Factors and mechanisms of queen behavioral flexibility in the ants

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“Yeah, but your scientists were so preoccupied with whether or not they could, they didn't stop to think if they should.” – Dr. Ian Malcom, Jurassic Park

“But, one thing is for certain: there is no stopping them; the ants will soon be here. And I, for one, welcome our new insect overlords”. – Kent Brookman, The Simpsons

“Vergessen Sie es,er tut er sowieso. Der Schicksaleisbrecher hat gerade den Hafen verlassen und wir beide sind nur Eisschollen, die nichts weiter tun können, als die Bugwelle zu bestaunen, die unaufhörlich näher kommt. Im Ozean des Leidens.” – Bernd das Brot

“Forget it, he'll do it anyway. The icebreaker of fate has just left the harbor, and we are both just ice floes that can do nothing but marvel at the bow wave that is steadily approaching. In the ocean of suffering.” – Bernd the Bread

"Remember kids, the only difference between screwing around and science is writing it down" – Adam Savage

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Summary

A key aspect of eusocial societies is the division of labor, where individuals specialize in reproductive and non-reproductive tasks. Within eusocial insect colonies, non-reproductive workers perform essential tasks, such as foraging, nest maintenance, defense, and brood care, while reproductive queens focus primarily on egg-laying.

Ants, in particular, exhibit extraordinary diversity in their social systems. The ant queen has been traditionally seen as a specialized reproductive unit, yet during colony foundation, queens can perform diverse behaviors including brood care. Its behavior becomes increasingly specialized as workers emerge. However, recent research has shown that this specialization is reversible and queens can express several non-reproductive behaviors upon changes in the social environment. Despite this, we still lack a fundamental understanding of how queen behavior is shaped across the ant phylogeny, and how social context and underlying molecular processes modulate behavioral flexibility.

To address these questions, I employed three complementary approaches. First, I conducted a broad phylogenetic investigation of queen behavioral flexibility across 38 ant species, using standardized behavioral assays. This analysis revealed that behavioral plasticity in queens is widespread, though it varies greatly across lineages. The social structure of species explained some of the variation. Second, I focused on intraspecific variation of queens in polygynous queens of the Amblyoponinae *Stigmatomma pallipes* showing that queens differ in mobility and mating status. Crucially, by generating a high-quality reference genome and comparing tissue-specific transcriptomes, I reveal that unmated queens express gene expression profiles closely resembling those of workers. Therefore, elucidating that the molecular underpinnings of behavior are not bound by morphology alone. Third, I investigated the molecular mechanisms regulating queen behavioral reversibility in the ant *Lasius niger*. Using gene expression profiling, I identified 123 genes that differed in the brains of queens depending on the presence or absence of workers. Among these, I identified a subset of genes that are known to modulate behavioral plasticity in insects, such as *Krüppel-homolog 1* (*Kr-h1*), and parts of the insulin-like signaling pathway.

Together, this thesis demonstrates that queen behavioral flexibility is a widely conserved yet not fixed trait in ants, which is shaped by both social context and molecular regulation. These insights deepen our understanding of how specialized roles, whether in insect societies or other complex systems, are maintained and modulated. Ultimately, this work contributes to a broader framework for understanding how division of labor emerges and is maintained in biological systems, emphasizing the importance of variation within a highly specialized role.

Zusammenfassung

Ein wesentlicher Aspekt eusozialer Gesellschaften ist die Arbeitsteilung, bei der sich die Individuen auf reproduktive und nicht-reproduktive Aufgaben spezialisieren. In eusozialen Insektenkolonien übernehmen nicht-reproduktive Arbeiterinnen Aufgaben wie Nahrungssuche, Nestpflege, Verteidigung und Brutpflege, während sich die reproduktiven Königinnen in erster Linie auf die Eiablage konzentrieren.

Insbesondere Ameisen weisen eine außergewöhnliche Vielfalt in ihren sozialen Systemen auf. Die Ameisenkönigin wurde traditionell als spezialisierte Fortpflanzungsorgan angesehen, doch während der Koloniegründung können Königinnen vielfältige Verhaltensweisen ausüben, darunter auch die Brutpflege. Mit dem Auftauchen der Arbeiterinnen wird ihr Verhalten zunehmend spezialisiert. Jüngste Forschungen haben jedoch gezeigt, dass diese Spezialisierung reversibel ist und Königinnen bei Veränderungen im sozialen Umfeld verschiedene nicht-reproduktive Verhaltensweisen zeigen können. Dennoch fehlt uns noch immer ein grundlegendes Verständnis darüber, wie das Verhalten der Königin im Laufe der Ameisen-Phylogenie geprägt wird und wie der soziale Kontext und die zugrunde liegenden molekularen Prozesse die Verhaltensflexibilität modulieren.

Um diese Fragen zu beantworten, habe ich drei sich ergänzende Ansätze verwendet. Zunächst habe ich eine umfassende phylogenetische Untersuchung der Verhaltensflexibilität von Königinnen bei 38 Ameisenarten unter Verwendung standardisierter Verhaltenstests durchgeführt. Diese Analyse ergab, dass Verhaltensplastizität bei Königinnen weit verbreitet ist, obwohl sie stark variiert. Die soziale Struktur der Arten erklärte einen Teil dieser Variation. Zweitens konzentrierte ich mich auf die intraspezifische Variation von Königinnen bei polygynischen Königinnen der Amblyoponinae *Stigmatomma pallipes* und zeigte, dass sich Königinnen in ihrer Mobilität und ihrem Paarungsstatus unterscheiden. Durch die Erstellung eines hochwertigen Referenzgenoms und den Vergleich gewebespezifischer Transkriptome konnte ich zeigen, dass unverpaarte Königinnen Genexpressionsprofile aufweisen, die denen von Arbeiterinnen sehr ähnlich sind. Damit habe ich aufgezeigt, dass die molekularen Grundlagen des Verhaltens nicht allein durch die Morphologie bestimmt werden. Drittens untersuchte ich die molekularen Mechanismen, die die Verhaltensreversibilität der Königin bei der Ameise *Lasius niger* regulieren. Mithilfe von Genexpressionsprofilen identifizierte ich 123 Genen, die sich im Gehirn der Königinnen je nach Anwesenheit oder Abwesenheit von Arbeiterinnen unterschieden. Unter diesen habe ich eine Untergruppe von Genen identifiziert, von denen bekannt ist, dass sie die Verhaltensplastizität bei Insekten modulieren, wie z. B. Krüppel-Homolog 1 (*Kr-h1*) und Teile des insulinähnlichen Signalwegs.

Insgesamt zeigt diese Arbeit, dass die Verhaltensflexibilität der Königin ein weit verbreitetes, aber nicht festgelegtes Merkmal bei Ameisen ist, das sowohl durch den sozialen Kontext als auch durch molekulare Regulation geprägt wird. Diese Erkenntnisse vertiefen unser Verständnis davon, wie spezialisierte Rollen, sei es in Insektengesellschaften oder anderen komplexen Systemen, aufrechterhalten und moduliert werden. Letztendlich trägt diese Arbeit zu einem umfassenderen Rahmen für das Verständnis bei, wie Arbeitsteilung in biologischen Systemen entsteht und aufrechterhalten wird, wobei die Bedeutung der Variation innerhalb einer hochspezialisierten Rolle hervorgehoben wird.

General introduction

1. Major Evolutionary Transitions and Sociality

1.1 Defining Major Evolutionary Transitions

Throughout evolutionary history, the transition to complexity has been a rare exception rather than the rule. Most organisms exist as independent units, from single-cell bacteria to solitary animals, that rarely come together to exchange genetic information. Yet despite their rarity, the emergence of cooperative and integrated systems has repeatedly transformed the planet. For example, the evolution of eukaryotic cells through endosymbiosis enabled the structural and metabolic complexity that underlies all higher life-forms (Vellai and Vida 1999). The emergence of multicellular organisms allowed for the specialized division of labor among cells and gave rise to the macroscopic ecosystems present in the visible biosphere (Grosberg and Strathmann 2007; Ispolatov et al. 2012). In some of these multicellular lineages, a comparable division of labor occurred among previously solitary individuals, leading to the emergence of social groups. This is observed most strikingly among insects and mammals, such as humans, which have reshaped the global environment (Clutton-Brock et al. 2009; Gowdy and Krall 2013)

These recurrent leaps in organization are known as major evolutionary transitions (METs) (Smith and Szathmary 1997; West et al. 2015). They describe events in which formerly independent entities combine to form new, higher-level units capable of reproduction. Each transition, from self-replicating molecules forming protocells, to symbiotic prokaryotes merging into eukaryotic cells, to the evolution of multicellular organisms and cooperative social groups, represents a shift in the level at which natural selection operates. What unites these transitions is the eventual loss of autonomy of the lower-level units: once integrated, they can no longer persist independently and only reproduce as a part of the whole (Smith and Szathmary 1997). Mitochondria no longer divide freely outside the cell, somatic cells maintain the organism but irreversibly forgo reproduction, and members of cooperative groups often forgo direct reproduction in favor of the collective.

1.2 Rules and Mechanisms of Sociality

This pattern introduces a fundamental evolutionary paradox: how do independent components relinquish aspects of their autonomy and reproductive potential for the benefit of the collective, even when they could, in principle, survive and reproduce on their own? This question has puzzled scientists since the 19th century, from Darwin’s reflections on sterile

workers in social insects (1859) to later insights about sociality by Fisher (1930), Haldane (1932), and Hamilton (1964). Today, several well-established evolutionary mechanisms explain this phenomenon. Fisher and Haldane formalized the concept of inclusive fitness, noting that helping relatives can propagate shared genes. Building on this foundation, Hamilton formalized kin selection: a cooperative act is favored when the benefit to a relative *B*, weighted by genetic relatedness *r*, exceeds the cost to the actor *c*:

$$rB > c$$

Beyond kin selection, cooperation can also evolve through other mechanisms that do not depend on genetic relatedness (Nowak 2006; Axelrod and Hamilton 1981). One such mechanism is mutualism, which arises when individuals gain immediate advantages from helping others, such as increased survival, resource acquisition, or predator avoidance , for example between clownfish and sea anemones (García Jiménez 2024; Boucher et al. 1982; Dugatkin and Mesterton-Gibbons 1996; Connor 1995). In some cases, cooperation emerges as a by-product of selfish actions, where helping others is unavoidable but also beneficial to the group. Reciprocity provides another pathway to cooperation, explaining cooperative acts that yield delayed fitness benefits, and can be differentiated in two kinds. In direct reciprocity, individuals help others expecting future return, whereas indirect reciprocity relies on social reputation (Trivers 1971; Nowak and Sigmund 1998). Both forms require repeated interactions and the ability to recognize or track members of the group and are particularly relevant in socially complex animals.

