., 1996; Bloch & Hefetz, 1999; Brown et al., 2003; Takahashi et al., 2008
Apinae	Stingless bees (e.g., *Melipona*)	Queens 2–6× heavier than workers; shape also differs. Some *Melipona* spp. Queens are raised on same food as workers; overproduction of queens leads to conflict.	Grüter, 2020; Oliveira et al., 2022
Xylocopinae: Allodapini	*Exoneura tridentata* (allodapine bee)	Bimodal size morphs among females: Larger morphs are more reproductive and stay in the nest.	Houston, 1977
Xylocopinae	Carpenter bees	Subtle size differences between reproductive and helper: Subtle	Shell & Rehan, 2022

		morphological dimorphism with reproductive skew.	
		WASPS	
Vespinae	Yellowjackets, hornets	Distinct queen and worker castes; different numbers of ovarioles; two distinct size classes of female offspring; the smaller females become workers, and the larger become queens.	Bell & Sumner, 2013; Hunt, 2007; Ross, 1991
Wasps	General biology	Workers are not irreversibly sterile—no wasps are known to lack spermathecae.	Bell & Sumner, 2013
Eumeninae	*Zethus miniatus*	Behavioural reproductive division, not full morphological caste: Some females act dominantly as reproductives, others assist in brood care.	Krombein, 1978
Paper wasps (Polistinae)	*Belonogaster grisea*	Two morphs: larger "queen-like" females and smaller "worker-like" females: Larger females tend to mate and found nests; smaller ones forage more often.	Pardi & Marino Piccilo, 1970, 1981
Termitidae ("Higher termites")		Irreversible caste differences determined before adulthood; extreme queen–worker dimorphism. Queens in Macrotermitinae can lay up to 20,000 eggs/day.	Boomsma, 2023; Inward et al., 2007; Bourguignon et al., 2014; Engel et al., 2016; Evangelista et al., 2019; Grasse, 1949

Archotermopsidae, Stolotermitidae, Kalotermitidae, Stylotermitidae, Rhinotermitidae		Workers retain capacity to develop into reproductives or defenders even in late instars.	Abe, 1987; Korb, 2019
APHIDS			
Hormaphidinae	General biology	Morphologically specialised, non-dispersing sterile “true” soldier nymphs.	Abbot & Chapman, 2017
Hormaphidinae	*Tuberaphis styraci*	Soldier caste with venomous secretions	Kutsukake et al., 2004
Eriosomatinae	General biology	Morphologically specialised nymphs for defence but can disperse and reproduce later – not completely sterile.	Abbot & Chapman, 2017
THRIPS			
	Kladothrips spp.	Morphologically specialised non-dispersing soldier caste. Soldiers mate with siblings and defend gall.	Crespi et al., 1992, 1997; Abbot & Chapman, 2017
	Koptothrips intermedius	Morphologically specialised helpers that act as “medics”. Defence against fungal pathogens rather than predators.	Turnbull et al., 2012
	General biology	No species with obligately sterile soldier castes	Abbot & Chapman, 2017

Table 5. Non-reproductive division of labour: behavioural

Taxonomic Group	Example Species	Behavioural Worker Specialisation	References
BEES			

Apinae	*Apis mellifera* (Western Honeybee)	Temporal polyethism: 4 age-based castes: cleaning → nursing → nest-building, maintenance, nectar processing, and nest guarding → foraging	Johnson, 2009
Apinae	Stingless bees	Temporal polyethism or unclear: Some show age-based roles; others show size-linked or no clear pattern	Grüter et al., 2023; Inoue et al., 1996; Mateus et al., 2019
Apinae	Bumblebees	Size-based task variation: No discrete subcastes, but size influences role	Goulson, 2010; Mares et al., 2005; Cumber, 1949; Holland & Bloch, 2020
ANTS			
Leafcutter ants	*Atta*, *Acromyrmex*	Temporal polyethism: Young: inside tasks; Older: foraging, waste, defence	Hölldobler & Wilson, 1980
	Leptothorax unifasciatus	Spatial task division: Workers specialise in zones of the nest	Sendova-Franks & Franks, 1995
	Temnothorax albipennis	Specialists and generalist: Some workers specialise in tasks; others are flexible	Chittka & Muller, 2009
TERMITES			
Termitidae: Neocapritermitinae	*Neocapritermes taracua*	Temporal polyethism: Young: care; Old: foraging and defence	Yanagihara et al., 2018

Table 6: Non-reproductive division of labour: morphological

Taxonomic Group	**Example Species**	**Nature of Morphological Worker Specialisation**	**References**

BEES			
Apinae	Stingless bee: *Tetragonisca angustula*	Soldier guards: Guards ~30% larger; distinct task allocation	Grüter et al., 2017, 2012
Apinae	Other stingless bees (10 Neotropical spp.)	Minor & major workers: Three spp. show bimodal size distribution	Grüter et al., 2017, 2012
Apinae	*Bombus terrestris*, *B. impatiens*, *B. lucorum*	Continuous size variation: 8–10x mass variation, size predicts in-nest vs foraging roles	Goulson, 2010; Holland & Bloch, 2020; Mares et al., 2005; Cumber, 1949
ANTS			
Leafcutter ants	*Atta cephalotes*	Multiple worker sub-castes Head size difference up to 3.9 mm; soldiers defend, minors tend brood/fungus	Bell-Roberts et al., 2024
	Cardiocondyla batesii	Monomorphic <0.01 mm head size difference among workers	Chapter 3 (this thesis)
	Formica spp.	Continuous variation Size correlates with behavioural role	West & Purcell, 2020
TERMIES			
Termitidae: Macrotermitinae	*Macrotermes bellicosus*	Major and minor workers: Majors forage; minors tend fungus and brood. Caste is set early in development	Gerber et al., 1988; Lys & Lethold, 1991; Okot-Kotber, 1985

Termitidae: Apicotermitinae		Secondary loss of soldier caste	Revely et al., 2024
Termitidae: Nasutitermitinae	*Trinervitermes bettonianus*	High polyphenism: Differentiated workers, but not obligately sterile	Revely et al., 2024

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Chapter 3: Larger colony sizes favour the evolution of worker size variation in ants

Abstract

Division of labour is central to the evolution of complex life, and the size-complexity hypothesis is a leading explanation for what drives complexity. It predicts that entities of larger sizes will require greater complexity to function. Previous research has identified correlations between number of cells and number of cell types, as well as social insect colony sizes and number of castes or worker size variation. However, these factors can be correlated with other variables that could also influence the evolution of division of labour, necessitating causal analyses to resolve the relationships. Here, I test the relative likelihood of alternative causal hypotheses for the evolution of worker size variation across 152 species of ants. I found support for the hypothesis that larger colony sizes favoured the evolution of greater variation in worker size. In contrast, we did not find consistent support for the effects of either queen mating frequency or number of queens per colony in driving this relationship. Overall, the results reinforce the size-complexity hypothesis, suggesting that colony size is a key factor in the evolution of non-reproductive division of labour.

Introduction

Division of labour is a fundamental characteristic of complex systems, leading to irreversible mutual-dependence and playing a key role in major evolutionary transitions, such as from unicellular life to multicellularity, or from solitary insects to superorganisms (Boomsma, 2023; Bourke, 2011; Szathmáry and Smith, 1995; West *et al.,* 2015). To understand what causes major transitions in complexity, we must understand factors that drive or constrain the evolution of division of labour.

The size-complexity hypothesis proposes that larger entities, whether individual organisms or colonies of insects, require increased complexity to function effectively. It predicts that increases in size will lead to increased division of labour (complexity). For example, larger organisms need more cell types for specialized tissues, organs, and regulatory systems to maintain efficient function, while larger colonies require more intricate specialisation among the workers, such as brood care, foraging, waste disposal, and defence (Boomsma, 2023; Bourke, 2011; Bell and Mooers, 1997; Bonner, 2004; Fisher *et al.,* 2020; Cooper *et al.,* 2022; Oster and Wilson, 1978).

Support for the size-complexity relationship has been found in multicellular systems: Organisms with larger numbers of cells have more cell types (Bell and Mooers, 1997; Bonner, 2004; Fisher *et al.,* 2020). Support has also been found by studies on social insects: Ant species with larger colony sizes have workers with greater size variation and more sub-castes (Ferguson-Gow *et al.,* 2014; Lecocq de Pletincx *et al.,* 2021; Dornhaus *et al.,* 2012; Bonner, 1993; Anderson and McShea, 2001; Wills *et al.,* 2018). However, despite these correlations, there are still challenges to the size-complexity hypothesis.

In ants, relatedness is predicted to influence the evolution of division of labour and is determined by how many males the queen mates with, as well as the number of queens per colony. When colonies are formed by a single monogamous pair, relatedness among colony members is maximal, which may reduce conflict and increase cooperative behaviours such as the specialisation to non-reproductive roles (Oster and Wilson, 1978; Fjerdingstad and Crozier, 2006; Frumhoff and Ward, 1992). The opposite prediction has also been made – that when multiple mating by the queen reduces relatedness between workers, they are more likely to police each other's reproduction, forcing each other to specialise to non-reproductive roles (Fjerdingstad and Crozier, 2006; Ratnieks, 1988). Additionally, colony size can be correlated with queen mating frequency, making it difficult to disentangle their effects (Lecocq de Pletincx *et al.,* 2021; Dornhaus *et al.,* 2012; Boomsma and Ratnieks, 1996).

The relationship between colony size and worker division of labour can be open to alternative causal explanations. For example, while the hypothesis predicts that larger colony sizes favour the evolution of greater division of labour, it is also possible that greater division of labour enables colony sizes to grow larger. The additional correlates queen mating frequency and queen number complicate this further, as it is also possible that an increase in colony size favours increased queen mating frequency, and this favours the evolution of more division of labour among workers. In this case, colony size and division of labour would be correlated, and would give the false impression that colony size directly favours increased division of labour. Disentangling these relationships requires the careful testing of alternative causal models with control for phylogenetic relationships between species.

I examined division of labour in worker ants, by examining size variation. Division of labour in ants could be measured in a few different ways, such as behavioural differences, reproductive dimorphism, or number of castes, but here I use the measure of size variation among workers. I chose this measure as it encapsulates the morphological differences that characterise extreme specialisation and mutual dependence. Additionally, the majority of ant species do not possess more than one worker caste, and so using this measure allows us to study differences not captured by only looking at discrete number of sub-castes. There is evidence that different-sized workers perform different roles even in species that do not have multiple worker sub-castes (West and Purcell, 2020).

Ants provide an excellent model for testing the size-complexity hypothesis because they are a single monophyletic clade showing substantial variation in division of labour, including worker size variation, across species. They also show variation in the colony characteristics hypothesized to influence division of labour, and there is an impressive body of literature describing the colony characteristics and life history of ants from around the world.

I analysed data from 152 species of ant to examine the roles of colony size, queen mating, and number of queens per colony on the evolution of worker size variation. I performed phylogenetic regressions to see how the variables correlate with each other, and to provide an overview to help guide further causal analyses. I then examined the likely causal relationships using phylogenetic path analysis. This is part of a larger project in which we also carried out analogous analyses examining the number of worker castes (Bell-Roberts *et al.,* 2024).

Methods

To test the roles of colony size, queen mating frequency, and number of queens per colony in the evolution of worker size variation, I performed a phylogenetic comparative analysis. I first tested which factors correlate with greater worker size variation and then used phylogenetic path analysis to examine the causal relationships that underly these correlations. This also allowed us to test how robust our results are to different methods.

Data Collection

We had previously collected data for several variables to analyse the number of worker castes (Bell-Roberts *et al.,* 2024): (i) Colony size (number of adult workers in a mature ant colony), (ii) effective queen mating frequency (the estimated number of mates weighted by the proportion of offspring sired by each male (Boomsma and Ratnieks, 1996; Nielson *et al.,* 2003), and (iii) the observed number of queens per colony (Bell-Roberts *et al.,* 2024). The data was compiled from major reviews, comparative studies, books, and then a literature search using Web of Science (Blanchard and Moreau, 2017; Hughes *et al.,* 2008; Hölldobler and Wilson, 1990; Burchill and Moreau, 2016). We also incorporated data collected by the Global Ant Genomics Alliance consortium (Boomsma *et al.,* 2017).

We collected data on worker size variation for all species for which we had information on colony sizes or queen mating frequency. To calculate worker size variation for each species, we measured the width of ant heads using scaled images on the AntWeb online database (antweb.org; California Academy of Sciences, 2023). We searched for the names of each target species in the online database and downloaded front-view images of the ants' heads and using ImageJ v1.53i, we measured the width at the widest point excluding the eyes (Schneider *et al.,* 2012). 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 = worker head width standard deviation / worker head width mean).

In total, we measured 1,064 worker ant heads (Figure 1). The number of workers measured per species ranged from 2 to 33, with a mean of 7. The number of workers measured was not correlated with the degree of size variation within that species (Supplementary Figure S1). In the scatter plot, CV values were widely dispersed across the full range of sample sizes, and the fitted regression line was nearly flat, indicating no meaningful association between how many workers were measured and the estimated degree of size variation. Overall, species with larger samples did not appear more variable than those with smaller samples, meaning that the observed differences in worker size variation among species reflect biological patterns rather than uneven sampling effort.

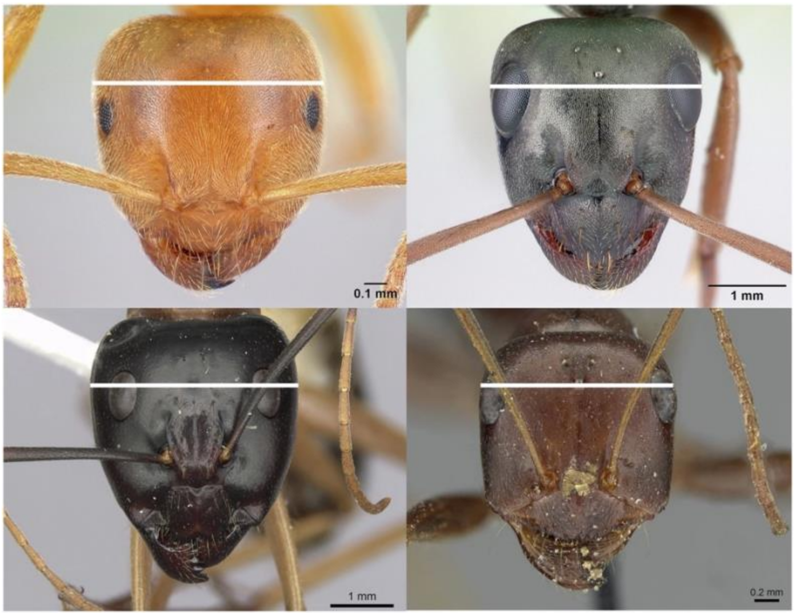


Figure 1. Measurement of ant head width. We downloaded front-view images of ant heads from www.antweb.org and measured at the widest point excluding the eyes (horizontal white line) using the image-processing software ImageJ. © California Academy of Sciences.

For the analyses, we excluded any species in our dataset that had the following distinct life history traits which represent evolutionarily derived elaborations of the ancestral full-sibling-colony state at the root of the ant clade. These included (i) species that form supercolonies (vast networks of connected nests); (ii) socially parasitic species that may lack a worker caste; (iii) species that can parthenogenetically produce queens and/or workers; (iv) species with gamergates (mated workers that reproduce sexually); and (v) species that use interlineage hybridisation for genetic caste determination. These species likely experience different selection pressures for the evolution of our traits of interest.

Phylogeny

To control for the effects of shared ancestry, we used a phylogenetic tree created by Economo *et al.* (2018) comprising of a molecular backbone calibrated using fossil records, with non-sequenced species placed probabilistically at the tips, giving a sample of 400 possible topologies. In the Bayesian correlational analyses, we repeated the analyses across all 400 and combined the posterior samples. For methods using frequentist statistics, it was not possible to run over all 400 trees, so we repeated with four Maximum Clade Credibility trees instead, representing 100 of each of their tree-building methods.

Correlations: Bayesian Phylogenetic Mixed Models

We fitted Bayesian Phylogenetic Mixed Models (BPMMs) with Markov Chain Monte Carlo (MCMC) estimation, using the MCMCglmm package v2.34 (Hadfield, 2010). Models were run for a minimum of 1,000,000 iterations, with a burn-in of 100,000, and a thinning interval of 1,000. We ensured there was a posterior effective sample size of at least 300 for all parameters, and the majority of estimates had an effective sample size of over 1,000. We used the coda package v0.19-4 to calculate the degree of autocorrelation between successive iterations in each chain (Plummer at al., 2006). We also fitted each model independently twice and used Gelman and Rubin's convergence test to compare within and between- chain variance (Gelman and Rubin, 1992). We modelled our traits as Gaussian continuous variables.

Causality: Phylogenetic Path Analysis

We used phylogenetic path analyses to evaluate alternative causal models for the evolution of worker size variation, including paths of direct and indirect influence among the variables (*"phylopath"* package version 1.1.3; Von Hardenberg and Gonzalez-Voyer, 2012). We modelled worker size variation as a continuous trait and performed the analysis first with all species for which data was available for all traits (n = 94) and then with only species that possess a single worker caste (monomorphic; n = 60). This technique uses frequentist statistics, meaning we cannot combine posterior samples run over 400 trees, and so we instead ran our analyses with four Maximum Clade Credibility (MCC) consensus trees and compared the results (Economo *et al.,* 2018).

We tested four alternative potential causal models for the relationships between our variables (Figure 2). Path analysis should be tested on models that are biologically plausible, not just on all possible combinations. We used the results of our correlational analyses to construct our models: Non-significant relationships were not included as direct paths, and where there were significant correlations, we alternated the direction of the arrow to test the most likely causal explanation.

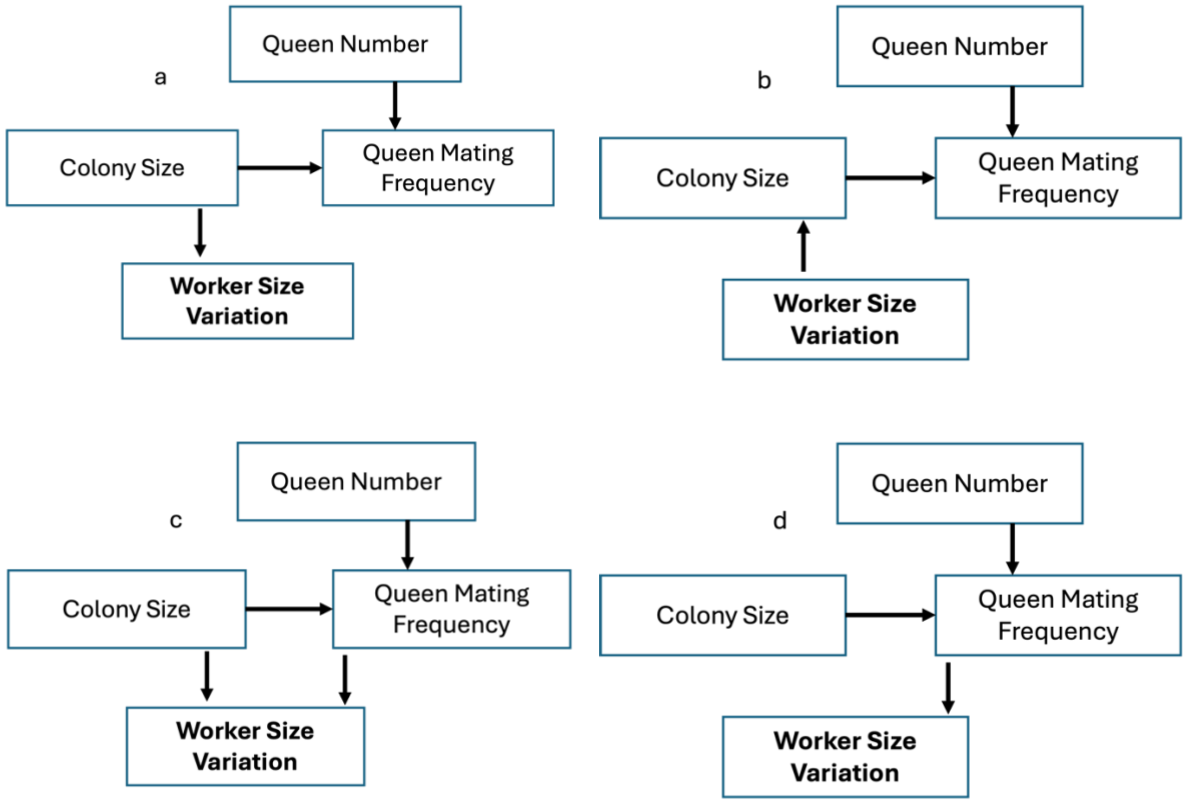


Figure 2. Potential causal models for the evolution of worker size variation.
Plots show directed acyclic graphs (DAGs). We designed four alternative causal models for the relationships between our variables and tested them using phylogenetic path analysis.