These mechanisms, kin selection, mutualism and reciprocity, often act together rather than in isolation (West et al. 2007; Nowak 2006). Kin-selected altruism may be reinforced by mutualistic benefits, and reciprocity can stabilize cooperation in groups with repeated encounters (Hamilton 1964). Additional factors, including population structure, multilevel selection, synergistic benefits from division of labor (or specialization), and enforcement or policing of cheaters, can further enhance the persistence of cooperation (Gardner 2015; O'Gorman et al. 2008; Wilson 1997; Singh and Boomsma 2015; Ratnieks and Wenseleers 2005; Wenseleers et al. 2004). Therefore, cooperation of the specialized high-level unit relies on a multitude of mechanisms.

1.3 Sociality across life

The interplay of these mechanisms can be systematically explored through the METs, which link increasing organizational complexity to the emergence of cooperation. At the level of independent single-celled units, cooperative behaviors are basic, often leading to facultative sociality (West et al. 2007). These systems, such as bacteria and archaea, display

sophisticated communication (*e.g.*, quorum sensing) and coordinated activity (*e.g.*, biofilm formation) (Bassler 1999; Miller and Bassler 2001; Harrison and Buckling 2009; Xavier and Foster 2007). In these aggregations, the units retain full reproductive potential, but they collectively benefit from coordinated defense or resource acquisition. The evolutionary threshold to multicellularity introduced the next stage of specialization. This transition is mirrored by organisms exhibiting a transient division of labor. Slime molds (*e.g.*, *Dictyostelium*) provide a striking example: free-living amoebae aggregate under stress into multicellular stalks where only a subset of cells becomes reproductive spores, while the rest undergo altruistic death to form the supportive structure (Gross 1994; Raper and Fennell 1952; Whittingham and Raper 1960; Strassmann and Queller 2011). This temporary cooperation perfectly illustrates the trade-offs of reproductive specialization and gains through kin selection and introduces other social concepts, such as the problem of cheaters (Khare and Shaulsky 2010; Matapurkar and Watve 1997).

Among fully integrated multicellular organisms (Metazoa), cooperation is widespread, yet generally remains at a level considered as a non-obligate sociality (Dugatkin 2002). Many vertebrates exhibit cooperative behaviors like alloparenting, cooperative hunting (*e.g.*, lionesses), sentinel behavior (*e.g.*, meerkats) or sharing of resources (*e.g.*, vampire bats) (Scheel and Packer 1991; Clutton-Brock et al. 2001; Crisp et al. 2021; Wilkinson et al. 2016). In these systems, individuals assist conspecifics while fully maintaining their own reproductive potential, though only few fully realize it (Keller and Reeve 1994; Clutton-Brock et al. 2001; Holekamp and Engh 2009). The benefits of cooperation often emerge from a combination of kinship and immediate mutualistic advantages.

Taken together, these examples demonstrate the repeated rise of systems in which formerly independent units become increasingly interdependent throughout evolutionary history. However, in all these cases, the underlying autonomy of individuals remains intact: cells of slime molds are innately solitary, vertebrate helpers can become breeders, and cooperative assemblies can dissolve without permanent loss of reproductive potential (Jack et al. 2008; Komdeur et al. 2008). Only in a very limited number of lineages did these cooperative forces culminate in a qualitatively different form of organization: a permanent division of labor built on irreversible reproductive specialization, similar to the shift from prokaryotes to eukaryotes (Sagan 1967; Smith and Szathmary 1997). At this point, the fitness interests of individuals and the collective become fused, and selection acts primarily at the level of the emergent group. This condition, called eusociality, represents the pinnacle of social integration (Nowak et al. 2010; Wilson and Hölldobler 2005).

2. Eusociality: Definition, Origins, and Taxonomic Distribution

2.1 Defining Eusociality

The term "eusocial" (from Greek *eu-* meaning "true") was first defined by Suzanne Batra (1966) to describe ground-nesting halictid bees in which reproductive division of labor occurs without morphological caste differentiation, specifically, species where foundresses recruit daughters as helpers while retaining their own reproductive capacity. Later Charles D. Michener (1969), who investigated social structure across the bees, identified three core features that set eusocial species apart: (1) a reproductive division of labor; (2) cooperative care of offspring; and (3) overlapping generations that allow parents and their adult offspring to cohabit and cooperate. E. O. Wilson (1971) applied the term more broadly towards social insects. These criteria remain the standard definition and mark the point at which social groups transition into functionally integrated societies (Wilson and Hölldobler 2005; Smith and Szathmary 1997).

Before the term 'eusociality' was formally described, the concept of the 'superorganism' was used to describe insect colonies that functioned as integrated wholes. This concept was first developed by William Morton Wheeler (1911; 1920)and was based on the idea that the division of labor within these colonies was analogous to the organs of a body. The term largely fell out of favor, primarily challenged by the rise of kin selection theory and evidence of within-colony reproductive conflict, which focused on individual fitness rather than the “collective good” (Crozier 1989). The concept has since seen a modern revival, where it is used to describe the profound functional integration of the most complex eusocial colonies, such as their colony-level metabolism and life-history scaling (Gillooly et al. 2010; Hou et al. 2010; Haber 2013). Key collective adaptations like social immunity and the resource-sharing behavior exemplify this integration (Holldobler and Wilson 2009). Today, while the framework remains debated, it is used to emphasize that the colony can be viewed as a single, adaptive unit of selection.

2.2 Core Features Across Eusocial Lineages

Although eusocial systems share the same defining criteria, their social organization can vary widely across taxa (Cronin et al. 2013; Nowak et al. 2010; Wilson and Hölldobler 2005). Colonies are generally composed of reproductive individuals, which produce the offspring, and functionally non-reproductive individuals, which perform tasks such as brood care, defense, foraging, and nest maintenance. In some species, these division of labor is rigid,

while in others, individuals retain the ability to switch between reproductive and non-reproductive states in response to ecological or social cues (Johnson et al. 2009; Kapheim et al. 2020; Amador-Vargas et al. 2015; Divieso et al. 2020). Cooperative brood care ranges from simple guarding of communal eggs to sophisticated provisioning systems involving coordinated behaviors and age-structured task allocation (Michener 1969; Walsh et al. 2018; Schultner et al. 2017; Queller 1997). Overlapping generations ensure sustained cooperation and enable division of labor to persist over the lifetime of the colony (Michener 1969; Mitesser et al. 2017).

Eusociality is rare, having arisen only a small number of times given the vast diversity of animals (Wilson and Hölldobler 2005; Smith and Szathmary 1997). Among vertebrates, it is restricted to a few exceptional cases, such as the naked mole-rat and the Damaraland mole-rat, where only one female typically reproduces while non-reproductive individuals assist in rearing young and maintaining the burrow (Burda et al. 2000; Burland et al. 2002; Jarvis 1981). In invertebrates, eusociality has also emerged in a limited number of snapping shrimps (*Synalpheus*), which defend shared sponge habitats using non-reproductive defenders (Duffy 1996; Duffy et al. 2000)

The most extensive and complex expressions of eusociality occur among the insects. While the Hymenoptera (ants, some bees and some wasps) and Isoptera (termites) account for the vast majority of eusocial species, research indicates that eusociality has evolved independently at least twelve times across the insect tree of life, including the Thysanoptera (gall-forming thrips), Hemiptera (gall-forming aphids), and Coleoptera (ambrosia beetles and possibly other weevils) (Kent and Simpson 1992; Shibao 1999; Chapman et al. 2000; Crespi 1992; Bourke 2011; Boomsma 2007; Fischman et al. 2011; Wilson and Hölldobler 2005). These repeated origins underscore that while eusociality is rare, specific ecological and genetic pressures can recurrently promote its emergence and persistence outside the major social orders.

Within this broader context, the Hymenoptera stand out as the insect order with the highest frequency of independent transitions to eusociality. Conservative phylogenetic reconstructions identify nine separate origins within the order: five in bees, three in wasps, and a single origin in ants (Wilson and Hölldobler 2005; Boomsma 2007). This repeated emergence reflects a confluence of genetic and ecological predispositions that make cooperative breeding particularly favored in hymenopteran lineages.

First, the haplodiploid sex-determination system of hymenopterans means that species may be more predisposed to be eusocial. This is due to females being diploid and males haploid, generating a relatedness asymmetry: sisters reared from a single, once-mated mother share,

on average, 75% of their genes, whereas each female shares only 50% with her own offspring. This asymmetry lowers the inclusive-fitness threshold for helping behavior, making kin selection more potent. However, this factor alone is insufficient to explain the evolution of eusociality (Trivers and Hare 1976; Andersson 1984; da Silva 2022), as evident by the vast majority of hymenopterans displaying solitary life-histories.

Second, many hymenopterans already exhibit progressive brood care and nest provisioning behaviors that furnish a behavioral scaffold for the evolution of non-reproductive helpers (Neff 2008; Flintham 2022). When suitable nesting sites are defensible yet scarce, such as wood cavities, underground chambers, or limited-resource patches, group defense becomes advantageous, favoring the retention of offspring in the natal nest (Emlen 1982).

Third, life-history traits that generate overlapping generations and multi-brood nest cycles increase the probability that daughters will remain as helpers, especially when solitary nest founding is unlikely. Seasonal or ecological pressures that produce female-biased early-season cohorts, further elevate the likelihood of daughter-stays. In some lineages, maternal manipulation via hormonal or nutritional cues may bias offspring development toward a non-reproductive phenotype, effectively “programming” them to assist rather than reproduce independently (González-Forero 2015; Kapheim, Nonacs, et al. 2015; Rees-Baylis et al. 2024).

Across all but one eusocial taxon, there still exist solitary and subsocial genera that vastly outnumber their eusocial counterparts. The one exception, where all extant species express social living: the ants (Formicidae) (Hölldobler and Wilson 1990; Boomsma and Gawne 2018).

3. The Ants

3.1 Ant Biology, Ecology, and Colony Structure

Ants belong to the insect order Hymenoptera, nested within the Aculeata (stinging wasps, bees, and ants), a lineage defined by the modification of the ovipositor into a stinger. The ant lineage split off from the other Aculeata during the mid-Cretaceous period around 120 to 140 million years ago (mya), followed with a quick diversification, driven partly by the rise of the angiosperms (flowering plants) (Ward 2014; Moreau et al. 2006). Ants share the traits of the order Hymenoptera, such as being holometabolous, with larval stages that differ morphologically and behaviorally from adults and require pupation (undergo complete metamorphosis) to reach adulthood. Like eusocial bees and wasps, larvae are typically limbless and immobile, and entirely dependent on care from other colony members. After pupation, the adult body is divided into a head (caput), mesosoma (thorax), a constricted petiole (sometimes with two segments), and a gaster (abdomen), which most often features a stinger or an acid pore (Hölldobler and Wilson 1990).