Results

We found that the largest variation in worker head size was 3.9mm in the American leaf-cutter ant *Atta cephalotes*, and the smallest variation in size was <0.01mm in the Palearctic ant *Cardiocondyla batesii*. Using the measure of worker coefficient of variation, *Pheidole spadonia* had the greatest amount of variation (0.77), and *Cardiocondyla batesii* had the least (<0.01).

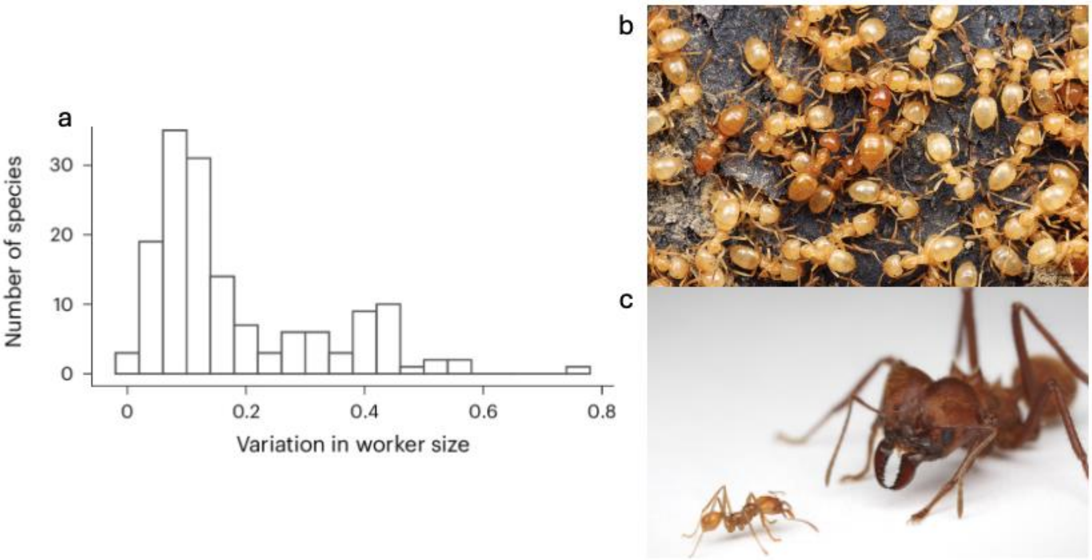


Figure 3. Variation in Worker Size (a) Distribution of worker coefficient of variation (CV worker size = worker head width standard deviation / worker head width mean; n = 152, (b) *Lasius claviger*; a species characterized by monomorphic workers with low size variation, and (c) *Atta cephalotes*; a species characterized by extreme size variation among workers. Photos by Alex Wild (https://www.alexanderwild.com)

Correlations

We found that ant species with larger colonies and higher queen mating frequency have greater variation in the size of their workers (Figure 4 a - b; BPMM: colony size: $\beta = 0.05$, CI = 0.03 to 0.08, n = 122, R2 = 0.14; mating frequency: $\beta = 0.12$, 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 (Figure 4 c; 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, we found no association between either queen mating frequency or number of queens per colony with variation in worker size (Figure 4 d – e; 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, $n_{species} = 68$).

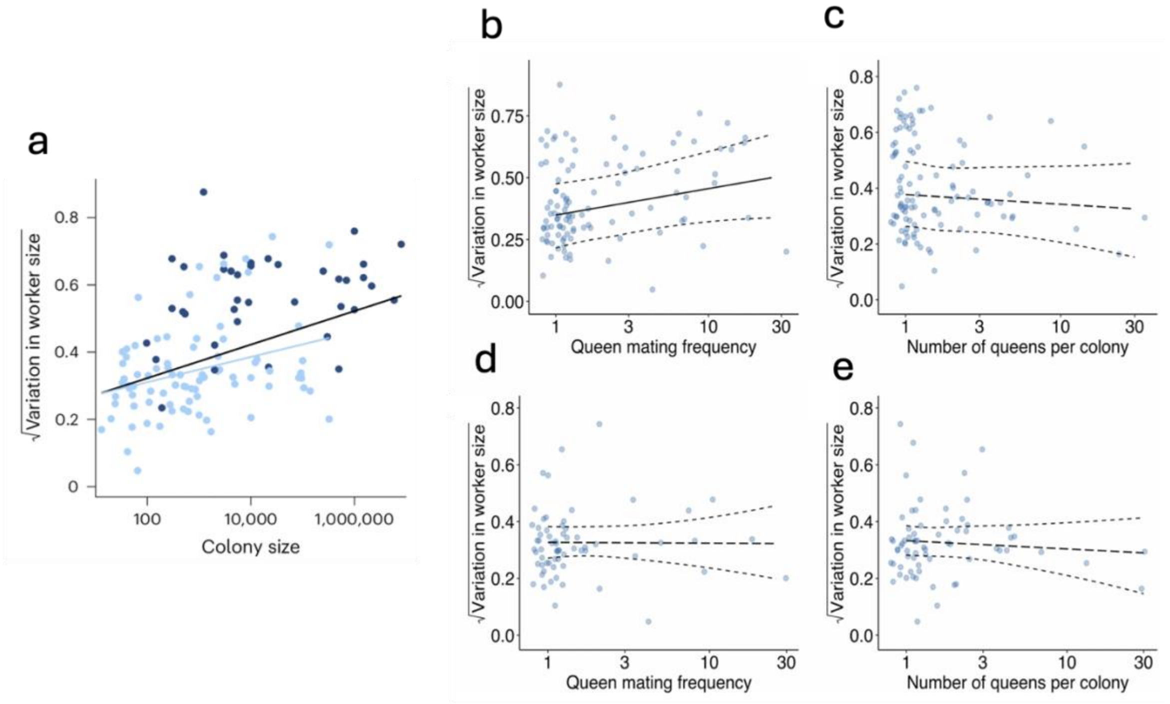


Figure 4. Species with larger colonies and higher queen mating frequencies have significantly greater variation in worker size. (a) We found a significant correlation between large colony sizes and the coefficient of variation in worker size when analysing all species for which data were available (dots and regression line in black, n = 122) and when analysing only species with a single worker caste (dots and regression line in light blue, n = 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)** Species with higher queen mating frequency have more variation in worker size when analysing all species for which data is available; **(c)** We found no significant association between number of queens per colony and worker size coefficient of variation; **(d - e)** When analysing our data looking at only species with a single worker caste, we found no significant correlations between either queen mating frequency and size variation or number of queens and size variation. 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 queen mating frequency and number of queens per colony are plotted on a $\log_{10}$ scale. Axes for variation in worker size are on a square root scale.

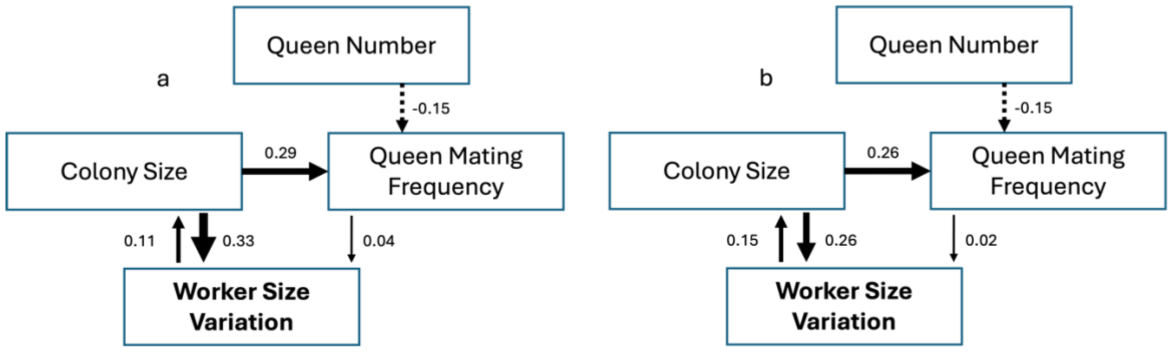
Phylogenetic Path Analysis

We identified correlations between larger colonies, higher queen mating frequency, and greater coefficient of variation in the size of their workers. To understand why these correlations exist, we tested a series of alternative causal models using phylogenetic path analysis. First, we analysed all species for which we had data, then we analysed only species with monomorphic workers. We found that these two categories of species have different causal relationships between their traits: When looking at all species, larger colony sizes likely facilitate the evolution of greater worker size variation, but in monomorphic species, size variation appears to precede and facilitate larger colonies.

All species with data

Larger colony sizes may facilitate the evolution of greater variation in worker size, but the direction of causality could also work in the opposite direction (Figure 5). Of our four alternative causal models, three were accepted (Figure 5, a – c). The rejected model contained no direct path from colony size to worker size variation, indicating that there must be a direct path for the model to be accepted (Figure 5, d). We repeated this analysis for four different MCC trees and then averaged the result of the accepted models for each run.

In contrast, we did not find support for the hypothesis that queen mating frequency influences worker size variation. The uncertainty of the best-supported model is reflected by the confidence intervals of the path coefficients between (i) colony size and worker variation and (ii) mating frequency and size variation, which are large and overlap with zero (Figure 5).



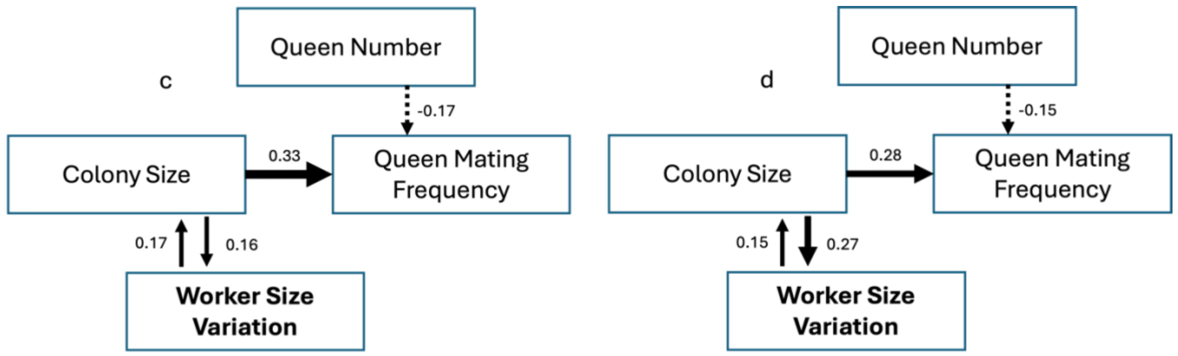
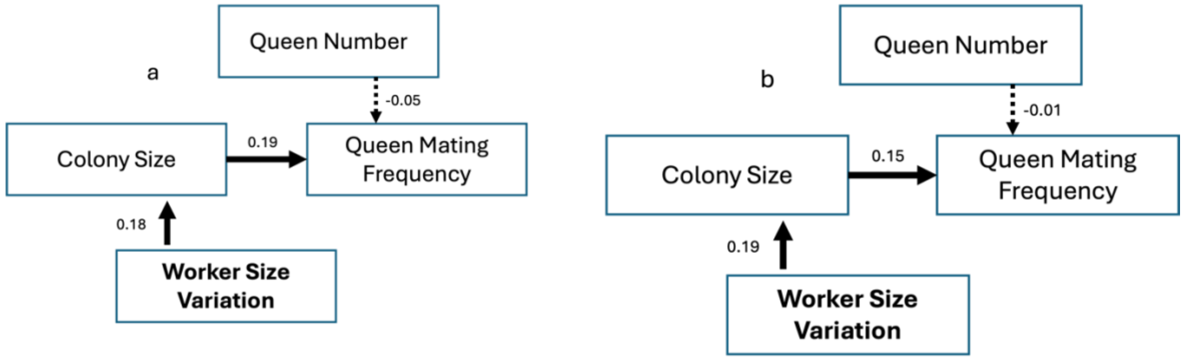


Figure 5. Larger colony size may facilitate the evolution of increased worker size variation. Here we display the results of the phylogenetic path analysis using four different MCC trees. Plots show the averaged results models that were accepted from our initial four potential causal models (shown in Figure 2). This analysis was performed using all species where data was available for all four traits (n = 94). Values represent standardised path coefficients and arrows indicate the direction of the relationship between variables, with heavier lines indicating larger coefficients. All traits were modelled as continuous variables.

Monomorphic species

We repeated the path analysis after filtering the dataset to only include species with a single worker caste (monomorphic) and found the contrasting result that greater variation in worker size favours the evolution of larger colony sizes (Figure 6). As before, we did not find support for the hypothesis that variation in worker size is influenced by queen mating frequency.



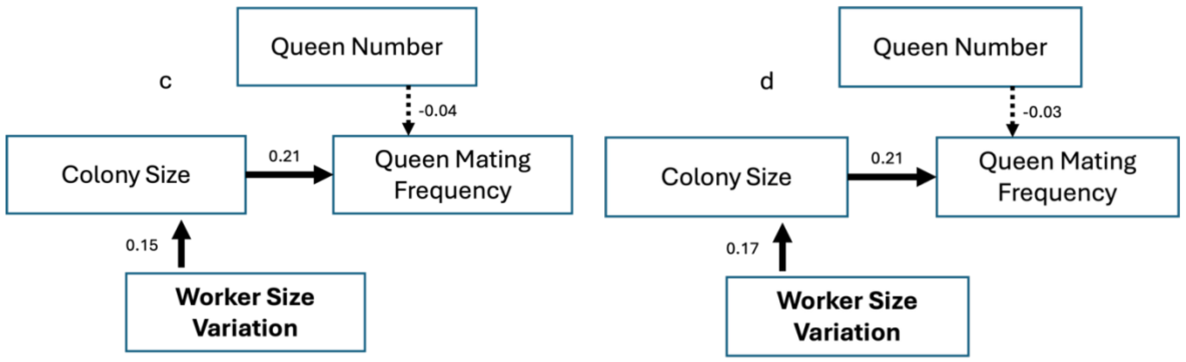


Figure 6: In monomorphic species, worker size variation precedes / increases colony size. Results of the phylogenetic path analysis using four different MCC trees. Plots show the averaged results models that were accepted from our initial four potential causal models (shown in Figure 2). This analysis was performed using only species with a single worker caste (monomorphic; n = 60). Values represent standardised path coefficients and arrows indicate the direction of the relationship between variables, with heavier lines indicating larger coefficients. All traits were modelled as continuous variables.

Discussion

We found that species with greater worker size variation have larger colonies and higher queen mating frequency. By testing alternative causal models with phylogenetic path analysis, we found evidence that larger colony sizes favoured the evolution of greater variation in worker size. However, there was some uncertainty with this result, and causality may also act in the opposite direction. When reanalysing using only ant species with monomorphic workers, we found the consistent result that worker size variation favours increased colony size, suggesting that variation in worker size preceded larger colony sizes.

Overall, our results support the size–complexity hypothesis but suggest that colony size is not responsible for the evolution of variation in worker size in species that lack discrete physical worker castes. Previous work identified positive correlations between increased division of labour and both cell number in multicellular organisms, and colony size in social insects (Bell and Mooers, 1997; Bonner, 2004; Fisher *et al.,* 2020; Ferguson-Gow *et al.,* 2014; Lecocq de Pletincx *et al.,* 2021; Dornhaus *et al.,* 2012; Fjerdingstad and Crozier, 2006; Boomsma and Ratnieks, 1996). However, those results were open to multiple explanations. Our results support a distinct evolutionary pattern where larger colony sizes increase worker size variation and where size variation precedes larger colony sizes in species without multiple worker sub-castes.

Why would larger colony sizes favour increased worker size dimorphism?
When including species with multiple physical worker castes, our measure of size variation encapsulates this discrete morphological specialisation as well as variation that would not meet the threshold for being considered discrete castes. This could mean that the result of colony size favouring greater worker size variation could mostly be driven by selection pressure for discrete castes (polymorphism). This is supported by our finding in the broader analyses that larger colony sizes precede and seem to facilitate the evolution of more worker subcastes (Bell-Roberts *et al.,* 2024).

There are multiple reasons why larger colony sizes could select for increased division of labour. In larger colonies, the number of tasks that need to be performed is expected to be much higher, requiring greater division of labour to maintain colony efficiency. For example, nests of *Atta* leafcutter ants can be up to 600m^3 and visible from space by satellites (Hölldobler and Wilson, 1990). Comprising tens of millions of workers, these colonies have unique logistical challenges, such as how to transport waste out of all the chambers of such a gigantic city-like nest, how to defend its huge perimeter, and how to transport supplies into the centre of the colony. Having workers of varying sizes or multiple sub-castes may enable the colony to match worker capabilities to specific tasks. For example, larger workers are more effective in defence, while small workers might excel in brood care, enhancing overall colony efficiency (Bourke, 2011). Larger colonies may also be better able to maintain the correct ratio of worker sub-castes or sizes than small colonies, while generalist workers are preferred in small colonies (Cooper *et al.,* 2022; Liu *et al.,* 2021). It is also possible that larger colonies naturally exhibit greater worker size variation due to statistical effects; larger sample sizes tend to have a wider range of values, whereas smaller colonies may show less variation simply due to the constraints of a smaller population.

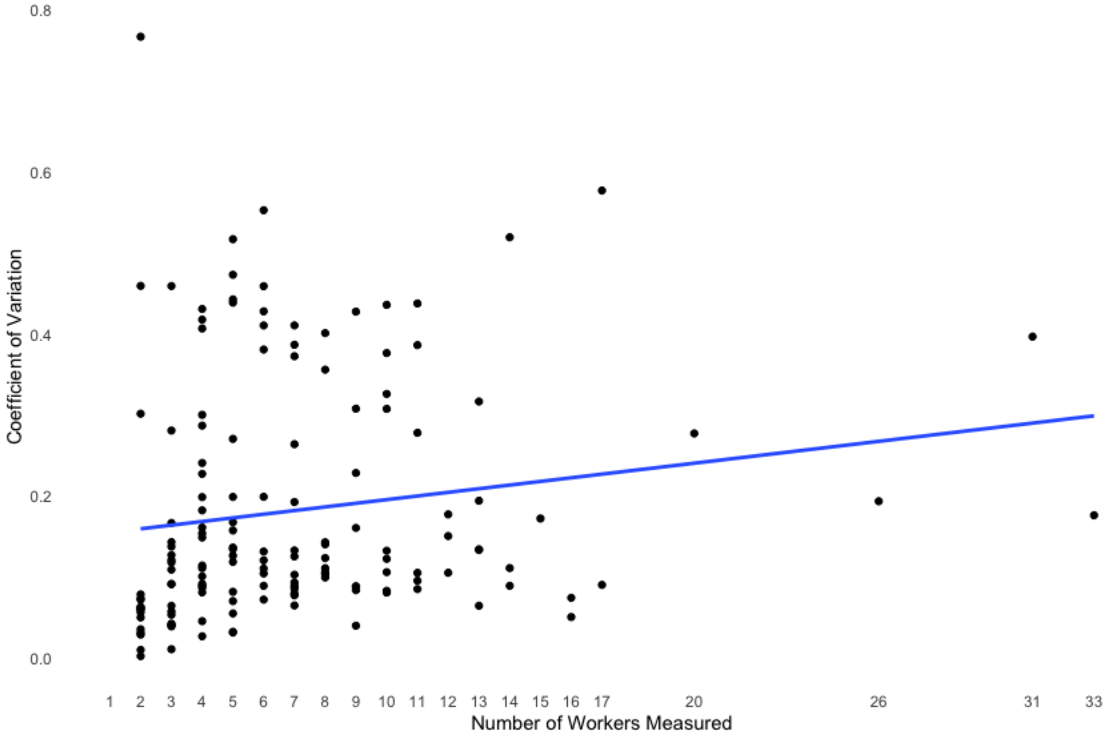
Why would monomorphic species show a distinct evolutionary pattern where size variation precedes larger colony sizes? When we analysed our data looking exclusively at species with monomorphic workers, we were looking purely at selection for variation that does not meet the threshold for discrete sub-castes. Continuous size variation among workers may allow some task specialisation without true polymorphism and could provide enough efficiency benefit to allow colonies to grow larger.

Worker size variation could provide variation upon which selection can act to produce discrete worker castes. However, caution should be taken when considering continuous variation as a stepping stone to discrete castes – although it may facilitate sub-caste evolution, it can arise as a unique trait with distinct advantages that may not always lead to discrete caste evolution. It is possible that developmental or evolutionary constraints restrict some lineages from evolving discrete castes, and so increased worker size variation can act as an alternative specialisation mechanism. Outside of the ants, worker-sub castes are very rare, even among other social insects – entirely absent in vespine wasps, while in bees the presence of two worker sub-castes has only been identified in some stingless bees (Grüter *et al.,* 2017; Grüter *et al.,* 2012). The presence of traits including stingers or wings has been hypothesised to constrain the evolution of multiple worker sub-castes in bees and wasps and stingers may also constrain the evolution of worker sub-castes in some ant lineages (Blanchard and Moreau, 2017; Molet *et al.,* 2012).

Queen mating frequency and worker size variation. We found a positive correlation between queen mating frequency and worker size variation, but when analysing the causal relationships behind this correlation we found that mating frequency is only correlated through its interaction with colony size (Figures 5 & 6). Colony size and queen mating frequency may be positively correlated due to the advantages of increased genetic diversity that results from multiple mating (polyandry; Boomsma and Ratnieks, 1996; Baer and Smid-Hempel, 1999; Cremer *et al.,* 2007; Hamilton, 1987; Hughes and Boomsma, 2004; Ugelvig *et al.,* 2010; Schmid-Hempel, 1998; Loope *et al.,* 2014; Naug and Camazine, 2002; Yasui, 1998).

In summary, we found mixed support for the size-complexity hypothesis in ants. Examining worker size variation, we found that larger colony size favours the evolution of greater division of labour, but division of labour may also facilitate the evolution of larger colony sizes, so causality can act in both directions. When examining number of worker sub-castes, the results were more straightforward– consistent evidence that larger colony sizes is what facilitates increased worker specialisation. The generality of this rule 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, or the difference between queens and workers in superorganismal social insects (reproductive division of labour; Fisher *et al.,* 2020; Fisher *et al.,* 2013; Howe *et al.,* 2024). Additionally, further analyses could test the hypothesis that continuous worker size variation precedes discrete worker caste evolution, and whether the presence of a stinger can constrain the evolution of division of labour among workers.

Supplementary Material



Supplementary Figure S1. The number of workers measured per species is not meaningfully correlated with the degree of size variation in that species.

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Chapter 4: The evolution of greater queen-worker dimorphism is facilitated by larger colony sizes in ants

Abstract

Explaining variation in the extent of division of labour remains a major problem for our understanding of how complex life evolved. Ants show remarkable variation in their extent of reproductive division of labour, from workers who can reproduce sexually and are approximately the same size as queens, to workers that are completely sterile and 300x smaller than their queens. Examining data from 546 species of ant, we found that: (i) the ancestral ant worker likely had full reproductive potential, though was effectively sterile in the presence of a queen; (ii) the loss of worker reproductive potential generally followed a sequential step-by-step process, via reduced capacity for sexual reproduction, then the production of males only, and finally complete sterility; (iii) the independent evolution of complete sterility has occurred approximately 17 times, with only 42% of ant species having sterile workers; (iv) reproductive size dimorphism has increased to higher levels around 9 times. Exploring potential causality, we found support for the size-complexity hypothesis, that increased colony size has favoured increased division of labour between queens and workers, examining both queen-worker size dimorphism and the loss of reproductive capacity in workers.

Key words: division of labour, worker sterility, ants, the size-complexity hypothesis

Teaser Text: Why are some ant workers fertile while others are completely sterile? By analysing 546 species of ant, we uncover the evolutionary steps leading to extreme caste specialisation. We find that ancestral ant workers likely had full reproductive ability, that sterility evolved gradually many times over, and that both loss of worker reproductive potential and increased queen-worker size dimorphism are facilitated by large colony sizes, shedding new light on how complex cooperative systems can evolve.

Introduction

Division of labour has played a pivotal role in driving evolutionary transitions to more complex life (Boomsma, 2022; West et al., 2015; Bourke, 2011; Maynard-Smith and Szathmary, 1995). A major evolutionary transition in individuality is when individuals join to form a new higher-level, and more complex entity. For example, genes formed genomes, cells formed multicellular organisms, and animals formed superorganismal societies. Division of labour is fundamental to these transitions as it leads to individuals becoming reliant upon each other for reproduction (mutual dependence). Consequently, a major aim is to understand where division of labour has, or has not, evolved.

Ants show considerable variation in the extent of reproductive division of labour between the queens (reproductives) and their workers (helpers) (Hölldobler and Wilson, 1990; Vizueta et al., 2025; Matte and LeBouef, 2025). The workers of some species are born with the potential to mate and produce diploid offspring, while in other species they are completely sterile. Similarly, some species have workers and queens of approximately equal size, while others have queens more than 300 times larger than the workers (Figure 1). How can we explain this variation? How often has the extreme reproductive division of labour represented by sterility evolved in ants?

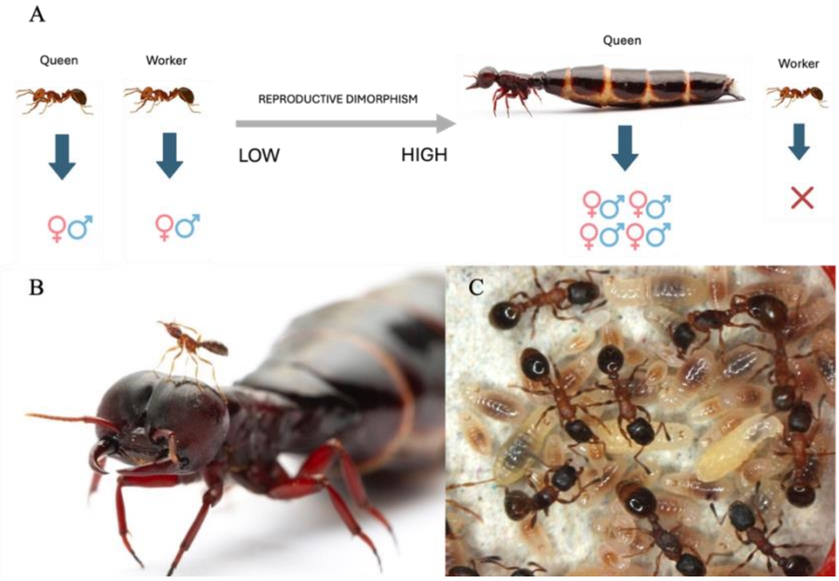


Figure 1. Reproductive dimorphism varies across species. (A) Reproductive dimorphism in ants can arise via size dimorphism and differences in reproductive capacity. (B) *Dorylus* worker perches on the head of a queen, illustrating extreme reproductive dimorphism. (C) *Leptothorax* queens and workers provide an example of minimal reproductive dimorphism. Photos: A & B: Daniel Kronauer, C: Walter and Heinze, 2015.

The size-complexity hypothesis provides a potential explanation for variation in the extent of reproductive division of labour across ants. It predicts that in lineages that have undergone a major transition to a higher-level organism, larger numbers of lower-level subunits select for increased division of labour (Bell-Roberts *et al.,* 2024; Bourke, 1999; Bourke, 2011). Consistent with this hypothesis, ant species with larger colonies have a greater queen-worker dimorphism (Fjerdingstad and Crozier, 2006; Kramer and Schaible, 2013; Ferguson-Gow et al., 2014; Matte and LeBoeuf, 2025; Bourke, 2011). Larger colony sizes have been hypothesised to favour increased size dimorphism between workers and queens because of reduced reproductive replacement opportunities for workers and increased fecundity required for queens (Bourke, 1999). However, the possible influence of colony size on the other key aspect of reproductive division of labour – worker sterility – has not been tested.

The possible influence of colony size on reproductive division of labour remains unclear. First, as discussed above, the influence of colony size on the evolution of worker sterility has never been tested. Second, the observed correlations between colony size and reproductive size dimorphism are correlational and open to multiple interpretations (Cornwallis and Griffin, 2024). While larger colony sizes could have favoured greater size dimorphism, the reverse causal relationship is also possible, that greater size dimorphism favoured larger colony sizes. For example, increased size dimorphism could have increased colony efficiency, or smaller workers could lead to larger colonies. Alternatively, greater size dimorphism could have been favoured by another factor that is correlated with colony size. For example, the number of males a queen mates with or the number of reproductive queens per colony, could influence the evolution of both reproductive division of labour and colony size (Cooper and West, 2018). Matte & LeBoeuf (2025) examined the evolution of queen-worker size dimorphism but could not determine whether larger colony sizes favoured increased size dimorphism or the reverse. Ultimately, distinguishing between these competing explanations requires phylogenetic analyses examining order of evolutionary change and most likely causal explanation.

We investigated the evolution of reproductive division of labour across 543 species of ants, examining both worker sterility and queen-worker size dimorphism. For worker sterility, we considered both the ‘reproductive potential’ of workers, in terms of what they are physically capable of at birth (Figure 2), and the ‘realised reproduction’ of workers, in terms of their level of reproduction within a normally functioning colony. Our aims were to: (1) determine how often extreme reproductive division of labour has evolved in ants, as represented by loss of worker reproductive potential or extreme queen-worker size dimorphism; (2) use phylogenetic comparative analyses designed to test causal hypotheses about how the evolution of these two different aspects of reproductive division of labour have been influenced by three potential explanatory variables – colony size, the number of males that queens mate with (polyandry) and the number of queens (polygyny) (Oster and Wilson, 1978; Fjerdingstad and Crozier, 2006; Frumhoff and Ward, 1992).

Materials and Methods

Measuring Reproductive Division of Labour

We measured reproductive division of labour in two ways: the ability of workers to reproduce and queen-worker head size dimorphism.

Measuring Worker Reproduction. We used a Web of Science search to gather data on worker reproduction for all species where we had colony size or queen mating frequency data (see below). We used the search terms:
(((ovar*) OR (steril*) OR (reproductive-constraint*) OR (ants)) AND (species name)).
We assigned each species to one of four categories of worker physical reproductive potential: 1= full capacity to produce male and female offspring, 2 = reduced capacity to produce male and female offspring, 3 = capacity to produce male offspring only, 4 = no capacity to produce offspring (Figure 2). These categories reflect physical differences in anatomy: reduced ovariole number (2), the lack of a spermatheca (3), or the absence of ovaries (4).

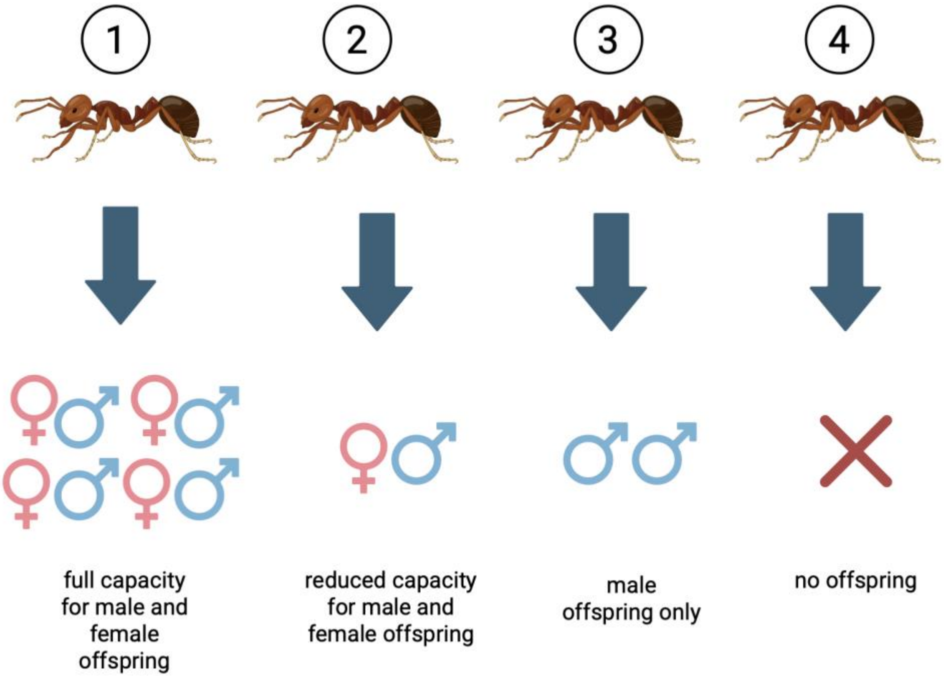


Figure 2. Variation in physical reproductive capacity of ant workers. Ant species were assigned to one of four categories of worker physical reproductive potential. 1= full capacity to produce male and female offspring, 2 = reduced capacity to produce male and female offspring, 3 = capacity to produce male offspring only, 4 = no capacity to produce offspring. The numbers indicate degree of sterility but do not imply any particular evolutionary order, i.e. they are categorical, not ordinal (inspired by Khila & Abouheif, 2010).

The concepts of 'full' and 'reduced' reproduction are defined relative to a more reproductive (queen) caste. In species where workers can sexually reproduce but a queen caste with greater reproduction is present, the workers would be categorised as having 'reduced' capacity. In contrast, in species with no caste with greater reproductive capacity, sexual workers could be considered to have 'full' capacity.

We categorised worker reproduction in two ways. First, we considered the 'reproductive potential' of workers, in terms of their physical potential to reproduce at birth. Second, we considered the 'realised reproduction' of workers, in terms of how much they reproduce in normal colonies. The reproductive potential and realised reproduction of workers can differ for several reasons, including behavioural interactions with or policing by other workers or queens (Bourke, 1999).

Measuring Queen-Worker Head Size Dimorphism. We used head size to estimate the difference in size between queens and workers as it is the most reported measure of queen and worker size in the literature and correlates with body size (Ferguson-Gow et al., 2014; Hölldobler and Wilson, 1990; Kaspari, 1993; Vaino et al., 2004; Weiser et al., 2004; Matte and LeBoeuf, 2025). We used the AntWeb online database (antweb.org) to find scaled images of all the species where we had previously found colony size, queen mating frequency, and queen number data (Bell-Roberts et al. 2024). Usually, several images of each specimen were included in the website's species pages to aid identification. This included front-view images of the ants' heads, which we downloaded and measured at the widest point excluding the eyes using the image-processing software ImageJ. The number of workers measured per species ranged from 1 – 37, with a mode of 5. The number of queens measured per species ranged from 1 – 10, with a mode of 1.

Some species have multiple worker castes, which can vary in size. We are interested in the evolution of extreme specialisation, so we measured maximum size dimorphism within a species, by using the minimum worker head width (i.e. smallest individual worker). Specifically, we measured reproductive size dimorphism as:

Reproductive size dimorphism = mean queen head width / minimum worker head width

A value of reproductive dimorphism equal to 1.0 implies that workers and queens are the same size, while values >1.0 imply that queens are larger than workers. We were able to compile head size information for a total of 1,062 workers and 132 queens.

Analyses

We began by testing alternative models of state evolution to build a picture of the evolutionary history of worker reproductive potential. We then used phylogenetic regressions to test for correlations between the variables that we measured. Finally, we used three methods to test hypotheses about the likely causality of these correlations: (i) phylogenetic path analysis, (ii) transition rate analysis between pairs of traits, and (iii) ancestral state reconstruction. We used different methods to both examine different but closely related questions, and to test the robustness of our results. We always analysed with the maximum number of species, where data for all the variables being considered was available. Consequently, the number of species varied across analyses because of variation in the data availability. We retained continuous variables as continuous, except when the form of analysis required that we binarize them.

Before running the analyses, we filtered out any species without the required data and any species known to be supercolonial, socially parasitic, clonal, or hybrids (Bell-Roberts et al., 2024; Helanterä, 2022). These species have very different life histories, meaning that we would need to make different predictions, and our variables cannot always be measured in the same way. We categorised all species by whether the reproductive state of their workers was explicitly described (high confidence subset) or inferred from congeners in cases of no known intrageneric variation (all species subset) and repeated all analyses with these two subsets. Each analysis used the largest set of species that had data for both the predictor and response, so sample sizes vary across analyses.

As trait similarity may be the result of shared ancestry, all our methods controlled for phylogeny (Dewar et al., 2025). We used different numbers of phylogenetic trees depending on the method employed. For Bayesian regressions and ancestral state reconstructions, we incorporated phylogenetic uncertainty directly by running models across 400 posterior trees. Phylogenetic path analysis, however, relies on frequentist statistics and does not readily allow for integration across a posterior sample of trees. For this reason, we used four maximum clade credibility (MCC) trees generated from different tree-building methods. This provided an alternative way of testing whether our results were consistent across plausible phylogenetic relationships.

Order of State Evolution. The variation that we see in ant worker reproductive potential could have arisen in different ways. It is possible that states 1 (full sexual capacity) and 4 (no offspring) represent extreme ends of a continuum, and that to reach either extreme, species must pass through the intermediate states as steps on a ladder of increasing or decreasing social complexity. However, an alternative possibility is that the four states could be non-ordinal categories, where one state can transition directly into any other (SI Figure 1).
To test which model has stronger support, we used MultiState in BayesTraits (Pagel, 1994). The free model was run with the following priors: HyperPriorAll exp 0 0.1, scaletrees 0.001, burn-in 3,000,000, iterations 30,000,000, stones 100 10000. For the restricted (sequential) model, we ran with the same priors except that we restricted non-sequential transitions to zero. We also tested two intermediate models in which non-sequential transitions could occur but were rarer than the sequential transitions. We compared the models in a pairwise manner (simpler model vs complex model; Supp Info Table 1).