Ants are globally widespread and are found across nearly all terrestrial ecosystems. With over 16,900 described species and subspecies, ants are a dominant life form, collectively numbering an estimated 20 quadrillion individuals worldwide (Antweb.; Schultheiss et al. 2022). This population accounts for a massive collective biomass, estimated to be around 12 million tons of dry carbon, a figure that surpasses the combined biomass of all wild birds and mammals (Schultheiss et al. 2022). Ants, thus, shape ecosystems globally, both through sheer numbers and their overwhelming collective mass. They are key ecological players: they aerate soil, disperse seeds, act as efficient scavengers, regulate invertebrate populations and can be the main herbivores and predators in their ecosystems (Blanton and Ewel 1985; Gammans et al. 2005; Handel and Beattie 1990; Frouz and Jilková 2008; Dejean et al. 2025; Brady et al. 2014)

Despite the relatively simple nervous system of a single ant, colonies exhibit remarkably complex behaviors. Building rafts, constructing complex nests, foraging trails, ritualistic territorial conflicts, raiding and tool-use emerge from the repeated interactions of many individuals rather than from the intelligence of any single ant (Schneirla 1944; Banschbach et al. 2006; McDonald 1984; Ettershank and Ettershank 1982; Van Wilgenburg et al. 2005; Robinson 2014; Mlot et al. 2011; Mueller et al. 1998). These feats are accomplished through decentralized self-organization (Bonabeau et al. 1997; Detrain and Deneubourg 2006). Here, simple local interactions between colony members, governed by instinctual rules and indirect communication or the lack thereof, provides the basis of this organization. These interactions

give rise to positive or negative feedback loops, which result in the rise of these complex behaviors (Czaczkes et al. 2013; Grüter et al. 2012; Gordon 2019).

As a hallmark of eusociality and a MET, ant colonies are divided into morphologically distinct reproductive and non-reproductive castes (Wheeler 1911). Reproductives include queens, who are typically the primary egg layers, and males, whose main function is mating. Non-reproductives, referred to as workers, are exclusively female. They perform tasks such as brood care, foraging, and nest maintenance. In many species the adult stage of the worker caste can be morphologically plastic. They can vary in size, shape, and sometimes function (Qiu et al. 2022; Wheeler 1986). This can range from smaller differences in size between minor and major workers to morphologically distinct soldier caste, specialized fungus tenders, living doors and food storages (Duncan and Lighton 1994; Baroni Urbani 1998; Huang and Wheeler 2011; Fujioka et al. 2019; Hasegawa 1993; Weber 1972; Gerstner et al. 2011). This plasticity is not limited to the workers; queens and males can also exhibit variation in morphology. For instance, ergatoid (worker-like) males can resemble workers and stay inside the nest and are equipped with sharp mandibles compared to their winged counterparts (Heinze et al. 1998). Furthermore, the size difference between the queen and workers (queen:worker dimorphism) can vary greatly within and between species (Wolf and Seppä 2016; Cole 1985; Wilson 1953; Miyazaki et al. 2005). Such variation is widespread across ant species, highlighting that the remarkable diversity of colony structures and life histories is a shared feature throughout the ant phylogeny.

3.2 Phylogeny of Ants

The extant species of ants fall into several major evolutionary lineages, separated into three distinct clades: the leptanilloids, the poneroids, and the formicoids (Moreau et al. 2006; Ward 2014; Urbani et al. 1992; Economo et al. 2018). While these clades are genetically determined, members can often be recognized by the distinctive morphology of the petiole.

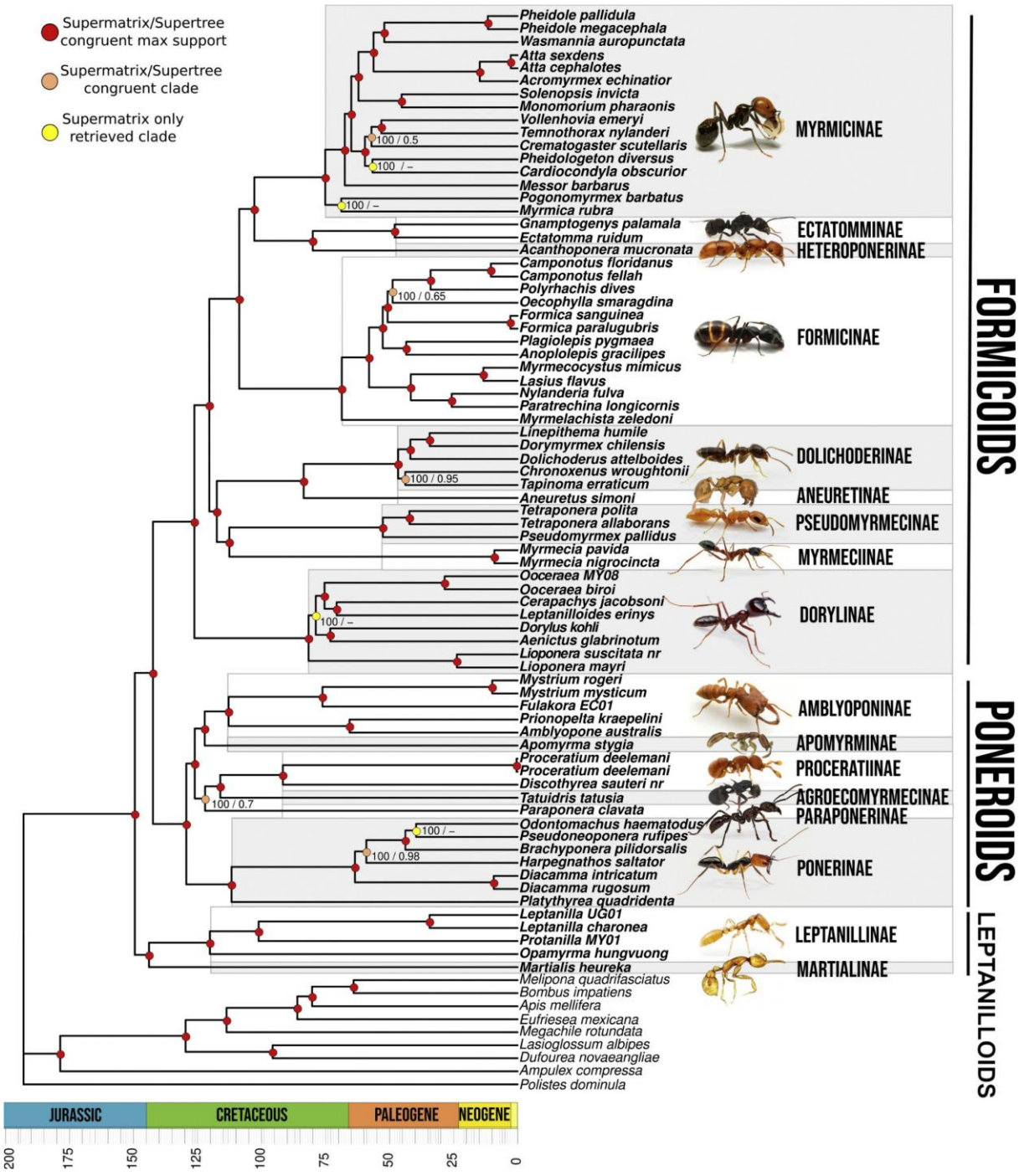

Figure 1: Phylogenetic tree of the ants, adjusted from Romiguier et al. 2022.

The leptanilloid clade, constituting around 1% of all ant species, consists of the subfamilies Martialinae and Leptanillinae. The leptanilloid split off from the other ants approximately 115–100 mya (Moreau et al. 2006). The Martialinae consists of a single species, *Martialis heureka*, which, despite being the sister species to all other extant ants, is eusocial (Rabeling et al. 2008). The subfamily Leptanillinae consists of several cryptic species that, despite their phylogenetic distance, can express sophisticated behaviors such as coordinated raiding (Masuko 1990; Sasaki et al. 2025).

The poneroid clade, the second largest and comprising approximately 11% (~1,600 species) of all ant species, includes several subfamilies, such as the Ponerinae, Apomyrminae, Amblyoponinae, and Paraponerinae (Ward 2014; Economo et al. 2018; Moreau et al. 2006). Similarly, this clade split off from the other ants approximately 110-100 mya (Moreau et al. 2006). The Ponerinae is the largest subfamily within this clade, consisting of the majority of species (~1,400 species) in the poneroid clade (Doré et al. 2025). One famous member is the genus *Odontomachus*, known for their trap-jaws (Gronenberg 1995; Gronenberg et al. 1993). The Amblyoponinae are particularly notable for their larval hemolymph-feeding behavior; queens (and sometimes workers) pierce larval integuments to feed on hemolymph (Masuko 1986; Traniello 1982; Wheeler 1900). Poneroid ants often maintain simpler colony structures, low queen:worker dimorphism, and strong predatory behaviors, contrasting with the more refined social systems of other clades (Doré et al. 2025; Schmidt and Shattuck 2014).

The formicoid clade contains the vast majority of extant ant species, accounting for approximately 88–90% of the total (Moreau et al. 2006; Ward 2014; Economo et al. 2018). This clade consists of several large subfamilies, including the Dorylinae, the Formicinae, and the Myrmicinae. This clade exhibits the greatest diversity in terms of caste morphology and colony organization. It features key innovations, such as trophallaxis (the direct exchange of fluids across colony members), agriculture, complex symbioses with other insect species, high queen:worker dimorphism, and diverse forms of parasitism (Matte and LeBoeuf 2025; Camargo et al. 2007; LeBoeuf 2017; Novgorodova 2005; Borowiec et al. 2021). These parasitic strategies range from temporary social parasites that usurp host colonies, to slavemaker species that steal brood, and inquilines, whose queens invade host nests to produce only reproductives due to having lost the worker caste (Mori et al. 2001; 2000; Bourke and Franks 1991; Dahan and Rabeling 2022; Chernenko et al. 2013).

The Dorylinae (army ants) are defined by elaborate raiding patterns, the building of living bivouacs, temporary nests formed from the living bodies of the colony, and nomadic behaviors (Mizuno et al. 2023; Schneirla 1944; Brady et al. 2014). These ants are only found

in the tropics and display remarkable dimorphism between workers and queens, as well as several worker subcastes (Brady 2003; Brady and Ward 2005). This subfamily also includes the clonal raider ant *Ooceraea biroi*, which has lost the queen caste and instead has workers that reproduce equally through parthenogenesis (Tsuji and Yamauchi 1995; Ravary and Jaisson 2002).

The Formicinae are characterized by the loss of the stinger and the presence of an acid pore, from which they can spray the namesake formic acid. Prominent members include the wood ants (*Formica* species), which are ecological keyholders in their respective temperate regions that maintain seasonal aphid farms (Laakso and Setälä 2000; Punttila et al. 2004; Elton 1932). Furthermore, this subfamily contains some social parasites, for example, in the *Lasius* and *Polyergus* genera. The latter, known as slave-making ants, raid *Formica* species and use their hosts as a workforce, while their own workers are morphologically adapted solely to raiding and cannot partake in normal colony upkeep (Mori et al. 1995).

The Myrmicinae is the largest subfamily across the ants, including over 6,700 species (Moreau et al. 2006; Brady and Ward 2005; Economo et al. 2018). It is characterized by the addition of a second petiole segment (postpetiole) and the loss of the cocoon around the pupating larvae. Unlike the Formicinae, Myrmicinae generally retain a functional stinger at the tip of the gaster for defense and subduing prey. Species can be highly diverse, from specialized predators to farmers. The subfamily includes prominent members, such as the leafcutter ants (*Atta* and *Acromyrmex* species), who cultivate a symbiotic fungus (Gerstner et al. 2011; Kleineidam et al. 2001). It is in this subfamily that the first supergene, a cluster of genes inherited together, responsible for social polymorphism in the fire ant *Solenopsis invicta*, was discovered (Helleu et al. 2022; Ross and Shoemaker 2018; Wurm et al. 2011; Keller and Ross 1998; Ross et al. 1996). The subfamily includes many prominent research subjects relevant to the study of social evolution, including *Cardiocondyla obscurior*, *Temnothorax nylanderi*, and *Wasmannia punctata* among others (Errbii et al. 2024; Jaimes-Nino et al. 2022; Modlmeier et al. 2013; Negroni et al. 2019; Okada et al. 2013; Breton et al. 2004; Clark et al. 1982; Ortiz-Alvarado and Rivera-Marchand 2020).

This phylogenetic architecture reveals both deep shared ancestries, where eusocial organization is conserved across these lineages, and striking ecological and morphological variation. It sets the stage for understanding how different social systems emerge and diversify, and how polymorphism in colony structure and caste roles arises from this rich evolutionary background.

3.3 Colony foundation, Social Structure and the Queen Caste

Ant colonies exhibit remarkable plasticity in social organization, reflecting adaptations to ecological constraints, colony growth demands, and reproductive allocation. This variation shapes both colony performance and the morphology and behavior of every individual within it. Central to this plasticity are two intertwined traits: colony foundation and social structure, i.e. the number of queens (Smith et al. 2009; Tsuji and Tsuji 1996; Peeters and Ito 2001b). The center of these traits is the queen caste itself, which is the caste behaviorally and morphologically specialized in reproduction (Keller 1991; Hölldobler and Wilson 1990; Wheeler 1911)

The majority of ant colonies begin with a mated queen. Mating typically occurs during a nuptial flight, in which queens and males disperse over a wide area and mate across colonies. In some species, however, mating takes place within the nest, where dispersal is absent and inbreeding is common (Keller and Passera 1992; Seifert 2010). After mating, the queen sheds her wings and can initiate a colony through one of two primary strategies: independent and dependent colony foundation (Hölldobler and Wilson 1990; S.M. Hakala et al. 2019)

In independent founding, a single queen initiates a colony on its own. She excavates a nest, commonly a soil chamber, lays her first brood and raises those offspring to adulthood and can be semi-claustral or claustral(Norman et al. 2016, 201; Augustin et al. 2011; Haskins and Haskins 1955). In semi-claustral founding, common among poneroids, the queen must leave the nest to forage so as to be able to feed the first larvae (Peeters 1997). This activity exposes the queen to a high risk of predation and starvation. In claustral founding, the predominant mode in the formicoids, the queen seals itself inside the nest and raises the brood without leaving, alleviating some of the risks. It fuels larval development by metabolizing the lipids and proteins stored in its thorax and gaster (Wheeler and Buck 1996).

Both semi-claustral and claustral founding are extremely risky; empirical estimates place the proportion of queens that survive this bottleneck and give rise to a mature colony at well below one per cent (Hölldobler and Wilson 1990; Wilson 1971). Consequently, the founding queen must invest heavily in brood care, nest cleaning and defense until the first generation of workers can take over these tasks and then be able to behaviorally specialize in reproduction

Dependent founding avoids much of this peril by exploiting an already established colony (Keller 1991). The most common form is budding (or colony fission), in which a newly mated queen departs together with a contingent of workers from her natal nest and establishes a

satellite nest (Heinze and Tsuji 1995; Vargo and Porter 1989). A second, less common route is temporary social parasitism: a queen infiltrates a host colony, often using chemical mimicry, rubbing the scent of the host colony onto herself to evade detection, then usurps the resident queen and relies on the host workers to rear her own offspring (Shimada et al. 2025; Iwai et al. 2021; Chernenko et al. 2013; Buschinger 1986). Because workers in these systems are always present, dependent foundresses have no intrinsic need to engage in activities that independent one's encounter, such as digging, foraging or brood care.

These contrasting founding routes set the stage for the second major trait of variation: how many reproductive queens a colony ultimately maintains, a feature that profoundly shapes its social organization, reproductive output, and long-term stability (Tsuji and Tsuji 1996; Keller 1991; Hölldobler and Wilson 1990)

Monogyny, the condition of having a single reproductive queen in a mature colony, is the ancestral and most widespread social arrangement in ants (Heinze and Foitzik 2009; Hölldobler and Wilson 1990). While it can arise from both founding strategies mentioned above, such as independent founding or via the budding of a single queen from her colony (see army ants for example (Berghoff et al. 2008)) it can also emerge indirectly through secondary monogyny: multiple queens co-found a nest (pleometrosis), but after the first workers emerge, workers typically eliminate all but one queen (Teggers et al. 2021; Rissing and Pollock 2019; Sommer and Hoelldobler 1995). While one would assume that high relatedness would exist in monogynous colonies, due to monogamy, queens mated to a single male being ancestral (Boomsma 2009; Hughes et al. 2008), polyandry, that is queen being mated so several males, is common (Barth et al. 2014; Jaffé 2014; Holbrook et al. 2007; Crozier and Fjerdingstad 2001; Ratnieks 1990). Despite this, monogyny persists in many lineages because a single, long-lived queen enhances colony stability, synchronizes brood cycles through unified reproductive control, and focuses selection pressure on traits that maximize fecundity and longevity.

Polygyny, the coexistence of multiple reproductive queens, has evolved independently several times in the ants (Boomsma et al. 2014; Keller 1991; Ross and Carpenter 1991; Hölldobler and Wilson 1977). Primary polygyny occurs when several queens found a nest together and remain reproductively active in the mature colony, whereas secondary polygyny arises when daughter queens are adopted into an established nest (Rissing and Pollock 2019; Tsuji and Tsuji 1996). Polygyny reduces intracolonial relatedness, thereby, weakening the kin-selection benefits that favor strict monogyny, yet it endures because multiple queens buffer the colony against queen loss, allow rapid expansion, and facilitate the formation of supercolonies in which numerous nests share workers and queens (Heikki Helanterä 2022;

Rissing and Pollock 2019; Seifert 2010). In lineages where queen:worker dimorphism is modest, polygyny can compensate for the lower per-queen productivity of each individual (Peeters 1997). In contrast, in clades with pronounced queen:worker size differences, polygynous queens often display reduced longevity and fecundity relative to their monogynous counterparts, reflecting a shift in selective pressure from maximizing the output of a single queen to preserving the reproductive potential of several co-breeding individuals (Keller and Genoud 1997). Colonies with multiple queens present do not need to be functional polygynous. For example, in dominance hierarchies or the presence of multiple unmated queens can make it look like there are multiple mated queens present, when instead the colony is functionally monogynous (Ito 1993; Medeiros et al. 1992; Ito 1990; Buschinger 1979). Similarly, even among multiple mated queens, reproductive output can differ (Ross 1988).

Notably, founding strategy and social structure can be polymorphic within a species (Reber et al. 2010; Johnson 2010; Gill et al. 2009; Johnson et al. 2007; Hora et al. 2005; Helms Cahan and Fewell 2004; Johnson 2002). A clear example is found in species where these traits are linked to a supergene: a genomic region containing multiple tightly linked genes that are inherited together. In two such species, *Solenopsis invicta* and *Formica selysi*, the supergene fixes two distinct colony forms: monogynous colonies, which produce dispersing queens, and polygynous colonies, which generate non-dispersing queens that are typically readopted by the maternal nest (Mona et al. 2025; Chapuisat 2023; Brelsford et al. 2020; Hunt 2020; Ross and Shoemaker 2018). This further exemplifies the remarkable plasticity of ant social organization.

Colony-founding strategies and queen-number dynamics generate a continuous spectrum of selective pressures on the reproductive individual, and this pressure drives the remarkable morphological radiation seen in the queen caste. All ant queens already differ from workers in a suite of characters that reflect their reproductive role: they are typically larger; possess an expanded thorax; and a swollen gaster that houses enlarged ovaries. How these basic differences are elaborated, however, depends on the social and life-history context of the species.

In monogynous colonies founded independently, the typical form is an alate queen, which is fully winged and often provisioned with large lipid reserves to survive solitary founding and sustain long-term reproduction (Peeters and Ito 2001b). In species with dependent founding (budding or temporary social parasitism) (but also independent ones, see Johnson 2010), ergatoid queens can appear: wingless or reduced-wing forms that retain a worker-like body plan (Molet et al. 2009; Molet and Peeters 2006). This shift redirects resources from

dispersal and toward other functions, as these queens disperse less and may rely on an existing worker force for colony maintenance. In extreme cases, such as the dichthadiiform queens of army ants, this specialization reaches its limit whereby queens are completely wingless, hyperfecund, and entirely dependent on the workers (Hölldobler 2016; Peeters 2012). Meanwhile, in species with macrogyne/microgyne dimorphism (e.g., *Temnothorax* or *Myrmica*), another contrast has arisen: macrogyne queens, which are large and winged, invest in dispersal and independent founding, while microgynes, which are smaller, often wingless, and adopted by workers, prioritize rapid colony integration(Negroni et al. 2019; Choppin et al. 2021; Johnson et al. 2007; Elmes 1991). Similarly, ergatoid and alate queen can both appear in *Pogonomyrmex* species (Johnson 2010; Johnson et al. 2007). Furthermore, in a minority of lineages, the queen caste is functionally diminished: in *Harpegnathos saltator*, queens are superseded by mated workers (gamergates) (Opachaloemphan et al. 2023; Liebig and Poethke 2004; Peeters et al. 2000); in *Rhytidoponera*, queens coexist with numerous gamergates, rendering their reproductive role largely redundant (Molet et al. 2008; Tay and Crozier 2000; Komene et al. 1999; Crosland 1990; Haskins and Whelden 1965); and in *Diacamma,* queens are entirely absent, with a single dominant worker performing all reproductive duties (Kikuta and Tsuji 1999; Nakata 1995; Peeters et al. 1992).