Phylogenetic Correlations. We tested whether colony size, queen mating frequency (estimated number of mates weighted by the proportion of offspring sired by each male), or number of queens per colony were correlated with the worker reproductive potential or queen-worker dimorphism of ant species in a series of pairwise simple phylogenetic regressions. Colony size was $\log_{10}$ transformed and colony size, mating frequency, and queen number were analysed as continuous variables. Worker reproductive potential was analysed as categorical, divided between the four categories outlined in Figure 2 (1 = full capacity for male and female offspring, 2 = reduced capacity for male and female offspring, 3 = male offspring only, 4 = no offspring). Ants have a haplodiploid sex determination system where male offspring are haploid and hatch from unfertilised eggs, while female offspring are diploid and hatch from fertilised eggs. There are rare cases of species where diploid female offspring can be produced clonally but these were among species filtered out prior to our analyses. Species in categories 1 & 2 (capacity for male and female offspring) can therefore be considered the species with worker mating, while those in categories 3 & 4 (male offspring only & no offspring) have workers unable to mate.

We performed all regressions in R v 4.2.3. We controlled for phylogenetic non-independence by running Bayesian phylogenetic generalised linear mixed models (BPMMs) with the R package "*MCMCglmm*" version 2.34 (Hadfield, 2010; Cornwallis and Griffin, 2024). We used the package "mulTree" version 1.3.7 to repeat the analyses over all the possible ant phylogenetic trees produced by Economo et al. (2018). Economo et al. produced 100 posterior trees with each of four methods, giving a total of 400 possible trees. We repeated each regression analysis over all 400 trees to examine the effects of phylogenetic uncertainty. At present, only genus-level phylogenies exist for ants and species topology is largely unknown. Therefore, we want to know whether this uncertainty can change our results.

We ran all models for at least 11,000,000 iterations with a burn-in of 1,000,000 and a thinning interval of 5,000. We used the "*coda"* package version 0.19 – 4.1 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 (Plummer et al., 2006; Gelman and Rubin, 1992). We encountered convergence issues with the models when setting worker reproductive potential as the response variable (*categorical* family distribution), and so to test the robustness of results, we also ran the models with predictor and responses swapped. It is possible that the relationship effects could be in either direction. In those cases, we applied a *gaussian* family distribution to the colony size regression and *exponential* to queen number and mating frequency.

Phylogenetic Path Analysis. We used phylogenetic path analyses (*"phylopath"* package version 1.1.3; Von Hardenberg and Gonzalez-Voyer, 2012) to evaluate alternative causal models for the evolution of queen-worker dimorphism and worker reproductive potential, including paths of direct and indirect influence among the variables. Path analysis compares alternative causal models and disentangles direct and indirect effects between variables. This technique uses frequentist statistics, meaning we cannot combine posterior samples run over 400 trees, and so we instead ran our analyses with four Maximum Clade Credibility (MCC) consensus trees and compared the results.

We tested four alternative potential causal models for the relationships between our variables (Supplementary Material Figures 5 & 6). Path analysis should be tested on models that are biologically plausible, not just on all possible combinations. We used the results of our correlational analyses to construct our models: Non-significant relationships were not included as direct paths, and where there were significant correlations, we alternated the direction of the arrow to test the most likely causal explanation.

Correlated Trait Evolution and Transition Rate Analysis. We used *BayesTraits* V4.0 (Meade and Pagel, 2023) with Discrete MCMC estimation to test for correlated evolution between (i) worker reproductive potential and colony size, (ii) worker reproductive potential and queen number, (iii) queen-worker size dimorphism and colony size, and (iv) queen-worker size dimorphism and queen mating frequency. We compared the fit of a dependent model of evolution (in which the state of one variable is dependent on the state of the other), with that of an independent model in which traits evolve separately.

BayesTraits requires both traits to be modelled as binary variables: Worker reproductive potential (originally four categories) became (i) sex/no sex (categories 1, 2 vs 3, 4) and (ii) non-sterile/sterile (categories 1, 2, 3 vs 4). Queen-worker size dimorphism (initially a continuous variable) became low size dimorphism vs high size dimorphism, defined by whether values were above or below the median, excluding a 5% buffer zone. Colony size was transformed into a binary variable using the threshold of whether it fell above or below the median excluding a 10% buffer zone. We had more species with colony size data so could afford to discard more of them with a larger buffer zone. Queen number and queen mating frequency both used the threshold of above or below two. Having two or more queens per colony (polygyny) means that workers are not full siblings and therefore maximal relatedness cannot be achieved. Consequently, different predictions have been made about the expected reproductive behaviours of workers in single queen (monogynous) vs multiple queen (polygynous) colonies (Bourke, 2001). Similarly, two or more matings for queens (polyandry) reduces relatedness among workers, resulting in an average worker relatedness that is halfway between the theoretical maximum (0.75) and minimum (0.25) for single-queen colonies. Additional matings beyond two have a progressively smaller impact on relatedness, and mating with two males marks the point at which worker policing is favoured (Bell-Roberts et al., 2014; Ratnieks, 1988).

We scaled the tree, so the branches had a mean length of 0.1 (as recommended in *BayesTraits* V4.0 manual), enabling the algorithm to better explore parameter space and preventing rates from becoming very small or difficult to estimate. Scaling the branches does not affect results, since the parameter space of the transition rates is scaled by the same constant (Meade & Pagel, 2023). All models employed reversible-jump Markov chain Monte Carlo (rjMCMC) methods and an exponential prior, with a mean drawn from a uniform hyperprior ranging from 0 to 0.1. All models were run for 1 billion iterations with a burn-in of 1,000,000 iterations, sampling every 10,000 iterations. Analyses were repeated three times and the median log likelihood value was reported. We also repeated across all 400 posterior trees from Economo et al., 2018.

Trace plots were visually examined in *Tracer* V1.7.2 (Rambaut et al., 2018) to check chain mixing and convergence. Parameter acceptance rate was examined via the schedule file. All effective sample sizes were at least >3,000. Each model was run three times to check for consistency, and that with the median log marginal likelihood was chosen. Model support was evaluated using Bayes Factors (BFs). Natural log marginal likelihoods of both the independent and dependent models were estimated using a stepping stone sampler (Xie et al., 2011), set with 100 stones, running for 10,000 iterations and the default parameters of $\alpha = 0.4$ and $\beta = 1$ (Xie et al., 2011). BFs were interpreted following Kass & Raftery (1995): 0–2, minimal support; 2–6, positive support; 6–10, strong support; greater than 10, very strong support.

Ancestral State Reconstructions. To better understand the order of trait evolution and the relationship between colony size and our two measures of reproductive dimorphism, we carried out ancestral state reconstructions and analyses at transition points (Cornwallis et al., 2017; Hadfield, 2010). We examined: (i) whether colony size was higher at the ancestral nodes of lineages that had transitions to a state of high dimorphism versus those that didn't; and (ii) whether colony size was higher preceding or following transitions to high dimorphism. If colony size is higher in lineages that undergo the transition versus those that don't (part i), this would suggest that large colony size facilitated the transitions to high dimorphism. If colony size is higher after the transition to high dimorphism than it was before the transition, it would suggest that transitions in dimorphism facilitated the evolution of larger colonies.

For these analyses we transformed our measures of reproductive division of labour into binary variables (as described in *Correlated Trait Evolution*) but kept colony size, queen number, and queen mating frequency continuous. First, we reconstructed the ancestral states of worker sterility (sterile/non-sterile), worker sex (sex/no sex), and queen-worker size dimorphism (low/high) to find where these transitions occurred. We ran the analysis with all 400 posterior trees (Economo *et al.* 2018) with the *"corHMM"* package, version 2.8 (Beaulieu et al., 2013) and ran models with different rate categories (1-3 rates) and models of evolution (ER *equal rates* and ARD *all-rates-different*). We used AIC values to identify the best model. We then used Bayesian Phylogenetic Mixed Models (*BPMMs;* Hadfield, 2010*)* to examine (i) whether colony size was higher at the ancestral nodes of lineages that had transitions to a state of high dimorphism versus those that didn't and (ii) whether colony size was higher preceding or following transitions to high dimorphism.

Results

Worker Reproductive Potential

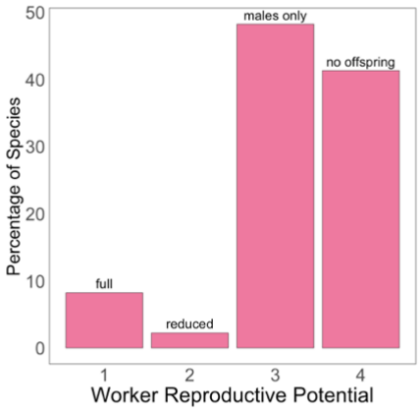
We categorised 546 species of ant by the reproductive potential of their workers. We found that 58% of ant species have workers with some potential for reproduction (Figure 3B). The majority of those are only able to produce male offspring (47% of all species). However, 11% of species have workers able to produce both male and female offspring, comprised of 10% sexually reproducing species, and 1% species clonally reproducing. We filtered out the clonal species and further divided the species where workers could reproduce sexually by whether they have ‘full’ or ‘reduced’ sexual capacity in relation to the queens (8 & 12% of the total respectively). The remaining 42% of the species had workers that were completely sterile.

We found a very different distribution of worker realised reproduction when compared to the distribution of worker physical capacity. Almost all species for which data were available (95%) show no worker offspring production in normal functioning colonies, with only 5% of species producing males (Figure 3C). This did not provide enough variation for correlational or causal analyses, and so we focused on worker physical reproductive potential.

Order of State Evolution. We found strongest support for a model of evolution where transitions in worker reproductive potential occur predominantly in a stepwise sequential manner, except for one non-sequential transition (Figure 3A). This suggests that ant workers usually lost their reproductive potential in ordered steps: first reduced capacity for sexual reproduction, then the production of males only and then complete sterility (1 → 2 → 3 → 4). The exception was one non sequential transition type, where states of full sexual capacity transitioned to no offspring (1 → 4), which can either be interpreted as a loss of all reproductive potential in one step, or as stepwise losses that happened so rapidly that the intermediate steps could not be detected. We reached this conclusion by testing different models against each other in each pairwise comparison (Supp Info Table 1).

A

B



C

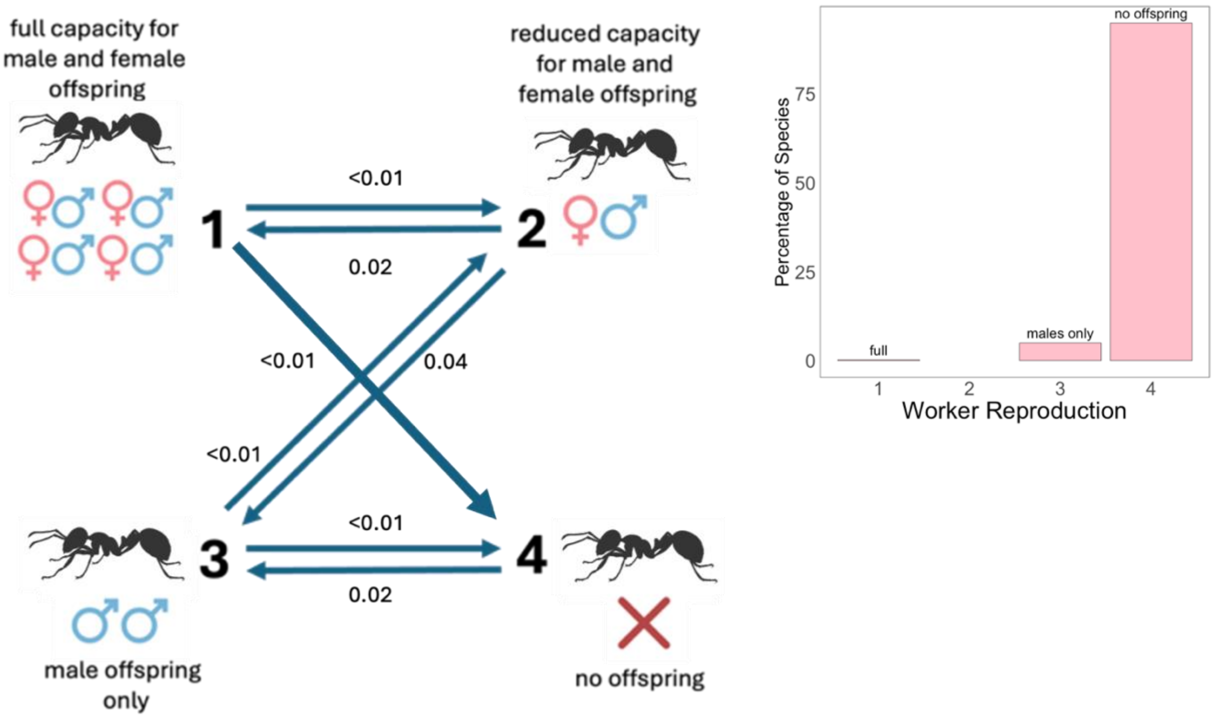


Figure 3. Worker reproductive potential. (A) Support for the mostly sequential model of trait evolution. Values are mean transition rates between states of worker reproductive potential at birth. We found strongest support for the model of evolution where ant workers lost or gained reproductive potential in a mostly sequential manner, except for one transition (full capacity → no offspring) where complete loss of potential occurred in one step. **(B) Distribution of worker reproductive potential** across 535 species of ant (their physical capacity) and **(C) Distribution of realised worker reproduction** across 522 species (the actual reproduction that workers do in a normally-functioning colony; not counting gamergate queens as workers) – not used in further analysing owing to lack of variation.

<u>Ancestral State Reconstruction of Worker Reproduction</u>. The ancestral ant worker most likely had full reproductive potential but was effectively sterile in the presence of a queen (most likely root state: = category 1 when looking at reproductive potential, category 4 when looking at realised reproduction).

The credible intervals for all states of reproductive potential include values close to zero, suggesting that while ‘full potential’ has the highest probability, there remains considerable uncertainty (Supplementary Material Figure 7; full reproductive potential = 0.59, CIs = <0.01 – 0.97, reduced reproductive potential = 0.11, CIs = <0.01 – 0.63, male offspring only = 0.11, CIs = <0.01 – 0.45, completely sterile = 0.13, CIs <0.01 – 0.71, n $_{\text{species}}$ = 389).

However, when examining realised reproduction, the root state of ‘no offspring’ was far more likely than the others (full reproductive potential <0.01, CIs = <0.01 – 0.01, male offspring only = 0.03, CIs = <0.01 – 0.07, no offspring = 0.78, CIs = 0.50 – 0.99, n $_{\text{species}}$ = 368; Supplementary Material Figure 8).

We estimated that there have been a mean of 5 gains and 16 losses of worker sexual capacity in ants (transitions between full or reduced sexual capacity versus male production only or no offspring; categories 1 & 2 ←→ 3 & 4); and a mean of 17 gains and 20 losses of complete worker sterility (transitions between full or reduced sexual capacity or male production versus no offspring/ complete sterility; categories 1, 2, & 3 ←→ 4) (Figure 4). The evolution of decreased reproductive potential in workers was associated with larger colony sizes but this association was not significant (Supplementary Material Figure 3).

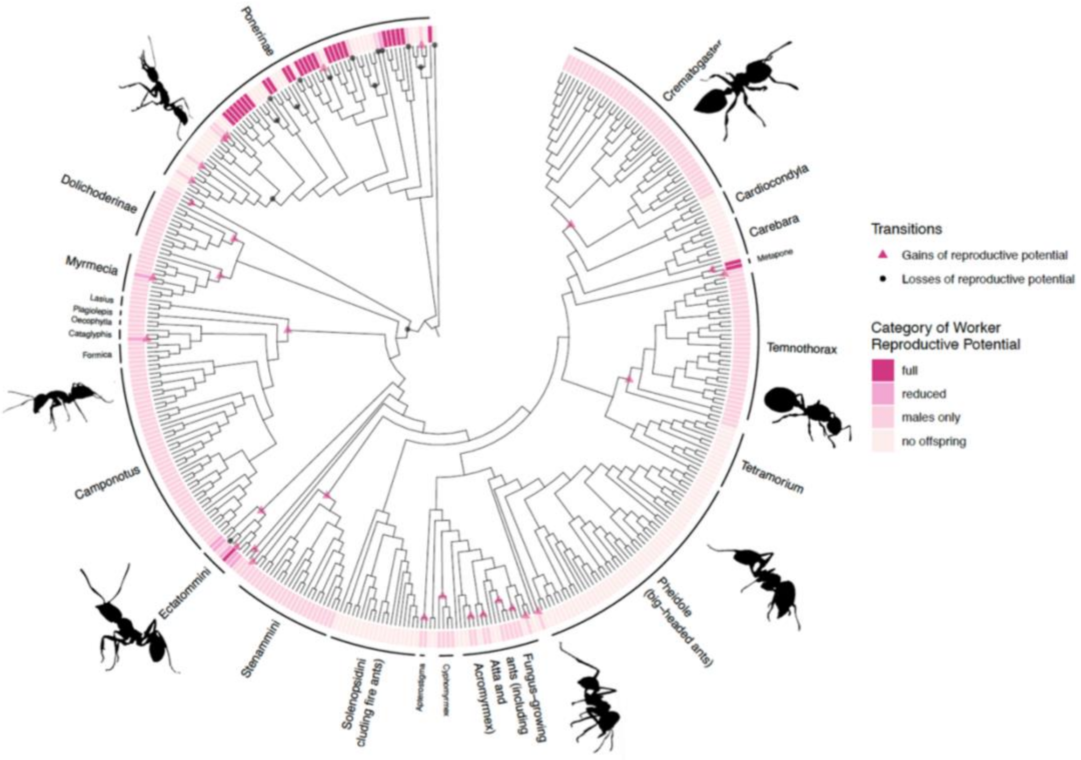


Figure 4. Ancestral state reconstruction of worker reproductive potential. We reconstructed worker reproductive potential as a four-level categorical variable using the all-rates-different model with two rate categories. Plot shows transitions of only one tree but we repeated across 400 possible phylogenies to calculate mean transition numbers (n species = 389).

Correlational Analyses. We found that ant species with less worker reproductive potential have larger colonies and more queens, but showed no correlation with queen mating frequency (BPMM: colony size and worker reproductive potential: pMCMC = 0.015, CI = 0.07 – 0.56, $n_{species} = 221$; queen number and worker reproductive potential: pMCMC = 0.04, CI = 0.02 – 0.81, $n_{species} = 98$; queen mating frequency and worker reproductive potential: pMCMC = 0.950, CI = -0.66 – 0.68, $n_{species} = 60$). Queen-worker size dimorphism was not a significant predictor of worker reproductive potential (pMCMC = 0.266, CI = -0.2765 – 1.0043, $n_{species} = 32$).

Phylogenetic Path Analyses. We found consistent support for the size-complexity hypothesis, but with a bidirectional relationship between colony size and worker reproductive potential. This suggests that larger colony sizes favoured the loss of reproductive potential, and the loss of reproductive potential favoured larger colony sizes (Figure 5A). This conclusion was robust across analyses with different levels of certainty (whether worker reproductive potential was inferred or described) or phylogenetic trees (Supplementary Material Datasheet tabs 4 & 4). In contrast, we did not find evidence of an effect of queen number on loss of worker reproductive potential. The results were similar when analysing loss of worker reproductive potential as binary, dividing species by which have workers that can mate and those with no worker mating (Supplementary Material Figure 4).

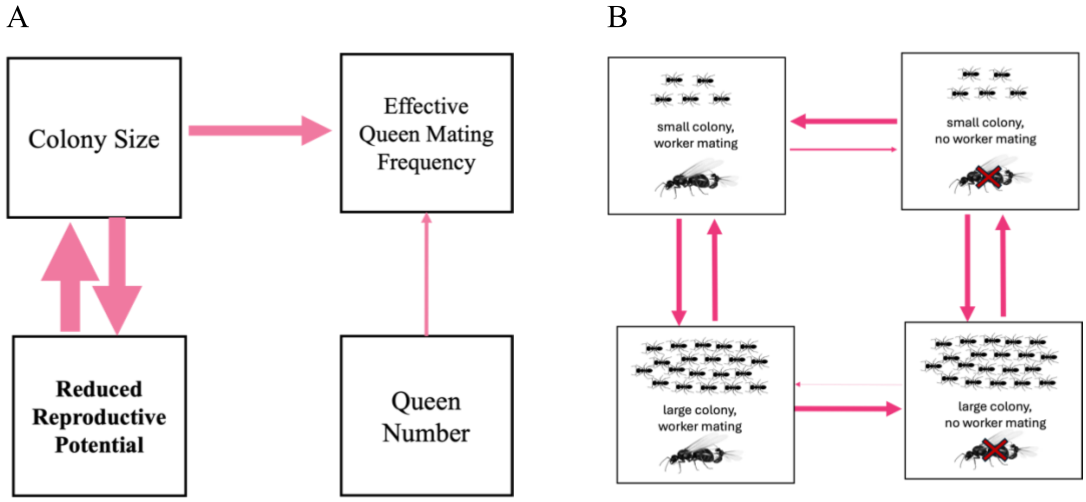


Figure 5. The best-supported causal models identified by phylogenetic path analysis and correlated evolution tests. (A) Path analysis. Best-supported causal model identified by phylogenetic path analysis: Reduced worker reproductive potential favours larger colony sizes, and larger colony sizes favour reduction of worker reproductive potential. We analysed reproductive potential as four categories (categories 1, 2, 3, 4; Figure 2). All other

variables are continuous. We display the results of our highest confidence data ($n_{species}$ = 44) and the FBD Crown MCC consensus tree, but we repeated the analyses for three others which can be found in Supplementary Material Datasheet. Arrows indicate the direction of the relationship between variables, with thicker lines indicating larger path coefficients. Dashed arrows would indicate a negative relationship, while unbroken lines indicate positive, **(B) Correlated evolution.** The best-supported causal models identified by correlated evolution tests: Larger colony sizes favour the loss of worker mating. Workers rarely lose sexual capacity in small colonies and rarely gain sexual capacity mating in large colonies. Values represent mean transition rates, with thicker arrows indicating higher rates. Here we display the results of our highest confidence data ($n_{species}$ = 124). Both reproductive potential and colony size are binarized, with reproductive potential analysed as (sex/no sex) and colony size as (below/above median colony size 214).

Correlated Evolution. We again found support for the size-complexity hypothesis with larger colony sizes having favoured reduced worker reproductive potential (Figure 4B). Correlated evolution analyses require worker reproductive potential to be analysed as a binary variable. This involved comparing species where the workers reproduced sexually (producing males and females, categories 1 & 2) or not (producing just males or no offspring, categories 3 & 4). We found evidence that larger colony sizes favoured the loss of worker sexual reproduction. This involved comparing species where the workers reproduced sexually (producing males and females, categories 1 & 2) or not (producing just males or no offspring, categories 3 & 4). The strength of the correlation between colony size and worker sexual reproduction was positive to strong (BF 2.6 – 5.6; Meade and Pagel, 2023). We found that ant workers more often lose the ability to reproduce sexually in large colonies, but rarely in small colonies (and the opposite with the gain of sexual reproduction). This was robust to different classifications of colony size and whether worker reproductive potential was explicitly described or inferred from genus-level information (Supplementary Material Datasheet tab 1)

In contrast, we found no evidence that loss of worker sexual reproduction influences colony size (Figure 4B). We also did not find evidence for colony size influencing a different aspect of reproductive potential (complete sterility) or for correlated evolution between queen number and worker reproductive potential. As an alternative analysis, we binarized reproductive potential by dividing between species where the workers could reproduce (categories 1-3) or were completely sterile (category 4). We found no evidence for correlated evolution between colony size and complete worker sterility, suggesting that the capacity for male production is not dependent on the size of the colony (median threshold BF = -5.7, mean threshold BF = -1.07). We also found no evidence for correlated evolution between queen number and worker sex or worker sterility, suggesting that the loss or gain of worker reproductive potential is not dependent on the number of queens in the colony (worker sex and queen number BF = -1.1, worker sterility and queen number BF= -3.8; Supplementary Material Datasheet tab 1).

Queen-Worker Size Dimorphism

We measured worker head size of 153 species, and queen head size of 132 species, and found that worker head size ranged from 0.29mm to 5.59mm, while queen head size ranged from 0.25mm to 5.26mm. After filtering, we have an overlap of 76 species for which we could calculate queen-worker dimorphism. We found that the reproductive size dimorphism, as measured by mean queen head width / minimum worker head width, varied from 0.84 (Harvester Ant *Veromessor pergandei*) to 7.03 (Driver Ant *Dorylus nigricans*; Figure 7B). Assuming that overall size is proportional to width^3 this means that means that the greatest dimorphism involves queens being approximately 350 times the overall size (volume) of workers.

Correlations. When looking at queen-worker size dimorphism, we found that ant species with greater queen-worker size dimorphism have larger colonies and higher effective mating frequency, while there was no significant correlation with the number of queens per colony (BPMMs: colony size pMCMC = <5e-04, CI = 0.03 – 0.08, $n_{species}$ = 54; queen mating frequency pMCMC = <5e-04, CI = 0.06 – 0.33, $n_{species}$ = 54; queen number pMCMC = 0.43, CI = -0.09 – 0.05, $n_{species}$ = 51). We found no correlation between worker reproductive potential and queen-worker size dimorphism (BPMM: pMCMC = 0.19, CI = -0.06 – 0.31, $n_{species}$ = 32). The significant correlations between queen mating frequency, colony size and reproductive dimorphism highlight the need to carry out analyses that can test the most likely causal explanation.

Correlations Between Potential Predictors. We found that colony size and effective queen mating frequency are correlated with each other, but that neither colony size nor mating frequency are correlated with queen number (colony size and mating frequency $\log_{10}$ MF pMCMC = 0.02, CIs = 0.19 – 1.96, $n_{species}$ = 57; colony size and queen number $\log_{10}$ colony size pMCMC = 0.81, CI = -0.08 – 0.06, $n_{species} = 91$; queen number and mating frequency queen number pMCMC = 0.19, CI = -0.12 – 0.64, $n_{species} = 57$).

Phylogenetic Path Analysis. We found strong support for the size-complexity hypothesis, with larger colony sizes favouring the evolution of increased queen-worker size dimorphism (Figure 6A). This result was consistent across phylogenetic trees. We also found a consistent but weak positive effect of queen mating frequency on size dimorphism.

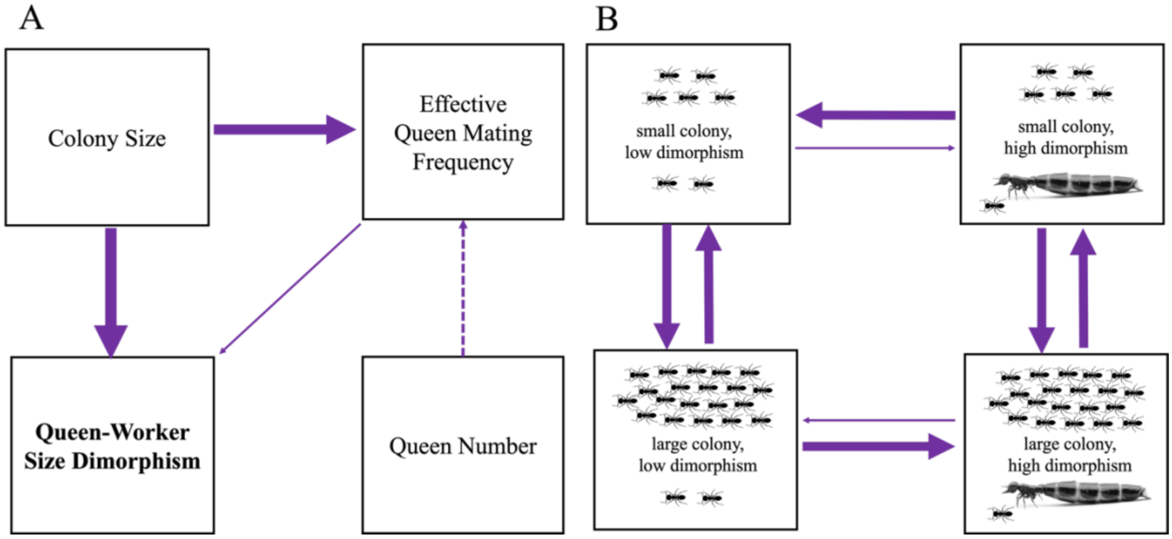


Figure 6. The best-supported causal models identified by phylogenetic path analysis and correlated evolution tests. (A) Path analysis. Larger colony sizes appear to have facilitated the increase of queen-worker size dimorphism. There is some evidence for a weak effect of queen mating frequency on size dimorphism. Here we display the results of one MCC consensus tree but repeated the analysis for three more which can be found in Supplementary Material. Arrows indicate the direction of the relationship between variables, with heavier lines indicating larger path coefficients. A dashed line indicates a negative relationship, while unbroken lines indicate positive relationships. **(B) Correlated evolution.** The co-evolution of queen-worker size dimorphism and colony size: larger colony sizes appear to have facilitated the increase of queen-worker size dimorphism. Arrows are weighted by mean transition rates, with heavier arrows indicating higher means.

Correlated Evolution. We again found support for the size-complexity hypothesis, with larger colony sizes favouring the evolution of increased queen-worker size dimorphism (Figure 6B).

The strength of the correlation between colony size and queen-worker size dimorphism was positive or strong depending on whether we used the median or the mean size dimorphism threshold for grouping species as having ‘large’ or ‘small’ size dimorphism (BF median threshold = 3.5 or BF mean threshold = 6.8, Supplementary Material Datasheet 1). This analysis required both colony size and queen-worker size dimorphism to be modelled as binary variables – we used the median to divide our data sets into binary but also repeated size with the mean to test the robustness of our conclusions (see methods). We found that ants rarely evolve greater queen-worker size dimorphism in small colonies, but more often do in large colonies. We also found that ants rarely lose high dimorphism in larger colonies but often do in small colonies. In contrast, we did not find support for a causal relationship between queen mating frequency and size dimorphism (BF = -1.9).

Ancestral State Reconstruction of Queen-Worker Size Dimorphism. When looking at the origins of queen-worker size dimorphism, we estimated a mean of 9 gains of high dimorphism, and 8 losses (Figure 7). The evolution of increased queen-worker size dimorphism was associated with larger colony sizes, but this difference was not significant (SI Figure 2). The most likely root state was high queen-worker size dimorphism (probability low = 0.49, probability high = 0.51, $n_{species}$ = 59, ‘low’ if dimorphism <= median of dataset, ‘high’ if > median; Supplementary Material Figure 9).

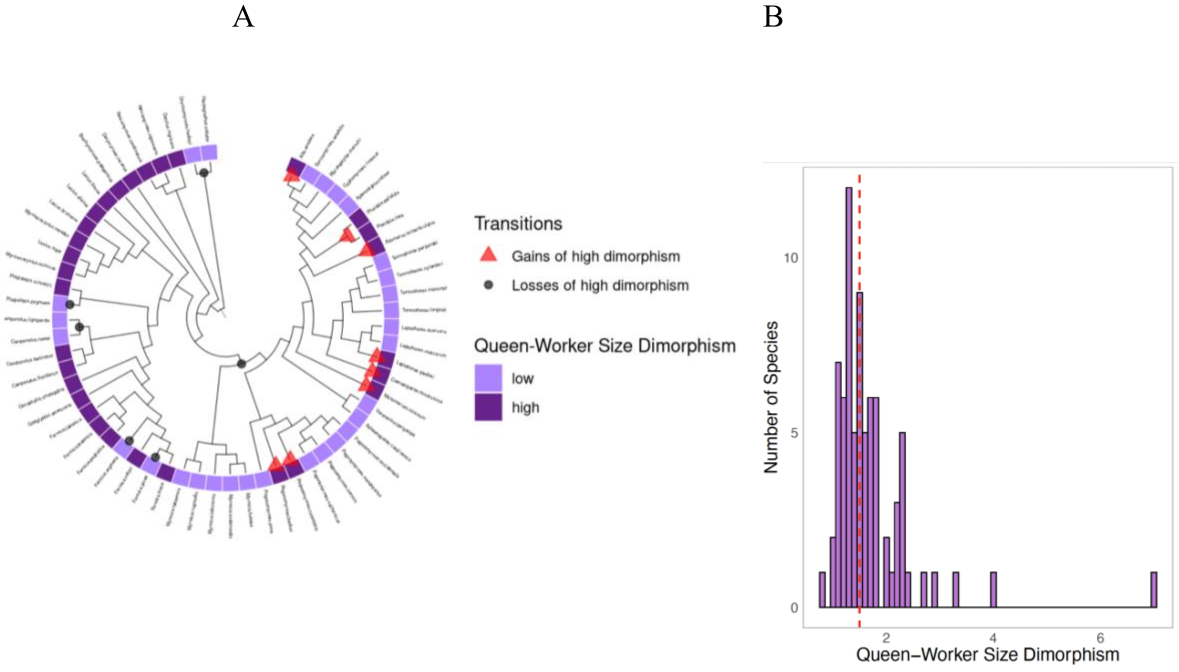


Figure 7. Queen-worker size dimorphism. (A) Ancestral state reconstruction of queen-worker size dimorphism. We reconstructed gains and losses of high size dimorphism using the equal rates model with one rate category. Plot shows transitions of only one tree, but we repeated across 400 possible phylogenies to calculate mean transition numbers ($n_{species} = 59$). **(B) Distribution of queen-worker head size dimorphism**. We calculated size dimorphism by mean queen head width divided by minimum worker head width ($n_{species} = 76$). Larger values indicate greater dimorphism, with queens relatively larger than workers. The median (1.50) is shown by a red dashed line.

Discussion

We found support for the hypothesis that increased colony size favoured greater division of labour between queens and workers in ants, examining both the loss of reproductive capacity in workers and queen-worker size dimorphism (Figures 5 & 6). Our analyses suggest that the 'ancestral ant worker' had full reproductive potential, with the ability to mate and produce either male or female offspring, though would not produce offspring in queenright colonies. The loss of reproductive potential appears to have then generally followed a sequential step-by-step process via a reduced ability to produce both males and females, then the loss of the ability to mate (male production only), and finally complete sterility (Figure 3A). Overall, the evolution of complete sterility has occurred around 17 times in ants, with 42% of examined species having completely sterile workers (Figure 3B). Analogously, the evolution of higher queen-worker size dimorphism, where queens can be up to 300 times size of workers, has occurred around 9 times in ants, and the common ancestor was more likely to have greater than average queen-worker size dimorphism.

The Ancestral Ant and Loss of Reproductive Potential

Our analyses suggest that the most recent common ancestor of ants likely had workers with full reproductive potential, consistent with recent findings (Meurville *et al.,* 2025). However, we also examined realised reproduction and found that these workers most likely produced no offspring in queenright colonies, despite their reproductive potential.

There was substantial uncertainty around our root state estimate of reproductive potential, indicating that further data or alternative modelling approaches with additional

Hymenopteran species may be needed to fully resolve that state. The closest relatives of the ant family are the Scolioidea and Apoidea (Peters *et al.,* 2017). All Scolioid wasps are solitary, and the most basally divergent lineage of Apoidea is Ampulicidae, which is also solitary (Melo, 2021). By examining realised reproduction as well as reproductive potential, we show that although the ability to reproduce was lost gradually, workers stopped reproducing earlier, and were already effectively sterile (Boomsma, 2022; Linksvayer & Johnson, 2019).

How Was Reproductive Capacity Then Lost in Ants?

We found strongest support for a model of evolution where the loss of worker reproductive capacity usually occurs in a sequential manner: first reduced capacity for sexual reproduction, then the production of males only and then complete sterility (Figure 3). Ancestral state reconstruction suggested that workers have lost the ability to reproduce sexually 16 times (gained 5 times), and that complete sterility has evolved 17 times (lost 20 times) (Figure 4). Both phylogenetic path analysis and correlated evolution analyses supported the hypothesis that larger colony sizes favoured this loss of worker reproductive potential (Figure 4). The path analysis further suggested that this relationship was bidirectional, with the loss of worker reproductive potential having also favoured larger colony sizes (Figure 4A).

Colony Size and Queen-Worker Size Dimorphism

Both phylogenetic path analysis and correlated evolution analyses supported the hypothesis that larger colony sizes had favoured increased queen-worker size dimorphism (Figure 6). Our ancestral state reconstruction suggested that higher queen-worker size dimorphism has evolved 9 times and been lost 8 times (Figure 7).

Matte & LeBoeuf (2025) have also found a relationship between larger colony sizes and queen-worker size dimorphism but could not determine whether larger colony sizes favoured increased size dimorphism or the reverse. There are a number of differences in the analytical methods that could explain this difference including: (a) different datasets - although we expect broad similarity as they incorporated data from the GAGA project which we had contributed to and cross-checked with (Bell-Roberts et al., 2024; Boomsma *et al.,* 2017); (b) different variables – they also included larval passiveness, adult diet, and worker polymorphism, whereas we included queen number and queen mating frequency; (c) different analysis methods - they used only phylogenetic path analysis whereas we used multiple methods to test robustness; (d) differences in number of phylogenetic trees used – we used 400 for all analyses except for path analysis, where we used four maximum clade credibility trees, whereas Matte & LeBouef (2025) used 200 posterior trees for path analysis using Multitree phylogenetic path analysis; (e) differences in the possible evolutionary models considered.

The Size-Complexity Hypothesis

There is now support for the hypothesis that larger colony sizes have favoured an increase in both reproductive and non-reproductive division of labour in ants. Examining reproductive division of labour, larger colony sizes appear to have favoured both a reduced reproductive potential (Figure 5) and greater queen-worker size dimorphism (Figure 6). Examining non-reproductive division of labour, we have previously found support for the hypothesis that larger colony sizes favoured more worker castes (Bell-Roberts *et al.,* 2024).

There are several potential hypotheses for why larger colony sizes would select for increased division of labour. In larger colonies, there is a lower chance for each worker to replace the queen if she dies, which could reduce the potential cost of becoming specialised to perform helping functions (Bourke, 1999; Bourke, 2011). Larger colonies may require a greater number of tasks, favouring increased specialisation (Bourke, 2011; Ferguson-Gow *at al.,* 2014). In larger colonies there is a reduced chance of stochastically having a lower than optimal number of individuals performing a task (Cooper *et al.* 2022; Liu *et al.* 2021). Larger colonies may be more likely to be associated with non-predatory diets that allow workers greater control of the allocation of resources to offspring (Matte & LeBoeuf, 2025). A combination of comparative and experimental methodologies may be required to test these different hypotheses.