Thus, the diversity of queen forms, or lack thereof, across ants reflects the interplay of founding strategy, queen-number dynamics, and an underlying genetic architecture. This genetic framework is the origin of both the morphological and behavioral plasticity that distinguishes castes. However, how and where genes are expressed and influenced by the social environment can have profound effects on different caste phenotypes.

3.4 Phenotypic Plasticity, Social Environment, and Molecular Regulation

The striking morphological and functional diversity among ant queens and workers is not merely a product of evolutionary divergence, but a direct consequence of caste determination: a developmentally plastic process that channels individuals with highly similar genetic backgrounds into radically different phenotypes based on environmental input (Smith et al. 2008; Bourke and Franks 1991; Wheeler 1986; Light 1942). Although queens and workers develop from eggs with a common genetic pool, their divergent fates are not necessarily genetically predetermined (Trible and Kronauer 2017; Abouheif 2021). Instead, caste-specific phenotypes emerge through differential gene expression, orchestrated by nutritional, social, and physiological cues that activate or repress developmental and reproductive gene networks (Smith et al. 2008; Wheeler 1986). At the molecular level,

experimental studies in ants and other eusocial Hymenoptera, have established that the social environment can act as a potent regulator of gene expression (Majidifar et al. 2024; Pulliainen et al. 2022; Manfredini et al. 2014; 2022; Mikheyev and Linksvayer 2015). In both queens and workers, gene expression profiles are highly responsive to social context. For instance, workers carrying out different tasks (e.g., nurses vs. foragers) show differences in gene expression related to metabolism, neurobiology, and aggression (Walsh et al. 2018; Das and de Bekker 2022; Chen et al. 2022; Kohlmeier et al. 2019). Furthermore, certain genetic machinery, such as the insulin/insulin-like growth factor signaling (IIS) pathway, juvenile hormone (JH) regulation and vitellogenin, have been recurrently utilized by social insects for the regulation of behavior and caste determination (Ferreira et al. 2025; Li et al. 2024; Miyazaki et al. 2021; Chandra et al. 2018; Kohlmeier et al. 2018; Corona et al. 2013; Libbrecht et al. 2013; Cahan et al. 2011).

During critical life stages, such as colony founding and mating, queens also exhibit significant transcriptional plasticity (Ferreira et al. 2025; Nagel et al. 2020; Lucas and Keller 2018). Studies show that queens founding colonies alone versus in groups, or placed in different group sizes, display wide differences in their transcriptional states in the brain (Helmkampf et al. 2016). This highlights how the initial social environment immediately influences the queen's metabolism, stress response, and reproductive allocation, often mediated by the regulation of a relatively small, conserved subset of regulatory genes.

While gene expression provides acute, flexible responses to social and nutritional cues, epigenetic mechanisms offer a complementary layer of regulation that can stabilize caste-specific phenotypes over time (Weiner and Toth 2012). These include chromatin remodeling, histone modifications (e.g., acetylation, methylation), and, in some species, DNA methylation, all of which alter gene accessibility without changing the DNA sequence itself (Bonasio 2014; 2012). In Hymenoptera, distinct caste identities are associated with stable epigenetic signatures around genes governing development, neurogenesis, and reproduction. Those signatures can emerge early and help “lock in” irreversible phenotypes (Bonasio 2014; Weiner and Toth 2012; Oldroyd and Yagound 2021). Crucially, epigenetic marks are not static: they can be dynamically remodeled in response to adult social context (*e.g.*, task shifts), allowing for both long-term commitment and short-term plasticity (Glastad et al. 2020; Alvarado et al. 2015). Thus, epigenetics serves not only as a developmental switch that translates early environmental input into lasting caste fate, but also as a modulator of ongoing behavioral and physiological flexibility in established colonies.

Despite these insights into developmental caste regulation, adult queen plasticity in established colonies remains critically understudied, a gap made more apparent when

contrasted with the well-documented transcriptional and behavioral flexibility of workers. We know a lot here about how gene expression changes during the transition between nurses and foragers, during queen loss, and between minor and major workers (Gilbert 2024; Liu et al. 2023; Das and de Bekker 2022; Kohlmeier et al. 2019; Simola et al. 2016). These examples underscore how worker phenotypes are not fixed but continuously remodeled by the social context. Yet for queens, the capacity to adjust behavior, physiology, or gene expression in response to social changes has only begun, most often focused on age and reproductive activities (Lenhart et al. 2025; Stoldt et al. 2025; Harrison et al. 2021; Negroni et al. 2021; Feldmeyer et al. 2022)., leaving a fundamental gap in our understanding about the flexibility of the reproductive core of the colony across the ants.

This gap is significant because queen plasticity may reflect fundamental trade-offs in social evolution. Queens are not merely static reproductive units; they are shaped by the life-history strategies of their species and can vary dramatically in morphology, behavior, and social role (Molet et al. 2007; Elmes 1991; Medeiros et al. 1992; Ward 1986). If queens were rigid in their morphology, life history and behavior, they would indeed be more akin to the germline in multicellular organisms, as they are often portrayed as in ants and, thus, the need for this variation would be limited. Therefore, the capacity or the lack for queens to respond flexibly to changes at the colony level may carry evolutionary consequences. For example, flexibility may enhance colony resilience, allow queens to assert reproductive control, or buffer against worker dominance, but it may also incur costs in terms of fitness, developmental constraint, or reduced specialization.

Having documented this diversity, a central question emerges: how is queen behavioral variability expressed across the ant phylogeny, which incorporates different colony structure, as well as founding strategies, and to what extent can queens adjust their behavior or gene expression in response to environmental cues? Observations such as claustral-founding queens reducing brood care once workers appear, or queens altering physiology after worker removal, demonstrate that plasticity exists (Majidifar et al. 2024; Ortiz-Alvarado and Rivera-Marchand 2020). It remains unclear whether this flexibility is an adaptive response driven by ecological pressures or is constrained by lineage-specific developmental programs. The molecular and behavioral mechanisms that enable, or limit, such adjustments, both during normal colony life and under changes, remain poorly understood. This knowledge gap hampers our ability to explain how the reproductive core of an ant colony maintains efficiency across diverse social systems.

On a broader level, studying the queen caste in ants offers a unique pathway to understanding major evolutionary transitions. Although solitary ancestral forms have been

lost during the evolution of eusociality, the queen's own life trajectory, from solitary foundress to integrated reproductive unit, preserves a living record of that transition. Therefore, its plasticity provides a direct window into how independent individuals become functionally specialized subunits within a larger collective. By decoding the mechanisms underlying its behavioral and physiological shifts, we gain not just an insight into ant societies, but also into the broader evolutionary logic of how autonomy gives way to integration past a major evolutionary transition. Because a queen's life trajectory spans from solitary foundress to integrated reproductive unit, its plasticity mimicking this transition, and its morphology and behavior revealing the adaptations made inside the eusocial system.

4. Aim of the Thesis

The goal of this thesis is to advance our understanding of the behavioral and molecular mechanisms that underlie queen plasticity in ants, thereby filling the knowledge gaps outlined in the introduction. To achieve this, I examined a broad suite of ant species, combining detailed behavioral assays with molecular profiling to tease apart how queen behavioral flexibility is shaped by phylogeny, life-history strategy, and social context.

The first chapter: “Queen behavioral plasticity across the ants” examines whether the socially regulated behavioural plasticity that has been reported for queens of a few species is common among the ants in general and whether it correlates with phylogenetic lineage or with specific life-history traits, such as colony foundation strategy and social structure. I hypothesize that queens of independently founding species, which must perform brood care during the founding stage, will retain a greater capacity for behavioral adjustment than queens of dependent-founding species, in which brood care is never required. To investigate queen behavioral plasticity, I assembled a behavioral dataset of 38 species spanning several subfamilies, obtained through field collections, collaborations, and colleagues. For each species, I recorded queen brood care behaviour in the presence and absence of workers, allowing us to quantify the extent of behavioral plasticity across the phylogeny.

The second chapter: “Unmated queens show worker-like behavior and gene expression in polygynous colonies of the ant *Stigmatomma pallipes*” focuses on the polygynous ant *Stigmatomma pallipes* to explore how queen specialization interacts with social organization. Here, we hypothesize that queens in polygynous colonies may display worker-like behaviour and gene expression patterns, with variation linked to mating status or dominance hierarchies. To elucidate the variation of queens in polygynous colonies of *S. pallipes*, I first investigated the activity and behavior of the queens. Furthermore, I evaluated the mating status and how it related to the variance in activity and behavior. To elucidate the transcriptomic variants between queens, as well as to compare with workers, we generated one of the first genome assemblies of this subfamily and used it to analyze transcriptomic data of the brain and the fat body. This approach allows me to reveal which factors influence queen variation in a polygynous system.

The third chapter: “Transcriptomic changes associated with the socially regulated behavioral specialization of ant queens” investigates the molecular basis of socially regulated behavioural specialization in *Lasius niger* queens. My hypothesis is that queen gene expression is dynamically modulated by social cues, such as the presence of workers or larvae, and that these transcriptional changes involve conserved pathways previously

implicated in caste or task differentiation. I, therefore, generated transcriptomic data for queens that had previously been shown to alter their brood care behaviour under different social conditions, identified differentially expressed genes, and linked these genes to known functional categories to infer the regulatory networks driving the behavioural shift.

Together, these three chapters provide a comprehensive assessment of queen behavioral plasticity, from its phylogenetic distribution, through its manifestation in a specific polygynous system, to the underlying transcriptional mechanisms, offering a solid foundation for answering how queens balance reproductive specialization in eusocial insects.

Evidence for social control of queen specialization across the ant phylogeny

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Abstract

The components of complex biological systems are specialized in performing specific tasks, thereby optimizing system effectiveness. This principle is exemplified in insect societies, where the reproductive division of labor between queens, which monopolize reproduction, and workers, which perform non-reproductive tasks, mirrors the germline-soma distinction in multicellular organisms. The notion that queens are intrinsically specialized as egg-laying machines has been challenged in three social insect species: their specialization appears to be shaped by the presence of workers. However, how widespread this social control of queen specialization is across the ant phylogeny remains unclear. To address this gap, we tested whether queen behavioral specialization is maintained by the presence of workers across 38 ant species. We further examined whether phylogeny and social structure influence queens' brood care behavior. Half of the species investigated (n = 19) expressed significant increased brood care in the absence of workers, therefore, showing queen behavioral flexibility. Among species lacking this flexibility, five nevertheless performed brood care regardless of worker presence. Across the studied species, queen behavioral flexibility was not phylogenetically conserved. Instead, social structure significantly influenced the extent of brood care expressed by queens. Our results suggest that behavioral flexibility is common across the ants, but, in some species, we revealed variance in the ability to be flexible. In addition to advancing our understanding of queen specialization and division of labor in ant colonies, our findings may offer novel, relevant criteria for assessing social complexity across insect societies.