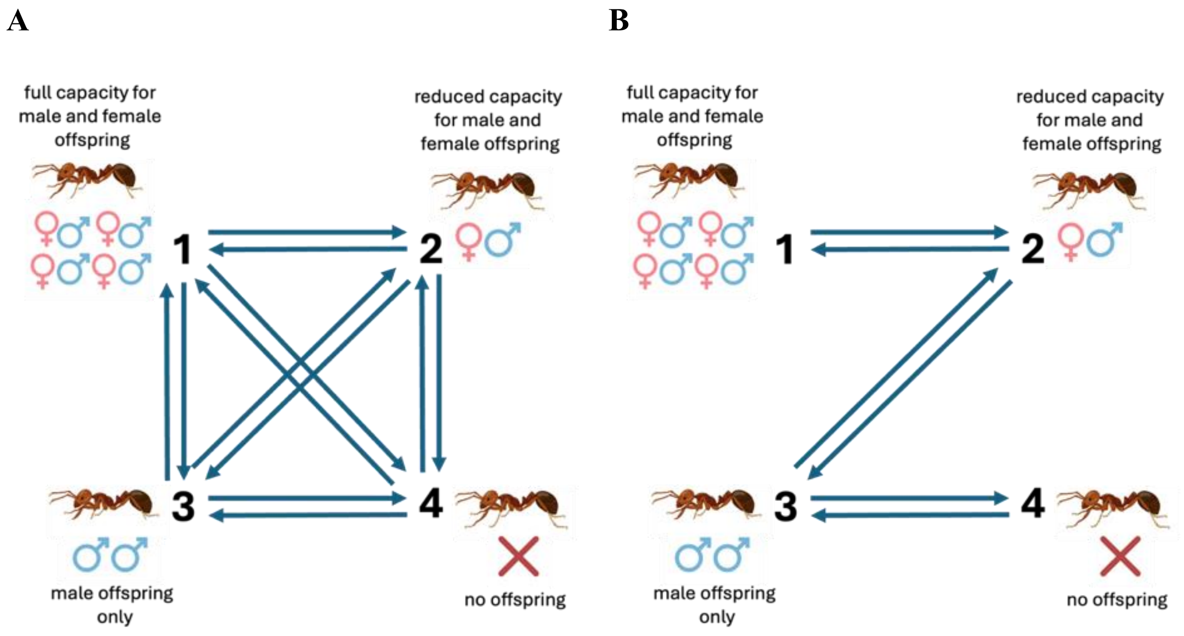
Queen Number and Reproductive Division of Labour

We found weak support for an influence of queen number on size dimorphism and worker reproductive potential. Overall, the relationship between queen number and reproductive division of labour was inconclusive, with different methods producing conflicting results. Phylogenetic path analysis indicated that higher queen number favours the loss of worker sexual reproduction, but that it is not important for overall reduced reproductive potential. Ancestral state reconstructions revealed that differences in queen number were not significant but were associated with a general trend towards fewer queens preceding the loss of worker sex (SI Figure 3 E). This result may reflect the increased uncertainty associated with this method which uses all 400 posterior trees, compared to averaged trees used in path analysis and the subset of the 400 used for tests of correlated evolution. In contrast, the tests for correlated evolution identified co-evolution between worker sex and colony size, but not between worker sex and queen number, or between complete worker sterility and either colony size or queen number (Figure 4B).

Conclusions

We have provided clear support for how colony size can influence the evolution of reproductive division of labour in ants, but there are at least three open questions: First, in ants, what came first – reproductive (sterility and queen-worker dimorphism) or non-reproductive (worker castes) division of labour (Molet *et al.,* 2012). Second, does colony (or group) size play a similar role in other insect lineages, including those that have not undergone a major transition to superorganismality? Third, does the number of cells have an analogous causal influence on division of labour in multicellular groups?

Supplementary Material



Supplementary Material Figure 1. Modelling evolutionary transitions in worker reproductive potential. (A) The ‘free’ model. All transitions between states are equally possible **(B) The ‘sequential’ model**. Non-sequential transitions are restricted to zero.

Ancestral State Reconstructions

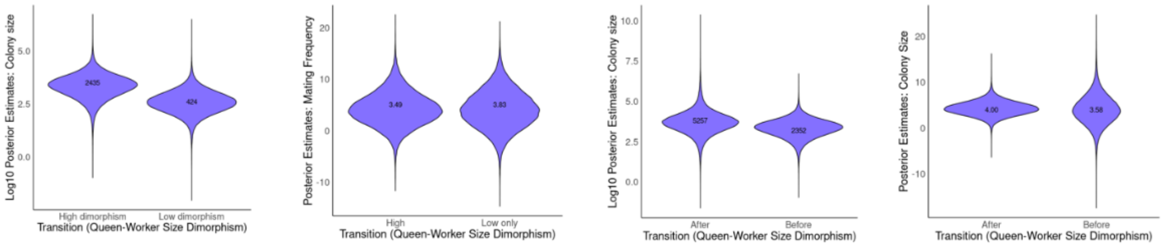
1. Colony size and queen-worker size dimorphism

We found no evidence that ancestral colony sizes were larger in lineages that transitioned to a state of high queen-worker size dimorphism when compared to lineages that did not have this transition (high dimorphism: posterior mode = 2435, HPD = 135 – 24054; low dimorphism retained: posterior mode = 423, HPD = 33 – 4287, $p_{\text{high vs low}} = 0.84$; Figure SI 1A). When looking at colony size before and after transitions to high dimorphism, we found no evidence that colony size may increase following transitions to high dimorphism (before high dimorphism: posterior mode = 2352, HPD = 134 – 24334; after high dimorphism: posterior mode = 5257, HPD = 138 – 147967, $p_{\text{after vs before}} = 0.67$; Figure SI 1B).

2. Queen mating frequency and queen-worker size dimorphism

We compared queen mating frequency in lineages that transitioned to high queen-worker size dimorphism with lineages where they remained in a state of low size dimorphism and found that there was no significant difference in queen mating (high dimorphism: posterior mode = 3.49, HPD = -2.29 – 10.72; low dimorphism: posterior mode = 3.83, HPD = -3.06 – 11.38, p transition to high > low = 0.50; Figure SI 1 C). We also compared queen mating frequency before and after transitions to high dimorphism within the lineages that had this transition and again found no significant difference (before: posterior mode = 3.58, HPD = -2.29 – 10.37; after: posterior mode = 4, HPD = 1.22 – 7.31, p after > before = 0.55; Figure SI 1 D).

a b c d



Supplementary Material Figure 2. Ancestral state reconstructions of colony traits and queen-worker size dimorphism. **(A)** Colony size in lineages that transitioned to greater size dimorphism compare dot lineages that retained smaller size dimorphism, **(B)** Colony size before and after transitions to greater size dimorphism, **(C)** Queen mating frequency in lineages that transitioned to greater queen-worker size dimorphism compared to lineages that retained smaller size dimorphism, (D) Queen mating frequency before and after transitions to greater queen-worker size dimorphism.

3. Colony size and worker sex

We found no evidence that colony sizes may be larger in lineages that lost worker sex when compared to those that retained sex (sex lost: posterior mode = 708, HPD = 25 – 19,957; sex retained: posterior mode = 252, HPD = 9 – 5650, $p_{\text{lost vs retained}}$ = 0.14; Figure SI 2A). We also compared colony size before and after losses of sex (‘sex.both’ vs no.sex.only.no.sex’) to test the alternative hypothesis and see if colony sizes increase following losses of sex, but again there was no significant difference (before loss: posterior mode = 708, CI = 25 – 19957; after loss: posterior mode= 1158, CI = 72 – 233690, $p_{\text{after vs before}}$ = 0.28; Figure SI 2B).

4. Colony size and worker sterility

Colony sizes may be larger when there are transitions to worker sterility than in lineages that retained some reproductive potential, but this difference was not significant (transitions to sterility: posterior mode = 2069, HPD = 57 – 90556; non-sterile: posterior mode = 596, HPD

= 32 – 10325, $p_{\text{sterility vs no sterility}}$ = 0.87; Figure SI 2C). When looking at colony size before and after transitions to sterility, we found no evidence that colony sizes are larger after transitions to sterility than before transitions (before transitions to sterility: posterior mode = 2069, HPD = 57 – 90556; after transitions to sterility: posterior mode = 1540, HPD = 78 – 50707, $p_{\text{before vs after sterility}}$ = 0.48; Figure SI 2D).

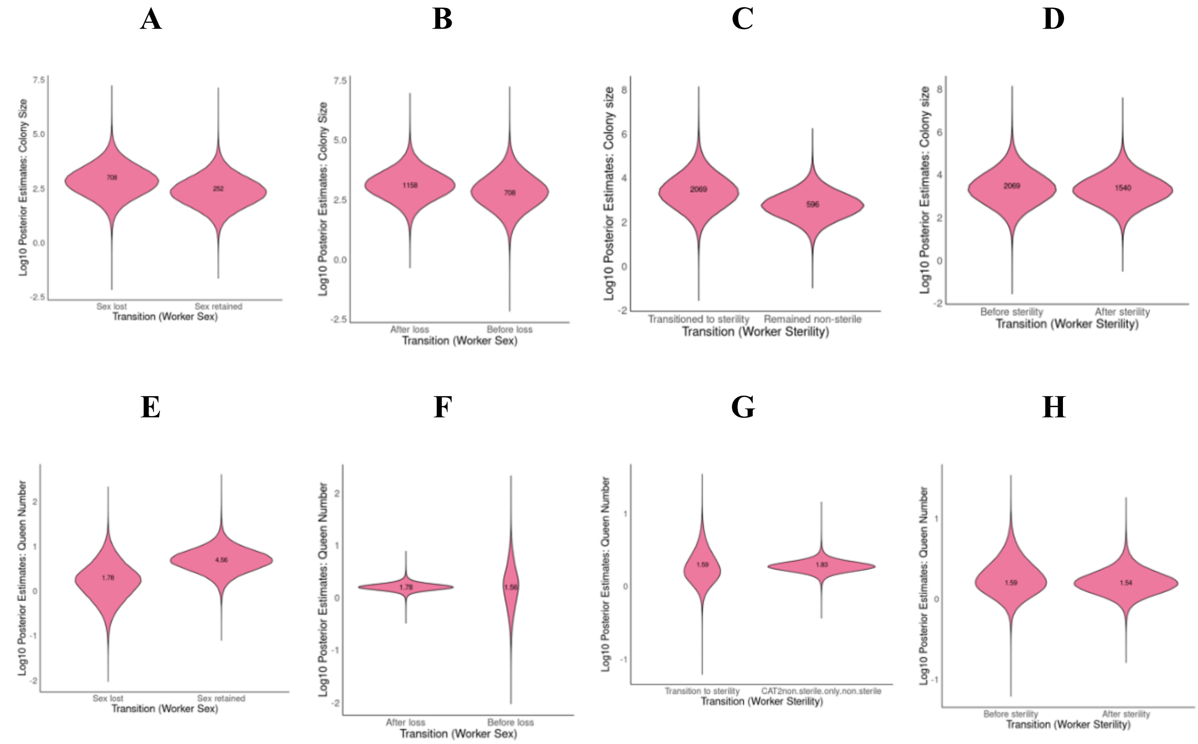
5. Queen number and worker sex

We found no evidence that there may be fewer queens per colony in lineages where workers lost their sexual capacity when compared to lineages where sexual capacity was retained (sex lost: posterior mode = 1.78, HPD = 0.23 – 10.70; sex retained: posterior mode = 4.56, HPD = 1.37 – 16.36, $p_{\text{sex retained > sex lost}}$ = 0.18; Figure SI 2E). We then compared queen numbers in lineages that lost worker sexual capacity before and after this loss, and found no significant difference, although lineages that lost sex had a much wider confidence interval than those that only had no sex (loss of sex: posterior mode = 1.78, HPD = 0.23 – 10.70; only no sex (after loss): posterior mode = 1.56, HPD = 1.15 – 2.26, $p_{\text{loss of sex > only no sex}}$ = 0.49; Figure SI 2F).

6. Queen number and sterility

We found no evidence that there were fewer queens in lineages that had transitions to worker sterility when compared to lineages that remained non-sterile (transitions to sterility: posterior mode = 1.59, CI = 0.70 – 5.35; remained non-sterile: posterior mode = 1.83, CI = 1.29 – 2.75, $p_{\text{non-sterile vs sterile}}$ = 0.45; Figure SI 2G). When comparing queen number before vs after transitions to worker sterility to see if queen number may decrease as a result of worker sterility, we also found no significant difference (before sterility: posterior mode = 1.59, CI =

0.70 – 5.35; after sterility: posterior mode = 1.54, CI = 0.79 – 3.36, p $_{\text{before vs after}}$ = 0.43; Figure SI 2H).

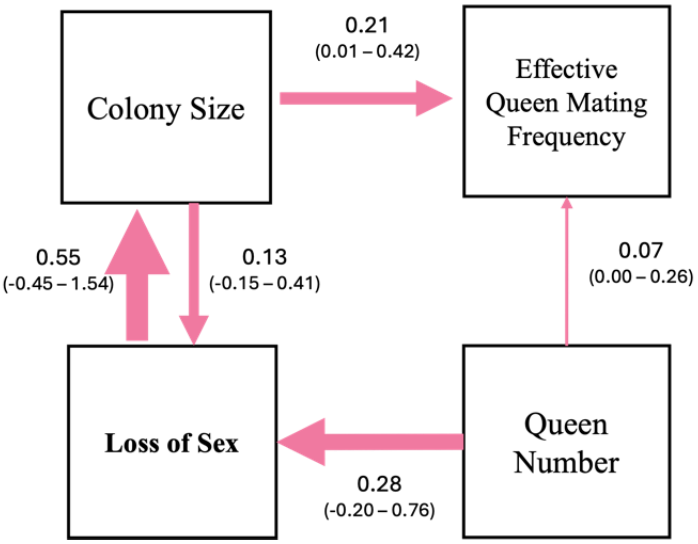


Supplementary Material Figure 3. Ancestral state reconstructions of worker reproductive potential. (A - H) Reconstructions of ancestral colony size and queen number at points of gains or losses of worker reproductive potential.

Phylogenetic Path Analysis

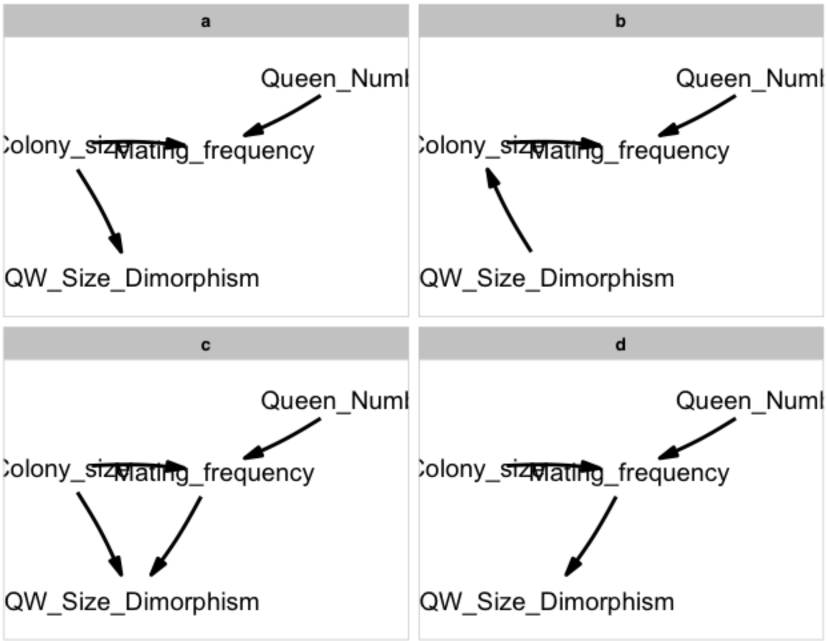
7. Worker sex analysed as a binary variable

We also analysed reproductive potential as binary sex/no sex (categories 1 & 2 vs. 3 & 3) to see if there are distinct factors influencing the loss of worker sex that would not be important in overall loss of worker reproductive potential. We found consistent evidence that loss of sex favours increased colony size, with the opposite causal direction also possible, although this direct was not as strong or consistent (Supplementary Material Figure 4). We found that this time, queen number was important: More queens per colony favours the loss of worker sex. All models showed this relationship, remaining consistent across subsets and phylogenetic trees (Figure 7B).



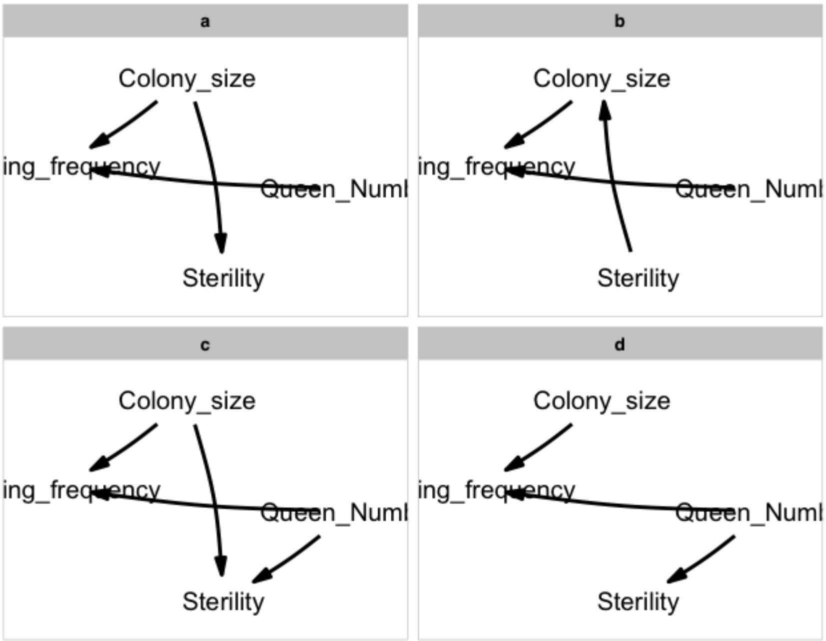
Supplementary Material Figure 4. Queen number favours the loss of worker sex, and loss of sex favours larger colony sizes, with causality potentially existing in the opposite causal direction. Worker reproductive potential was analysed as binary sex/no sex (full or reproduced sexual capacity vs. males only or no offspring; categories 1 & 2 vs. 3 & 4; Figure 2). All other variables are continuous. Here we display the results of our highest confidence data ($n_{\text{species}} = 44$) and the FBD Crown MCC consensus tree. Values represent standardized path coefficients with 95% confidence intervals. Arrows indicate the direction of the relationship between variables, with heavier lines indicating larger coefficients.

8. <u>Alternative causal models tested: Queen-worker size dimorphism</u>.



Supplementary Material Figure 5. The alternative causal models we tested using phylogenetic path analysis, based on the results of our correlational tests. We alternate the paths of influence to test whether a) colony size is responsible for increases in size dimorphism, b) size dimorphism is responsible for increases in colony size, c) colony size and queen mating frequency are both responsible for increases in size dimorphism, or d) only queen mating frequency is responsible for increases in size dimorphism.

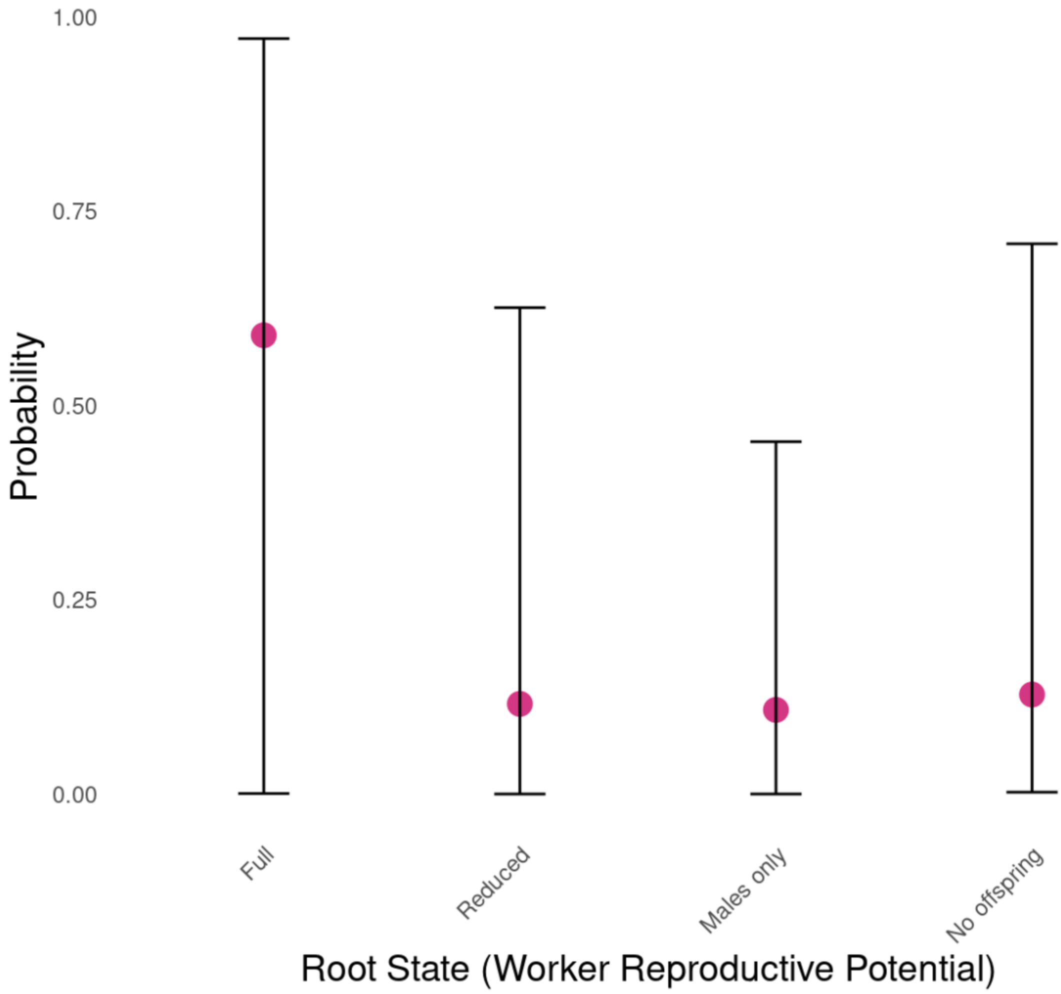
9. <u>Alternative causal models tested: Worker reproductive potential.</u>



Supplementary Material Figure 6. The alternative causal models we tested using phylogenetic path analysis, based on the results of our correlational tests. We alternate the paths of influence to test whether a) colony size is responsible for reduced worker reproductive potential (sterility), b) worker sterility is responsible for increases in colony size, c) colony size and queen number are both driving worker sterility, or d) only queen number is driving worker sterility.

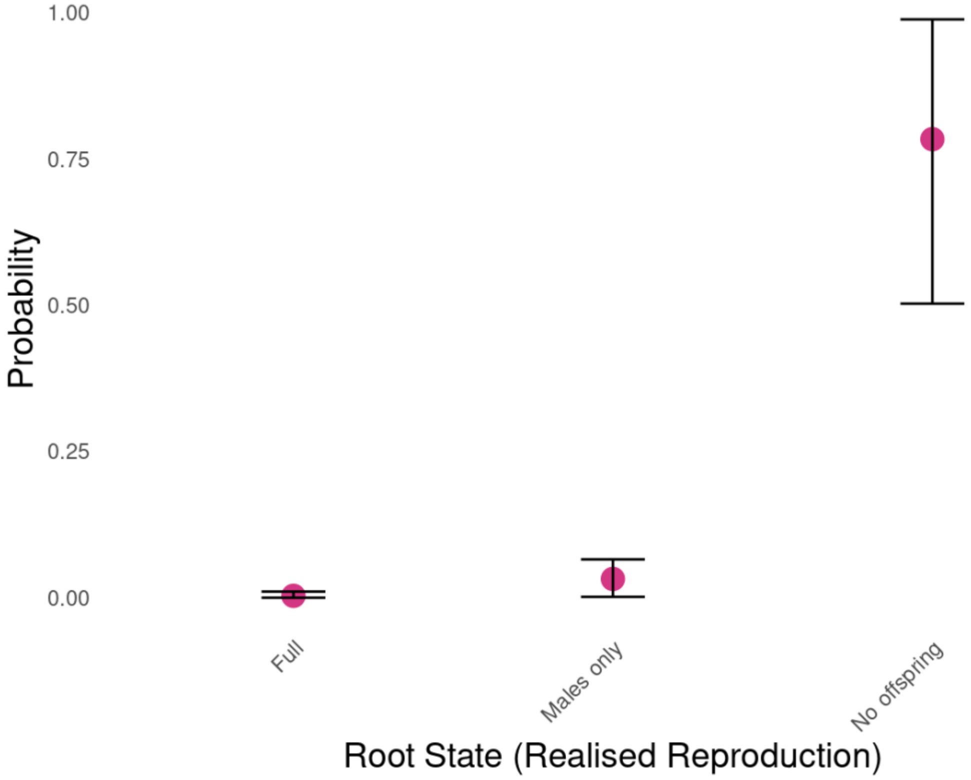
Root state estimates

10. Root state of worker reproductive potential.



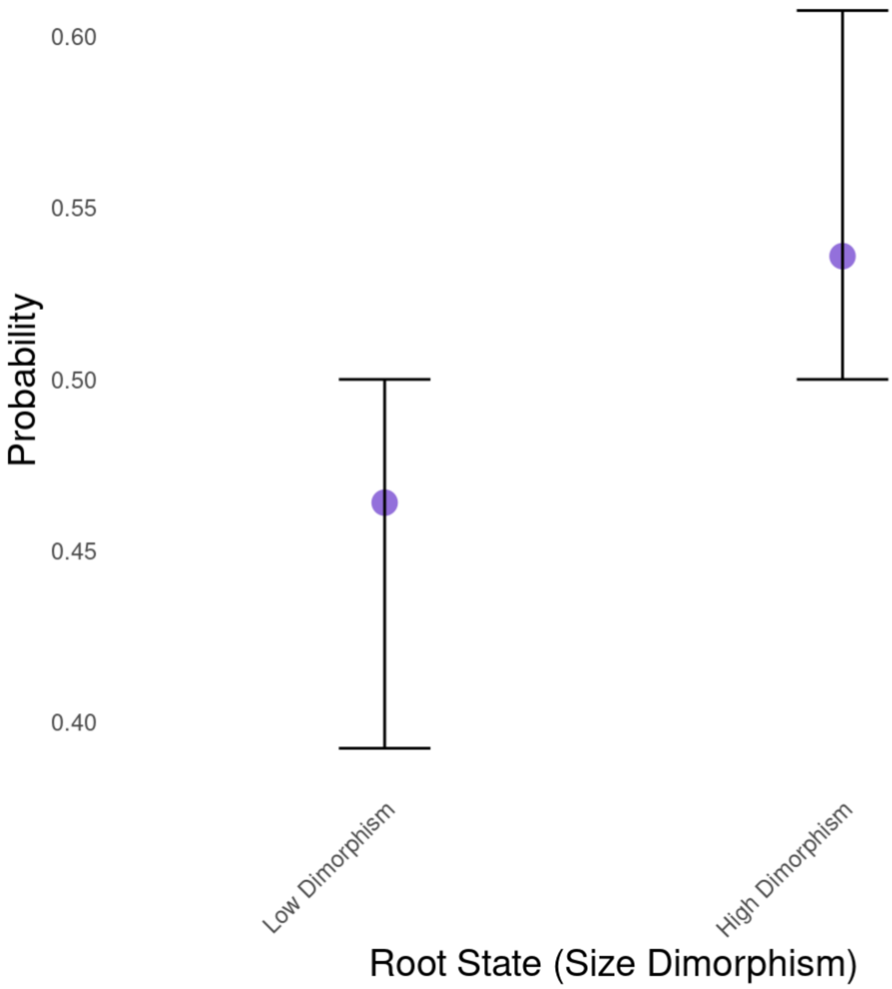
Supplementary Material Figure 7. The ancestral ant worker most likely had full reproductive potential, though all confidence intervals contain values close to zero, reflecting substantial uncertainty.

11. Root state of worker realised reproduction.



Supplementary Material Figure 8. It is very likely that ancestral workers were effectively sterile, producing no offspring in the presence of a queen or gamergate queen.

12. Root state of queen-worker size dimorphism



Supplementary Material Figure 9. The ancestral ant most likely had high queen-worker size dimorphism.

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Chapter 5: No evidence that mode of group formation predicts helper mating in Hymenoptera

Abstract

Examining 582 species of Hymenoptera, I test the prediction that reproductive division of labour is more likely to evolve in species with mother-offspring ("subsocial") group formation than those with same-generation ("semisocial") group formation. I found that 43%

of Hymenopteran species with subsocial group formation have lost helper mating, compared to only 8% of species with semisocial group formation. However, when controlling for shared ancestry in a phylogenetic regression, I found that this relationship was not significant. Results indicate that helper mating is highly phylogenetically clustered, and that variance in helper mating is explained mostly by shared ancestry rather than by group formation. Although the dataset spans a broad taxonomic range, variation in helper mating was insufficient to yield high statistical power. To more effectively test the relationship between group formation and reproductive division of labour, future work should examine reproductive skew or another measure that captures the subtler differences in reproductive division of labour across Hymenoptera.

Introduction

Complex life has evolved through a small number of major evolutionary transitions, in which individuals that could once replicate independently now can only replicate as part of a larger, more complex entity (West *et al.,* 2015). This applies to cells in multicellular bodies, and to insects in superorganismal colonies. In each case, formerly autonomous individuals lost self-sufficiency and, over evolutionary time, became irreversibly part of a larger whole. Consequently, a major question in evolutionary biology is to understand why these major transitions occurred. More specifically, what would lead individuals to give up their own reproductive potential and become sterile helpers?

Relatedness is predicted to influence whether an individual will act altruistically or selfishly. Helping is favoured when relatedness of the recipient to the actor (r) and the additional reproductive benefit gained by the recipient (B) is greater than the reproductive cost (C) to the individual performing the act ($r B > C$, Hamilton, 1964. The way that groups form affects the relatedness between individuals and is therefore predicted to influence the evolution of helping behaviours: Groups formed by offspring staying with their mother (subsocial group formation) have very high relatedness, and the adult offspring are more likely stay and rear their younger sisters instead of dispersing to rear their own daughters (sisters $r = 0.75 >$ daughters $r = 0.50$; Figure 1 A). In contrast, when groups are formed by individuals of the same generation coming together (semisocial group formation), the brood may be unrelated to some of the adults in the nest. At best, they may be nieces or nephews if the foundresses were sisters. Either way, relatedness is lower, and females are more likely to rear their own offspring (nieces r = 0.375 < daughters r = 0.50; Figure 1 B).

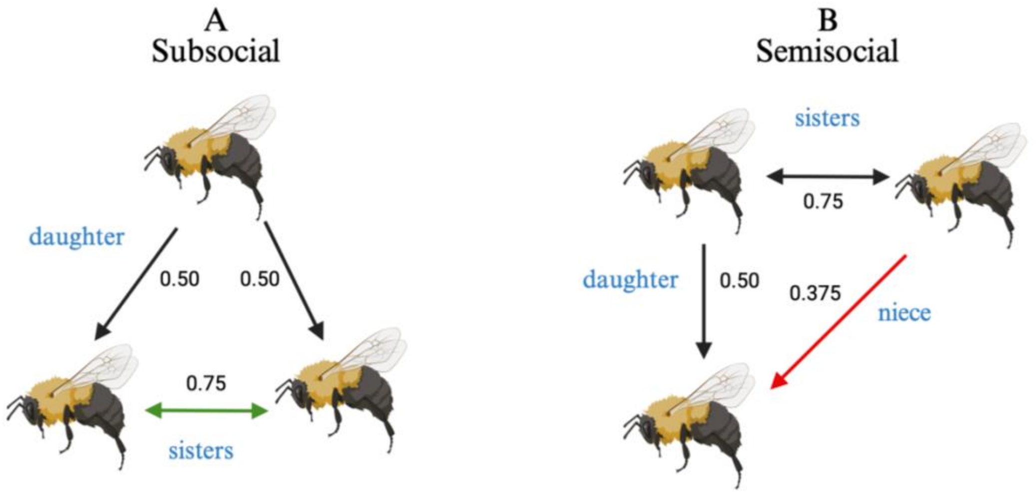

Figure 1. Insect groups can be formed in two main ways. (A) Groups formed by mother-daughter overlap have very high relatedness and so adult females may be more likely to show cooperation by helping rear their younger sisters instead of dispersing to rear their own offspring. **(B)** Groups formed by same-generation females, whether related or not, will have lower levels of relatedness overall: Helping behaviours may be less likely as females choose to rear their own offspring instead of nieces, nephews, or nonrelatives. The green arrow indicates high relatedness where an individual is predicted to exhibit helping behaviours, while the red arrow indicates low relatedness where helping behaviours are predicted to not evolve.

Previously, Reeve and Keller (1995) compared reproductive skew within 17 species of Hymenoptera and found that skew was greater when groups were matrifilial (mother-daughter associations) than when they were semisocial (same-generation e.g. siblings). Similar work on birds found that there was a clearer reproductive divide in groups formed through retention of offspring, while those formed through the aggregation of unrelated adults are smaller and lack specialisation (Downing *et al.,* 2020). Another study found that algal colonies are more likely to transition to multicellularity if they formed by cells staying with clonal parents compared with those aggregating with potential non-relatives (Fisher et al., 2013).

To date, no study has phylogenetically tested the relationship between group formation and cooperation in insects; a clade which includes extreme differentiation into reproductive and non-reproductive roles, with a physical loss of reproductive potential that is not found in groups such as birds (West *et al.,* 2015). This physical loss of reproductive potential is significant as it is analogous to the differentiation of cells into germ and soma in multicellular organisms. Group formation affects the relatedness of helpers to the brood they help rear and is predicted to influence reproductive division of labour across taxonomic groups and across different levels of biological organisation (Fisher *et al.,* 2013; Downing *et al.,* 2020; Kapsetaki *et al.,* 2017; Reeve & Keller, 1995). Hymenoptera (ants, bees, and wasps) show substantial variation in their degree of cooperation, from species that are completely solitary-living species (e.g. Megachilid leafcutter bees), to species with obligate colonial nesting (e.g. honeybees). Among the species that do live in groups, there is variation both in the amount of cooperative behaviour displayed, and their mode of group formation. In this study, I use comparative phylogenetic methods to test whether mode of group formation (subsocial vs semisocial) predicts the presence of helper mating in 582 species of Hymenoptera.

Methods

To test whether mode of group formation predicts the presence of helper mating in Hymenoptera, I ran a series of phylogenetically controlled regressions across 582 species of ants, bees, and wasps. I collected data from the literature and built a species-level supertree using 32 small, species-level trees and one family-level backbone tree.

Data collection

I compiled information on group formation and helper mating from the literature, primarily from textbooks such as The Social Behaviour of the Bees (Michener, 1974), The Solitary Bees (Danforth, Minckley, & Neff, 2019), The Evolution of Social Wasps (Hunt, 2007), The Biology of Social Wasps (Ross & Matthews, 1991), and Evolution of the Nests of Bees (Michener, 1964). Additionally, any species described as communal could be inferred to have helper mating as this is explicit in the definition of communal nesters (Michener, 1974). I incorporated data on ant reproductive potential from Chapter 4 of this thesis, along with information on broader insect sociality taken from an unpublished dataset created by Rosa Bonifacii. I had an overlap of group formation and helper mating data for 582 Hymenopteran species: 439 ants, 45 bees, and 98 wasps.

Classifying helper mating and group formation

Categorisation of helper mating was based on whether they have been recorded to mate in natural conditions. Several ant genera have gamergate workers with physical capacity for sexual reproduction. In natural colony settings, reproduction of these helpers would be supressed by the dominant reproductive. However, the helpers may have the opportunity to reproduce if the dominant reproductive dies, triggering aggressive fights and a hierarchical shift among helpers over becomes the replacement reproductive (Hölldobler & Wilson, 1990). I classified those species as having helper mating. Many bees and wasps retain reproductive organs such as the spermatheca but will not mate even in the case of queen death (Bell & Sumner, 2023; Holland & Bloch, 2020; Owen & Plowright, 1982; Bloch et al., 1996; Bloch & Hefetz, 1999; Brown et al., 2003; Takahashi et al., 2008). I classified these species as having no helper mating.

Subsocial. I classified species as subsocial if there are records of their groups forming via parent-offspring overlap, if they are described as 'matrifilial' (mother-offspring associations), or if they had a single foundress (Michener, 1971).

Semisocial. I categorised species as being semisocial if their groups were described as same generation, 'non-matrifilial', or initiated by multiple foundresses. This includes groups of non-relatives and groups of siblings. For ants, I categorised species with descriptions of primary polygyny as having semisocial group formation and assumed that all other ant species have groups formed subsocially (Hölldobler & Wilson, 1990; Hölldobler & Wilson, 1977). Primary polygyny describes cases where colonies are formed by multiple foundresses that remain alive long enough to reproduce and create a colony with multiple queens. Where multiple ant foundresses share a nest, this usually leads to them fighting to the death until only one remains alive to reproduce. Consequently, primary polygyny is very rare (Hölldobler & Wilson, 1977).

Previous researchers have defined 'semisocial' groups as either unrelated individuals coming together (Downing et al., 2020), or as the aggregation of any individuals of the same generation including sisters (Reeve & Keller, 1995). Whether formed by nonrelatives or sisters, these groups have lower relatedness than those formed via the subsocial route, hence our decision to combine them and test the role of parent-offspring overlap in the evolution of cooperation (Figure 1).

Species excluded or specially indicated

Some Hymenopteran species have lost their ability to mate but are swarm-founding so would not fit into either the subsocial or semisocial category of group formation (e.g. the Western Honeybee *Apis mellifera*; Peled et al., 2024; Wilson, 1971). I have excluded these from the analyses. Other species have descriptions of group formation that are ambiguous and it is not clear whether they should be considered subsocial or semisocial – I refer to these as the 'natal' species, as descriptions state that groups are formed when females remain on their natal nest (Danforth, 1989; Zucchi et al., 1969; Ayasse et al., 1990). These records don't tell us whether the mother is alive when this happens, in which case there is generational overlap (subsocial group formation) or if the mother is already dead before her vacant nest is inherited by her daughters (semisocial group formation). I indicated this in the dataset so that I could either exclude them or test how results changed depending on whether these species were classed as subsocial or semisocial. Finally, a number of species have both subsocial and semisocial group formation which is expressed facultatively. I grouped these species into a fourth distinct category which I excluded from the following analyses.

Creation of the Hymenoptera supertree

To control for similarity through shared evolutionary history, a species-level phylogeny for Hymenoptera is necessary. However, no such phylogeny yet exists. To resolve this issue, I created a Hymenoptera supertree: First, I built a backbone tree using the family-level Hymenopteran phylogeny produced by Blaimer *et al.* (2023). I pruned the backbone tree to only include one representative per family, then grafted on smaller, species-level phylogenies from a number of studies to get greater resolution at the tips. I used Open Tree of Life portal (opentreeoflife.org) to combine these sub-trees and create a species-level tree for all Hymenoptera. The resulting supertree is therefore a combination of 33 published phylogenies, and can be found at:
https://tree.opentreeoflife.org/curator/collection/view/jfturner6/hymenoptera

Phylogenetic regression

All analyses were performed in R version 4.2.3. I ran Bayesian Phylogenetic Mixed Models (BPMMs) with Markov chain Monte Carlo (MCMC) estimation, using the *MCMCglmm* package version 2.34 (Hadfield, 2010). Models were run for a minimum of 1 million iterations, with a burnin of 300,000 and a thinning interval of 1,000 To ensure model convergence, I used Gelman and Rubin's convergence test (Gelman & Rubin, 1992). I modelled both group formation and helper mating as categorical variables (family = "categorical").