Keywords

Social insects, behavioral plasticity, founding strategy, major evolutionary transition, monogyny, polygyny

Introduction

Major evolutionary transitions (METs), such as the emergence of cells, eukaryotic cells, multicellular organisms, and insect societies, are characterized by the repeated evolution of tightly integrated, specialized compartments that cooperate to form new higher-level units (West et al. 2015; Szathmáry and Smith 1995). In multicellular lineages, the germline is irreversibly committed to reproduction, while somatic cells perform the diverse tasks required for organismal survival. A striking parallel is observed in eusocial insects, where a reproductive queen caste, analogous to the germline, coexists with a non-reproductive worker caste, analogous to the soma, which maintains the colony (Helanterä 2016; Detrain and Deneubourg 2006; Wheeler 1911). Queens are morphologically and behaviorally adapted to be strictly reproductive and decoupled from colony maintenance tasks.

Despite these apparent specializations, queens do express non-reproductive behavior. Unmated queens have been reported to exhibit worker-like behavior, showing that morphological alone does not necessitate behavioral specialization (Vieira et al. 2012; Nehring et al. 2012; Floris et al. 2021). Furthermore, during the founding phase, queens need to express various behaviors, including foraging, brood care and digging (Hölldobler and Wilson 1990; Augustin et al. 2011; Woodard et al. 2013; Mizumoto et al. 2021). In a few species, the queen's behavioral specialization, upon which they focus on reproduction, is controlled by the presence of workers and is reversible (Majidifar et al. 2024; Woodard et al. 2013). While often investigated in the context of colony foundation, less is known about how behaviorally flexible queens are within established colonies and how certain life-history traits affect this flexibility.

Across the eusocial insects, the ants show high variability in social systems, founding strategies and queen caste morphology, making them an excellent system to investigate the flexibility of queen specialization (Hölldobler and Wilson 1990; Keller 1991; Tsuji and Tsuji 1996). Queen caste morphology can range greatly from individuals several times larger than the worker caste, to small worker-like ergatoid queens, some even smaller than the workers (Divieso et al. 2020; Molet et al. 2007; Peeters 1991). Similarly, the number of queens, the social structure of a colony, can differ across species and populations, from a single individual (monogynous) to several ones (polygynous) (Heinze and Foitzik 2009; Baroni Urbani 1998; Keller 1991; Hölldobler and Wilson 1977; Buschinger 1968). This social structure is often intertwined with the founding strategy. While queens often found their nest independently and, thus, have to express several behaviors previously mentioned above, in some species, queens found their nest dependently, in the presence of workers (Cronin et al. 2013; Vargo and Porter 1989; Vargo 1993; Hölldobler and Wilson 1977). It has been shown

that in two monogynous species with independent colony foundation, queen behavioral specialization can be proxied by the expression of brood care, is reversible, and depends on the social environment (Majidifar et al. 2024). These findings raise the question of how variable this behavioral specialization and social control is across the ants.

We hypothesize that queen behavioural flexibility in ants is more widespread than previously recognized, not because it is absent, but because historically, research has focused on worker flexibility, while queens have been relegated to be reproductive units (Beshers and Traniello 1996; Camargo et al. 2007; Seid and Traniello 2006; Tripet and Nonacs 2004). We further expect this flexibility to vary across the ant phylogeny, reflecting the diversity of life history traits among the ants, from varying queen-worker dimorphism to colony size. We expect queens from monogynous species, which must express brood care during the founding stage, to be more flexible than those from polygynous ones.

To test this, we conducted experimental investigation of queen behavioral flexibility across 38 species of ants, representing several subfamilies and spanning a broad phylogenetic and ecological range within the Formicidae. We elucidate that queen behavioral flexibility is common across the ant phylogeny and not restricted to specific clades. Furthermore, as hypothesized, monogynous species, on average, exhibited a stronger change in brood care behavior than polygynous species.

Methods

Species Collection

To investigate queen behavioral flexibility across ant species, we sampled a total of 69 species, consisting of 413 colonies and 474 queens, using four complementary approaches (Supplementary Table 1). First, we collected accessible species in Germany and France (n = 11 species). Second, we incorporated established laboratory colonies maintained by us and collaborators (n = 12 species). Third, during fieldwork at the Huyck Preserve, New York (42°30′56.23″ N, 74°08′21.34″ W) in May 2023, we examined common North American species either on-site or in Mainz, Germany (n = 11 species). Finally, to include underrepresented tropical taxa, queens were collected and filmed at the Panguana Biological Station, Huánuco, Peru (09°37′S, 74°56′W) in February 2024 (n = 35 species). The origins of the queens varied. European species and those from collaborators were often sourced from colonies established or maintained in the lab. For species from North America and Peru, queens and worker were directly collected from the field. The collection efforts included the turning of rocks, opening of sticks and acorns, excavation of soil, opening cavities in trees, and the disassembling of rotten logs and wood on the forest floor. Species were identified using a combination of resources for morphological species determination, collaborator expertise, and, for tropical taxa, DNA barcoding (CCBD, Canada), ensuring reliable taxonomic resolution.

Behavioral Experiments

To test whether queen behavior depended on the social environment, we conducted standardized brood care assays. Each queen was placed with five larvae, where possible being between the second and fourth instar, and five workers in a Petri dish lined with moistened blue plaster (50 mm diameter, nine mm height, for larger species: 90 mm diameter, 15 mm height, Falcon USA) and positioned under a camera (Sony FDR-AX33 Handycam, Sony Corporation, Japan). Depending on the location, several Petri dishes were filmed simultaneously as a batch. After a 24 h acclimation period, a batch was filmed for 60 minutes. Directly after filming, the workers were removed. Filming commenced two more times: 24 h and 48 h after worker removal for 60 min each, respectively. This yielded three recordings per queen: one with workers and larvae (with workers), and two with larvae only (24h after worker removal and 48 h after worker removal). This design provides each queen with an internal control for the presence of workers (with workers), while the other time points capture the queen's behavior in the absence of workers after identical handling and acclimation periods. We, hereby, followed the design established by Majidifar et al. 2024, which showed that the effect of worker is similar in this setup compared to a setup with

queens with and without workers at the same time point. Therefore, using this approach, we could increase the number of queens per species tested. To account for temporal- and spatial-positioning effects, the filming of queens of the same species was staggered across time and randomly placed under the camera. Colonies collected in Peru were filmed 24 h after collection, and queens were subsequently returned to their collection place when filming was completed.

Video recordings were analyzed with the BORIS software (Friard & Gamba, 2016) with scan sampling. In brief, each queen was observed for the first ten seconds of every minute, excluding the first and last five minutes of each recording to avoid behavioral fluctuations because of disturbances. For each scan, we scored the presence or absence of observed brood care, defined as active manipulation of larvae, including licking and transport (antennation alone was not considered brood care). This resulted in 51 scans per video and 153 scans per queen.

Statistical Analyses

We evaluated queen behavioral flexibility within species with at least three replicates, which resulted in a total of 38 species being used (Table 1).

Table 1: Investigated species for queen behavioral flexibility regarding brood care. For each species, the subfamily, the number of colonies investigated, the number of individual queens (replicates), and the country of origin are listed. Sometimes multiple queens from the same polygynous colony were used, therefore, explaining the discrepancy between the number of colonies and replicates.

Species	Subfamily	Colonies	Replicates	Origin
Stigmatomma pallipes	Amblyoponinae	19	19	USA
Diacamma rugosum	Ponerinae	3	3	Thailand
Odontomachus haematodus	Ponerinae	3	3	Peru
Mayaponera constricta	Ponerinae	4	5	Peru
Dolichoderus plagiatus	Dolichoderinae	15	15	USA
Linepithema humile	Dolichoderinae	18	18	France, Peru
Rhytidoponera metallica	Ectatomminae	3	3	Australia
Lasius flavus	Formicinae	3	3	Germany
Lasius niger	Formicinae	3	3	Germany
Plagiolepis cf	Formicinae	3	3	Peru
Formica fusca	Formicinae	4	4	Germany
Camponotus americanus	Formicinae	5	5	USA
Camponotus lateralis	Formicinae	5	5	France
Formica rufibarbis	Formicinae	6	6	Germany
Lasius americanus	Formicinae	7	7	USA
Anoplolepis gracilipes	Formicinae	15	15	Thailand
Paratrechina longicornis	Formicinae	15	15	Spain
Lasius emarginatus	Formicinae	19	19	Germany

Solenopsis geminata	Myrmicinae	3	3	Peru
Carebara panamensis	Myrmicinae	1	4	Peru
Pheidole pallidula	Myrmicinae	4	4	France
Tetramorium caespitum	Myrmicinae	5	5	Germany
Tetramorium simillimum	Myrmicinae	2	5	Peru
Messor capitatus	Myrmicinae	6	6	Spain
Crematogaster brasiliensis	Myrmicinae	3	7	Peru
Cyphomyrmex rimosus	Myrmicinae	9	11	Peru
Aphaenogaster picea	Myrmicinae	12	12	USA
Cardiocondyla obscurior	Myrmicinae	5	15	Japan
Myrmica punctiventris	Myrmicinae	15	15	USA
Pogonomyrmex barbatus	Myrmicinae	15	15	USA
Cephalotes spinosus	Myrmicinae	4	17	Peru
Monomorium pharaonis	Myrmicinae	5	17	Denmark
Wasmannia auropunctata	Myrmicinae	17	18	Spain
Crematogaster carinata	Myrmicinae	10	21	Peru
Solenopsis invicta	Myrmicinae	23	23	USA
Temnothorax americanus	Myrmicinae	24	24	USA
Temnothorax longispinosus	Myrmicinae	30	30	USA
Temnothorax rugatulus	Myrmicinae	30	30	USA

We modeled queen brood care activity with linear mixed-effects models using the lmer() function (lme4 package, Bates et al. 2015). The response variable was the square-root transformed rate of brood care, and the only fixed effect was the time point (with workers, 24 h without workers and 48 h without workers). To account for the repeated nature of the data, we included random intercepts for each queen and for each filming session or batch. In polygynous species, where several queens originated from the same colony, we added a further random intercept for colony of origin.

For species in which the sum of brood care events was close to zero, we summarized the observations to designate queens as: "1", if a queen was observed to perform brood care at least once during a time point; and "0", if otherwise (i.e., no brood care was observed). These binary data were analyzed with a binomial generalized linear mixed-effects model, keeping the same fixed and random effects as mentioned above.

When at least one of the two time points without workers (24 h and 48 h after worker removal) differed significantly in brood care expressed from the one with workers, we found evidence for brood care flexibility. Species that did not meet this criterion, where neither time point without workers differed in the expressed brood care from the one with workers; we could not find evidence for brood care flexibility. Therefore, using these observations, we assigned each species a binomial value which indicated the ability of the species to be

behaviorally flexible (1) or not (0). For species that did not display flexibility, we compared the brood care expressed in the presence of workers and tested whether it differed from zero. If the mean was significantly greater than zero, the species expressed brood care, regardless of worker presence. If the mean did not differ from zero, we found no evidence that queens expressed brood care in the presence of workers. Thus, we categorized species into three different categories based on: evidence of flexibility; no evidence of flexibility, with no brood care observed; and no evidence of flexibility, but with brood care observed.

To assess phylogenetic signal for queen behavioral plasticity, we first calculated the maximum log fold change (MLFC) in brood care behavior for each species. To do so, we first compared the amount of brood care expressed with workers between the two time points (at 24 h or 48 h without workers) and chose the one with the highest mean of brood care. We then calculated the MLFC between the chosen time point and the time point with workers. The MLFC was mapped as a continuous trait onto the ant phylogeny from Economo et al. (2018). Using the phylosig() function in phytools v.2.4-4 (Revell 2012) with the parameters (method="lambda"; and test=TRUE), we estimated Pagel's λ and performed a likelihood ratio test (LRT) against the null hypothesis of no phylogenetic signal (λ=0).

For discrete traits, such as the binomial flexibility and the assigned categories, the phylogenetic signal was tested using Geiger's fitDiscrete(v.2.0.11; Pennell et al. 2014)) with an equal-rates (ER) model: we compared a full model (estimating λ) against a constrained null model (λ=0) via LRT, where the test statistic LR = 2×(lnL_full - lnL_null) was evaluated against a χ^2 distribution with one degree of freedom.

To evaluate whether social structure, specifically monogynous versus polygynous, influenced the magnitude of queen brood care plasticity, we excluded five species with both social forms in the dataset (*Mayaponera constricta*, *Solenopsis invicta*, *Stigmatomma pallipes*, *Temnothorax longispinosus*, *Temnothorax rugatulus*) to ensure unambiguous assignment. The remaining 33 species were classified as monogynous or polygynous based on published records (Supplementary Table 2). For species lacking documented social structure, we inferred it from field observations, with nests containing multiple dealate queens being conservatively assigned to the polygynous category. To test if these two groups, monogynous and polygynous species, differed in log fold change, we utilized Welch's two-sample t-test, which does not assume equal variances between groups, thereby, accommodating observed heteroscedasticity in the response variable.

To assess whether queen behavioral flexibility differed between social structures *within* species, we reanalyzed four socially polymorphic species (*S. invicta*, *S. pallipes*, *T. longispinosus*, *T. rugatulus*), excluding *Mayaponera constricta* due to insufficient replicates. For each queen, we calculated the MLFC in the same fashion as described above but for

each queen instead.. We then compared MLFC distributions between monogynous and polygynous queens using two sample t-tests per species, treating individual queens as independent replicates.

Results

Queen behavioral plasticity found in half of the species investigated

To assess queen behavioral flexibility, we analyzed brood care dynamics across 38 ant species (with ≥3 replicates per species) using linear or binomial generalized mixed-effects models depending on the distribution of the data for each species (Supplementary Table 2). Each model tested the effect of worker removal by comparing brood care activity during worker presence against brood care activity in worker absence (24 h and 48 h post-removal). A species was classified as behaviorally flexible if the model output indicated a statistically significant increase in brood care at either post-removal time point relative to the control condition ($p < 0.05$). We found evidence for queen behavioral plasticity in 19 of 38 species (50%) (Figure 1, Supplementary Table 3).

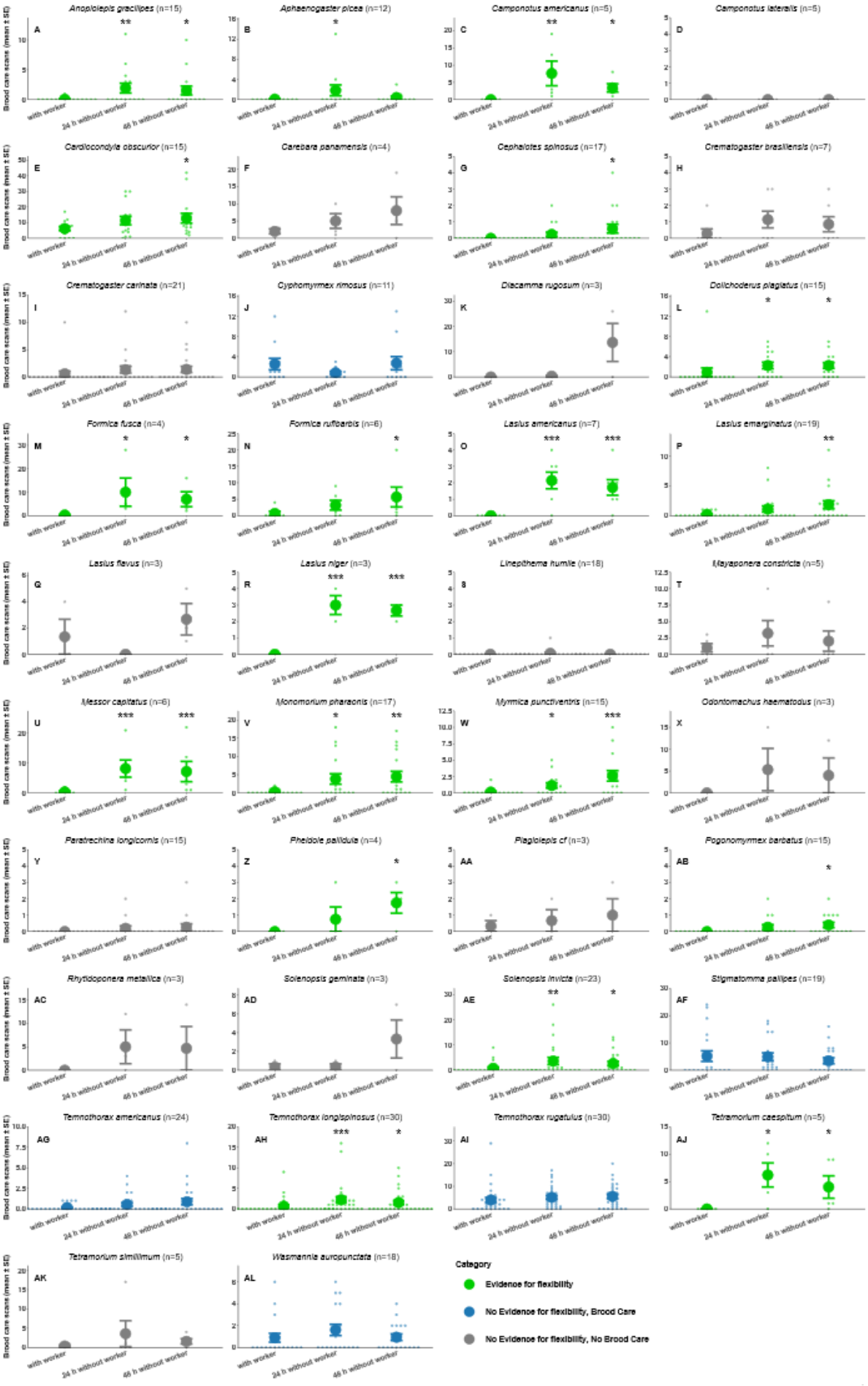
Anoplolepis gracilipes (n=15)
Aphaenogaster picea (n=12)
Camponotus americanus (n=5)
Camponotus lateralis (n=5)
Cardiocondyla obscurior (n=15)
Carebara panamensis (n=4)
Cephalotes spinosus (n=17)
Crematogaster brasiliensis (n=7)
Crematogaster carinata (n=21)
Cyphomyrmex rimosus (n=11)
Diacamma rugosum (n=3)
Dolichoderus plagiatus (n=15)
Formica fusca (n=4)
Formica rufibarbis (n=6)
Lasius americanus (n=7)
Lasius emarginatus (n=19)
Lasius flavus (n=3)
Lasius niger (n=3)
Linepithema humile (n=18)
Mayaponera constricta (n=5)
Messor capitatus (n=6)
Monomorium pharaonis (n=17)
Myrmica punctiventris (n=15)
Odontomachus haematodus (n=3)
Paratrechina longicornis (n=15)
Pheidole pallidula (n=4)
Plagiolepis cf (n=3)
Pogonomyrmex barbatus (n=15)
Rhytidoponera metallica (n=3)
Solenopsis geminata (n=3)
Solenopsis invicta (n=23)
Stigmatomma pallipes (n=19)
Temnothorax americanus (n=24)
Temnothorax longispinosus (n=30)
Temnothorax rugatulus (n=30)
Tetramorium caespitum (n=5)
Tetramorium simillimum (n=5)
Wasmannia auropunctata (n=18)
Brood care score (mean ± SE)
with worker
24 h without worker
48 h without worker
Category
Evidence for flexibility
No Evidence for flexibility, Brood Care
No Evidence for flexibility, No Brood Care

Figure 1: Expression of brood care behavior in the presence or absence of workers in 38 species of ants. Dots represent individual queens. Larger dots represent the mean brood care (+-se). Colors represent the different categories of brood care flexibility assigned to examined ant species.

In the remaining 19 species, queens did not show a significant change in brood care behavior upon the removal of workers at either time point (24 h and 48 h after worker removal). Therefore, we found no evidence for flexibility in these species. To test whether brood care behavior in the presence of workers in those 19 species exceeded what would be expected by chance, we performed a one sample t-test to compare the mean number of brood care events against 0. In five species, we detected significantly greater brood care expression than zero ($p < 0.05$).

Phylogeny does not determine queen brood care response

Across the 38 species analyzed, we quantified queen behavioral flexibility by calculating the maximum log fold change (MLFC) in brood care between the with-workers condition and the time points without workers (either 24 h or 48 h) that exhibited the highest mean brood care activity (Figure 2). We tested for a phylogenetic signal in this continuous measure of plasticity using Pagel's λ. We found no evidence of phylogenetic signal, with estimated λ being effectively zero (≈ 0.00008, LRT: $\chi^2_1 = 0.0$, $p = 1.00$), indicating that closely related species did not exhibit more similar brood care responses than expected under a null model of evolutionary independence. This pattern persisted for the discrete traits as well: for both, the binary flexibility variable and the categorial approach, the full model did not differ from a model with Pagel λ being equal to 0 (for both: LRT: $\chi^2_1 = 0.0$, $p = 1.00$).