Results

Summary

I found that 43% of Hymenopteran species with subsocial group formation have no helper mating, compared to only 8% of species with semisocial group formation, suggesting that there is some kind of relationship between group formation and helper reproductive capacity (Figure 2).

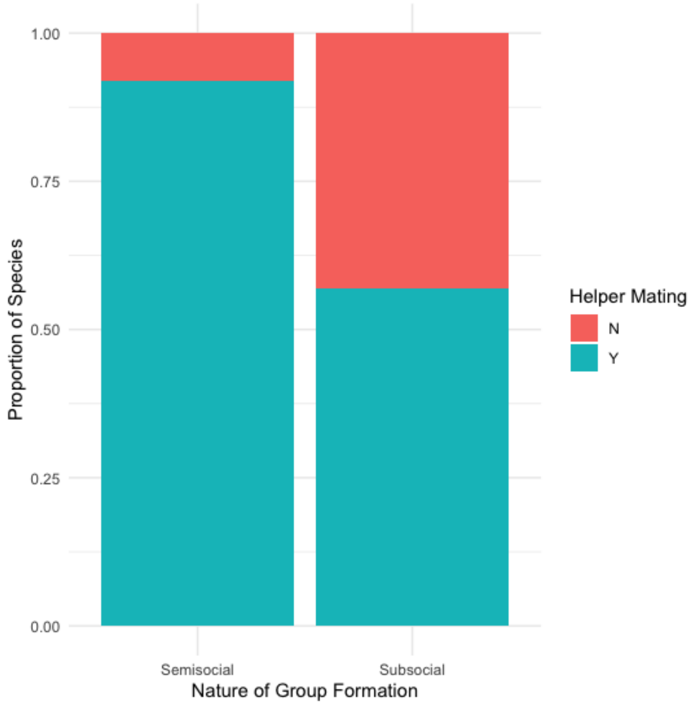

Figure 2. Helper mating is less common in species with subsocial group formation. Proportion of species with helper mating, categorised by whether they have same-generation (semisocial) or mother-offspring (subsocial) group formation (n species = 130). Here I do not include ants, species with ambiguous group formation (offspring stay at natal nest but it is not known if the mother remains alive), or swarm-founding species.

Phylogenetic regression

To test whether the apparent relationship between group formation and helper mating was still present after controlling for shared ancestry, I ran a series of phylogenetic regressions. I found that when controlling for phylogeny, group formation does not significantly predict helper mating. I repeated the analysis with ambiguous species classified differently and analysed both with and without ant species included. In all cases, shared ancestry (phylogenetic relationship) explained most of the variation in helper mating, and group formation was not a significant predictor (‘natal’ species classed as semisocial: posterior mean = -0.22, CI = -20.98 – 21.24, pMCMC = 0.88; ‘natal’ species classed as subsocial: posterior mean = 2.10, CI = -24.59 – 38.36, pMCMC = 0.92, n species = 130; ants added, and natal’ species are subsocial: posterior mean = -1.32 CI = -4.86 – 3.07, pMCMC = 0.51, n species = 528).

Phylogenetic distribution

To visualise the phylogenetic distribution of our traits, I plotted the data onto the Hymenoptera supertree. I found that absence of helper mating is mostly clustered within Vespinae, with only six species without helper mating in other clades (Figure 3).

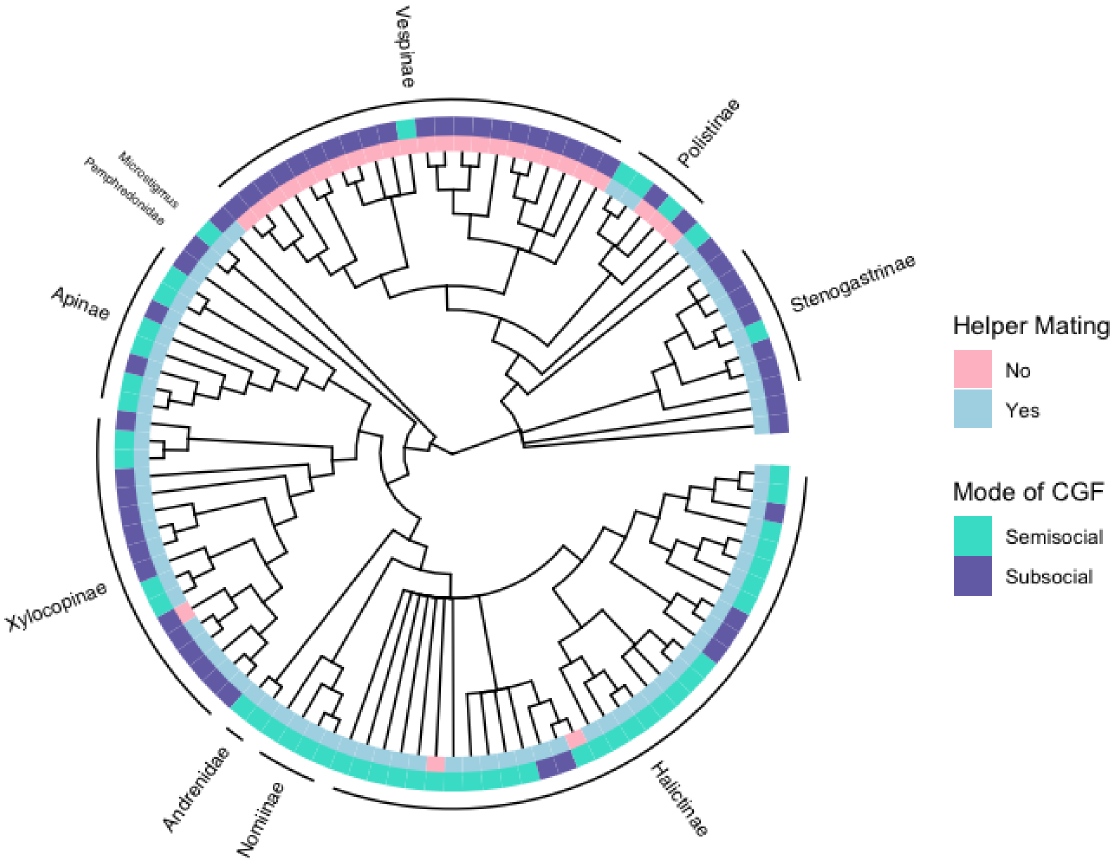

Figure 3. Phylogenetic distribution of helper mating and group formation across Hymenoptera. The distribution suggests that there are multiple independent transitions in helper mating and in group formation. Many species without helper mating are clustered within the wasp subfamily Vespinae. I filtered out ants, species with ambiguous group formation descriptions, and swarm-founders (n species = 115).

Discussion

Mode of group formation is not a significant predictor of helper mating

I found that Hymenopteran species with subsocial group formation have a higher proportion of helper sterility (absence of helper mating) than species with semisocial group formation (43% versus 8%; Figure 2). However, when I controlled for shared evolutionary history by analysing the relationship with a phylogenetic regression, it was not significant – although the trend was in the direction of subsocial species less likely to have helper mating.

Phylogenetic clustering of traits

The lack of significance in our results may be because of the way our traits are distributed – most species without helper mating are clustered within the wasp subfamily Vespinae (Figure 3). Within Vespinae, all but one species have subsocial group formation, which could explain the apparent relationship between subsocial group formation and absence of helper mating in the results without phylogenetic control. Outside of Vespinae, there are only six others without helper mating: three have subsocial group formation, and three have semisocial group formation (Figure 3). That means that there are not enough independent datapoints to draw reliable conclusions on the relationship between group formation and helper mating.

I aimed to increase variation in our social traits by adding ants into the analysis and managed to compile data for our chosen traits for 439 ant species. Ants are said to have a single origin of superorganismality at the base of their clade, but they have multiple losses and gains of helper mating, and some variation in group formation (Boomsma, 2023; Hölldobler & Wilson, 1977; see Chapter 4 of this thesis). However, in the current dataset they are quite uniform – after pruning and filtering, 100% of the ants have subsocial group formation. Helper mating is slightly more variable, with 9% of species categorised as having helper mating (38 of 418 species). Overall, despite having good taxonomic coverage of Hymenoptera (582 species of ants, wasps, and bees), there is not enough statistical power to draw reliable conclusions.

How can we increase variation in the response variable?

A clearer understanding of the relationship between helper mating and group formation requires more variation in our response variable. Most bees and wasps have helper mating, and those that do not are very clustered phylogenetically, meaning that we have very few independent data points. Since most of the data on bees and wasps was taken from textbooks, it may be possible to find more through a standardised literature search e.g. using Web of Science. However, it is unlikely that there are many more species that lack helper mating and are not already in the dataset – those with sterile helpers are very well-studied and so data is readily available and we have already included them. The data on ant group formation and reproduction was already collected via a standardised literature search (see Chapter 4) and so it is also unlikely there are many more ant species with data that we have not already included.

Collecting data for additional bees or wasps without helper mating is one way to increase representation of species in the less reproductive category, though there may not be any additional transitions represented by new species. An alternative approach is to change the response variable by lowering the threshold for inclusion into the less reproductive category. In other words, instead of looking at binary helper mating/ no helper mating, I could classify species by their reproductive skew (the difference in reproduction between breeders and helpers). Many species that have not lost helper mating entirely do have helpers with a lesser degree of reduced reproduction. These species would be in the category of greater reproductive potential currently, but when classified by reproductive skew, would be moved into the less reproductive category.

Conclusions

Overall, our results indicate that mode of group formation does not predict the absence of helper mating. However, we found that helper mating is highly phylogenetically clustered, and likely not the best way to capture variation in helper reproductive potential in Hymenoptera. To resolve the question of whether group formation can predict reduced reproductive capacity of helpers, future work should consider alternative response variables such as reproductive skew, which can capture subtler or continuous differences in helper reproduction. This will ultimately inform broader conclusions about the role of relatedness in the evolution of reproductive division of labour and cooperation across species.

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Chapter 6: Thesis Discussion

In this thesis, I aimed to explain the variation we see in degree of social complexity across insects. Specifically, I wanted to identify why some lineages have cooperation and division of labour while others remain solitary or have less pronounced division of labour. For example, some bees nest alone, with one female performing all duties including nest-building and brood provisioning (e.g. *Osmia* mason bees), while others (e.g. *Apis* honeybees) have colonies with thousands of sterile helpers rearing and defending their queen's brood. Division of labour can be between breeders and helpers but also among additional helper sub-castes. I collected data on different forms of division of labour in insects: size dimorphism between breeders and helpers, helper size variation, and helper reproductive potential (relative to the dominant breeder). I also gathered data on biological characteristics predicted to influence the evolution of division of labour: queen mating frequency, queen number, colony size, and group formation. I mapped these traits onto phylogenetic trees to find where transitions between states may have occurred, and I tested the relative importance of my predictor traits in the evolution of division of labour. My data chapters (3, 4, & 5) focus on species within the clade Hymenoptera, and chapters 3 & 4 on ants only, as this is where there was greatest availability of data and variation in key traits.

<u>Overview of insect social behaviour</u>. In Chapter 2, I give an overview of social behaviour across all insects. In particular, I try to draw attention to lineages with non-"eusocial" social behaviours, as these have been relatively neglected from popular study. These may include species with communal nesting behaviours or division of labour that falls short of the threshold for "eusociality" or "superorganismality". I include description of unusual cases of division of labour, such as nymphal soldiers in termites, aphids, and thrips, and the sterile defensive morphs of polyembryonic wasps. I highlight problems with popular terminology and the frameworks used for understanding insect sociality, such as inconsistent usage and implications of a social ladder or evolutionary sequence that is not empirically supported. Overall, I identify conceptual and empirical gaps in the study of social insects, and advocate for a trait-based approach. I intend to submit this chapter for publication as a review paper, after restructuring to pull the spotlight even more onto obscure social lineages. I will also add a phylogenetic tree figure with the key social traits mapped across insect lineages, and a finalised table indicating presence or absence of key social traits and the number of evolutionary origins of each. I hope to bring together the broad field of insect social

behaviour into a concise and accessible paper and hope that it will provide a useful reference for future comparative work.

The size-complexity relationship. In Chapters 3 & 4, I used phylogenetic comparative methods to explain differences in social complexity across ants. To measure social complexity, the traits I examined were: Worker size variation (Chapter 3), queen-worker size dimorphism (Chapter 3), and worker reproductive potential (Chapter 4). Chapter 3 has been published along with analyses on worker sub-castes in *Nature Ecology & Evolution* and Chapter 4 has been submitted to *Evolution*. In these chapters I found strong and consistent support for the size-complexity hypothesis, which predicts that larger systems have greater complexity (Bourke, 1999, 2011; Bell & Moores, 1997; Bonner, 2004). In the case of ants, it predicts that larger colony sizes have more division of labour among workers (size variation) and between queens and workers (size dimorphism and reproductive differences). I found that this is the case, and I tested alternative causal models to find the most likely causal explanation for this relationship. I found evidence that larger colony sizes facilitate: (i) the evolution of greater specialisation among workers; and (ii) greater specialisation into queen and worker castes. I found only weak evidence to support the alternative causal direction, which would be that greater specialisation (more division of labour) facilitates the evolution of larger colony sizes.

Relatedness and division of labour. In addition to testing the role of colony size on the evolution of division of labour, I tested the role of relatedness. I used two variables to indicate colony relatedness: number of queens per colony, and number of males the queen mates with. Both factors affect the relatedness of workers to the offspring that they are helping raise, in terms of whether they are full siblings, half siblings, or less. Relatedness has been predicted to influence cooperation and division of labour (Liu *et al.,* 2021; Oster & Wilson, 1978; Fjerdingstad & Crozier, 2006; Frumhoff & Ward, 1992). However, I found little support for this relationship in ants. Path analysis indicated that having more queens per colony may favour the loss of worker sex, but ancestral state reconstructions indicated that differences in queen number were not significant and may be in the direction of fewer queens preceding the loss of worker sex (see discussion in Chapter 4). The relationship between queen mating frequency and division of labour was also weak. Although there was a positive relationship between higher mating frequency and greater queen-worker size dimorphism, path analysis found only a very weak relationship between the two and indicated that mating frequency was much more strongly correlated with colony size, which is directly influencing size dimorphism. Testing alternative causal models revealed that the correlation between mating frequency and size dimorphism most likely exists because they are both correlated with the third variable: colony size. Additionally, multiple queen mating is a derived state in ants, evolving after their differentiation into queen and worker castes, so it would be interesting to test the role of relatedness in non-superorganismal lineages such as insects with transient, facultative, or non-morphological division of labour (e.g. "primitively eusocial" bees and wasps; see Chapter 5).

Reconstruction of the ancestral ant. I reconstructed the number of evolutionary origins of reproductive division of labour in ants: I estimate that there have been 5 gains and 16 losses of worker sexual capacity, and 17 gains and 20 losses of complete worker sterility in ants. These results are impactful as they affect the way we view ants in terms of their superorganismality. Ants are often described as superorganismal, with a single origin at the base of their clade (Hölldobler & Wilson, 2009; Boomsma, 2023; Bernadou *et al.,* 2021). Superorganismality is generally defined by a loss of individual totipotency and irreversible differentiation into germ and soma functions (queens and workers). By highlighting the diversity of specialisation across ants and showing that there are multiple origins of both worker sterility and worker sex, I challenge assumptions about when they became superorganismal. I reconstructed the most recent common ancestor of extant ant species and found that it most likely had workers with full reproductive potential. If worker sterility is the decisive criterion for superorganismality, then the ants that have not yet evolved this state cannot be considered superorganismal, and there were around 17 independent major transitions in this clade alone. The multiple gains of worker sex are also significant as they suggest that superorganismality, if characterised by sterile helpers, is a state that can be lost.

If we think of superorganismality in terms of morphological differentiation into queen and worker roles, instead of focusing on the reproductive capacity of the workers, then it may be consistent with the possibility of a single origin of superorganismality in ants. However, whether we define superorganismality by queen-worker differentiation or by complete worker sterility, we still have reason to say that superorganismality has been lost as several ant species have lost their queen caste and have reproductive gamergate workers instead. Here we must be careful in not calling this a reversion, as that would imply that these species have reverted back to an ancestral state of dominance hierarchies with no morphological divide. We do not have evidence yet to support this assumption as it is also possible that superorganismal clades arose directly from solitary ancestors (Boomsma, 2023; see Chapter 2 for the problematic implications of some social terminology).

Group formation. Finally, in Chapter 5 I examined the relationship between how groups are formed and their degree of reproductive division of labour (differentiation into reproductive and non-reproductive roles). To measure reproductive division of labour, I again looked at helper mating, as in Chapter 4, but this time across Hymenoptera. Group formation is predicted to influence cooperation and division of labour because of how it affects the relatedness within the group – how closely related helpers are to the offspring they rear (Fisher *et al.,* 2013; Downing *et al.,* 2020; Kapsetaki *et al.,* 2017; Reeve & Keller, 1995). Subsocial groups are formed when offspring remain with their mother and live alongside her (relatedness is high); Semisocial groups are formed when individuals from the same generation come together (relatedness is lower). By expanding the scope to all Hymenoptera, I could test the role of relatedness in lineages not considered superorganismal, including communal nesting bees or "primitively eusocial" wasps. I did not find evidence that mode of group formation predicts helper mating when controlling for phylogeny, likely owing to lack of statistical power despite having good taxonomic coverage. For a more reliable conclusion about the role of group formation in insect cooperation, an alternative response variable with more evolutionary transitions is needed. This could involve creating multiple categories of reproductive capacity in helpers (similar to the four categories in Chapter 4) or a continuous measure of reproductive skew. I would further improve Chapter 5 by running more phylogenetic analyses, such as ancestral state reconstruction, to test the robustness of the results and to estimate the number of evolutionary origins of my traits. By incorporating other potential explanatory variables, I could test how much of the possible relationship between subsocial group formation and loss of helper mating is due to group formation itself, or due to another correlated factor. Finally, I intend to resolve some remaining polytomies in the phylogenetic supertree, such as among *Lasioglossum* species, by incorporating additional phylogenies from the literature.

Conclusion. My thesis has provided strong support for the size complexity relationship, indicating that larger colony size facilitates the evolution both reproductive and non-reproductive division of labour. This has broad implications for the evolution of complex life, as division of labour allows increasing complexity across levels of biological organisation (e.g. division of labour between genes in a genome, cells in multicellular organisms, or organisms in a superorganismal colony). Further work could test if this relationship holds in multicellular systems i.e. Does the evolution of a larger body size (more cells) facilitate the evolution of greater division of labour (more cell types)? We know already that larger organisms have more cell types, but don’t fully understand why (Bell and Mooers, 1996). It would also be interesting to test if these same relationships are found in lineages that have not undergone a major transition in individuality, such as in species with only facultative cooperative breeding and transient division of labour (e.g. *Polistes* paper wasps). The relationship between relatedness and division of labour was more complex and inconsistent, and it is possible that relatedness plays a reduced role in lineages that have already passed a major evolutionary threshold and differentiated into queen and worker castes. Further research into “primitively” social species, where caste differentiation is absent or less extreme, may reveal a different role of relatedness in the evolution of social behaviour.

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