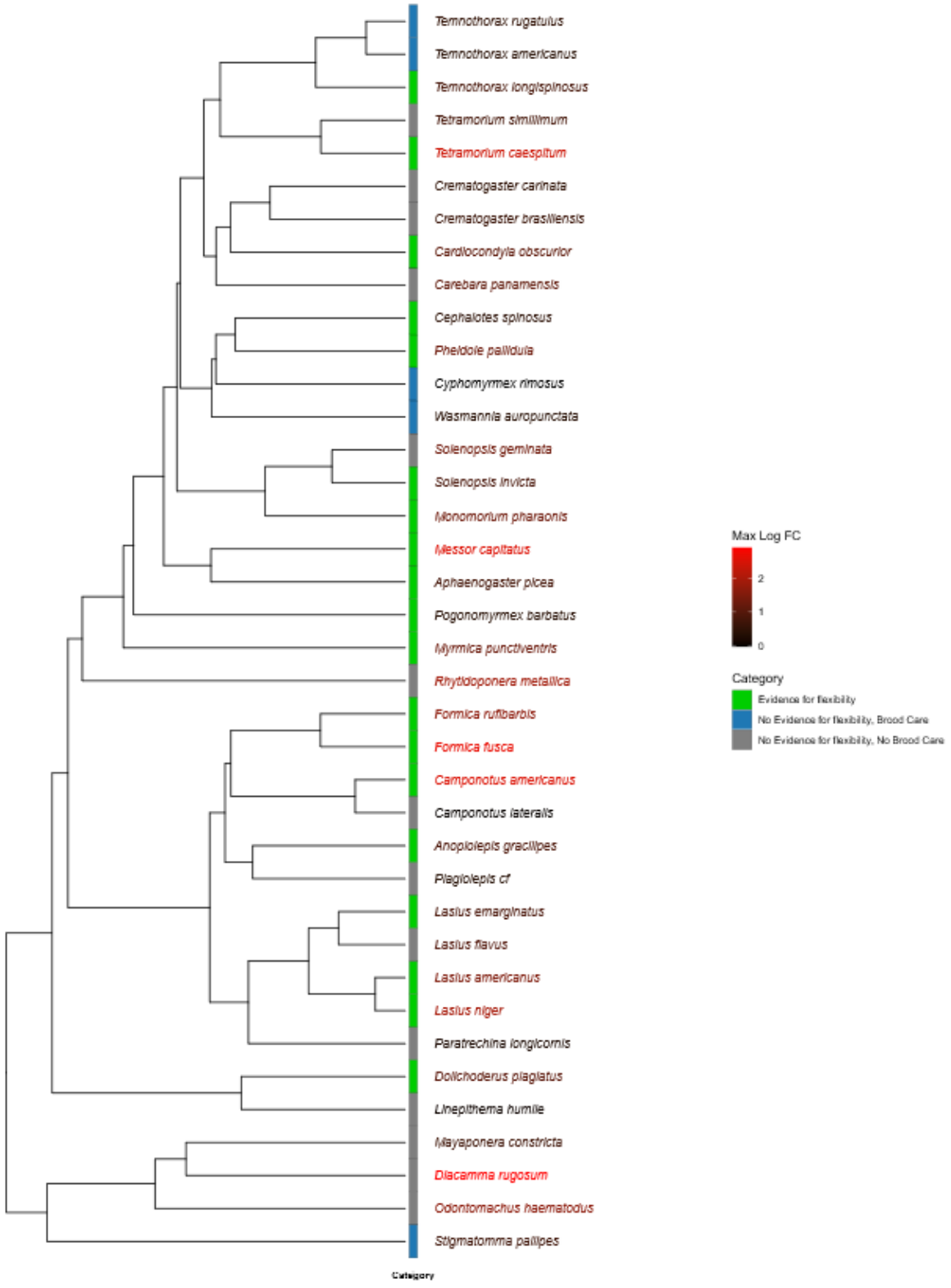

Figure 2: Phylogenetic tree of the 38 species indicating variation in queen behavioral plasticity across the ants. The color of species names indicates the magnitude of brood care increase in the absence of workers (maximum log fold change) of that species with black being low and red being high. Colored bars indicate categories based on significant differences in brood care between the presence and absence of workers.

Monogynous species express more brood care than polygynous species in the absence of workers

To assess the effects of social structure on queen behavioral flexibility, we compared the MLFC in brood care between species differing in social structure, specifically monogynous versus polygynous. Monogynous species (n = 21) exhibited significantly greater brood care plasticity than polygynous species (n = 12; Welch's t-test, t = 3.28, df = 30.2, p = 0.0026; Fig. 3). The mean MLFC was 1.45 (±0.19 SE) in monogynous species and 0.67 (±0.27 SE) in polygynous species.

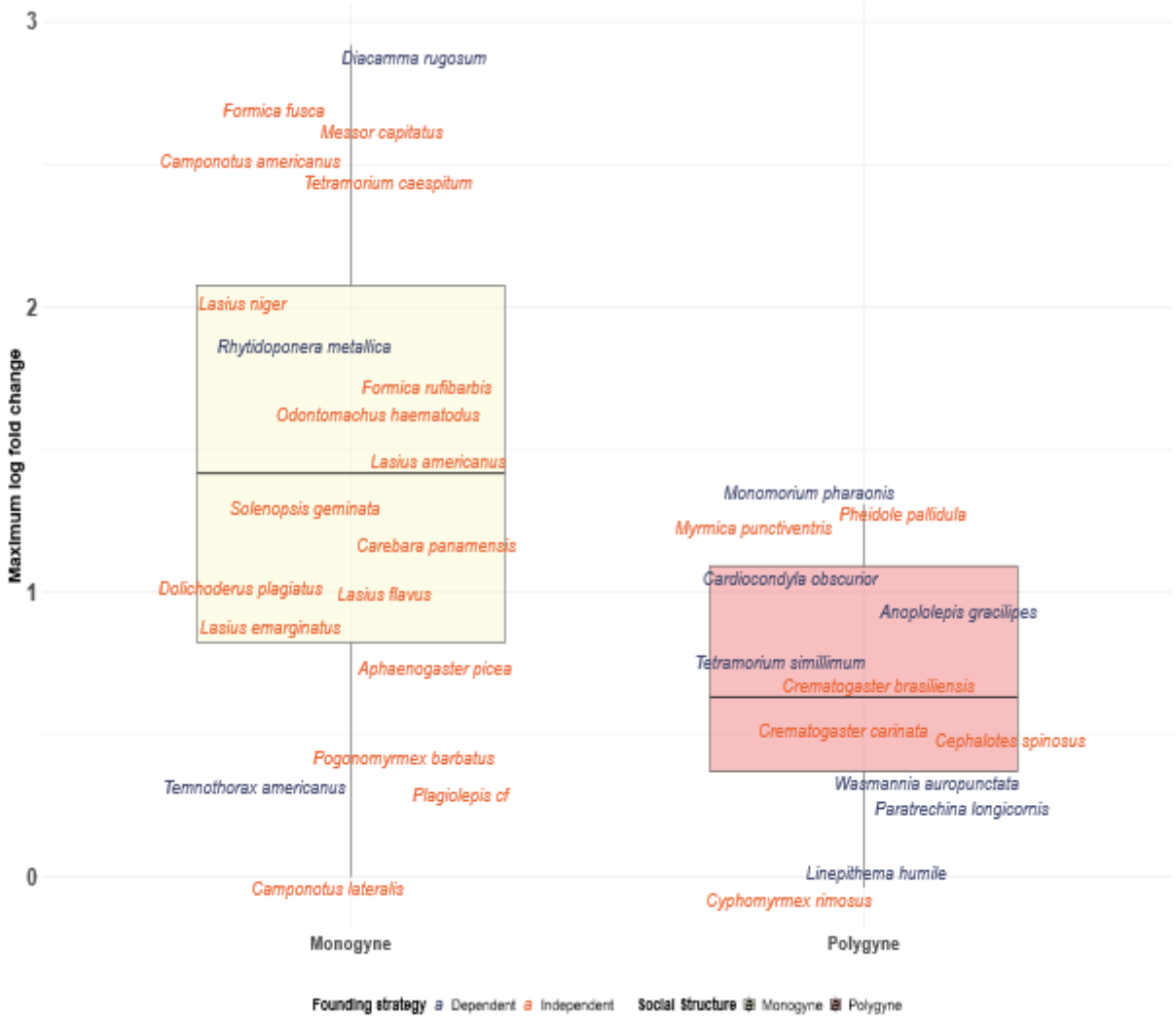

Figure 3: Monogynous species express greater changes in brood care magnitude than polygynous species. Colors of names indicate the founding strategy. Box plots and error bars indicate mean ± SE for each social structure, which is indicated by color. Maximum log fold change (y-axis) refers to the maximal magnitude of changes in brood care behavior in queens between the presence and the absence of workers.

To further understand the relationship between behavioral flexibility and colony structure, we investigated whether queens from socially polymorphic species modulate their brood care

behavior in response to colony structure (monogynous vs. polygynous). We compared MLFC distributions between social structures using t-tests for each of four socially polymorphic species. No significant differences were found in any species: in *Solenopsis invicta*, monogynous queens showed a mean MLFC of 1.49 versus 1.07 in polygynous queens ($p = 0.51$); in *Stigmatomma pallipes*, the values were 0.71 (monogynous) and 1.31 (polygynous, $p = 0.67$); in *Temnothorax longispinosus*, 1.43 (monogynous) vs. 0.78 (polygynous, $p = 0.15$); and in *Temnothorax rugatulus*, 0.94 (monogynous) vs. 1.32 (polygynous, $p = 0.53$).

Discussion

Queens in established insect colonies are often compared to the germline of a multicellular organism, which specializes in reproduction. While behavioral flexibility in queens has been shown in a few species, its evolutionary conservation has remained unknown. Here we show that across 38 ant species this flexibility is rather common, with half of the species expressing behavioral flexibility regarding brood care. We detected no phylogenetic signal for this trait. Furthermore, we elucidated that the social structure of the species plays a role in this flexibility. Queens from monogynous species express a stronger change in brood care behavior compared to polygynous ones. In socially polymorphic species, which express both social structures, we could only detect an effect of social structure in a single species *Solenopsis invicta*.

We found behavioral flexibility, upon which queens expressed brood care in the absence of workers, was not restricted to certain life-history traits, such as founding strategy and social structure. We detected it in both monogynous, independent and polygynous, dependent species. While in previous studies queen behavior variation across single species has been investigated (Helms Cahan and Fewell 2004; Molet et al. 2007) and similarly it has been shown that the queen behavioral specialization can be flexible upon changes in social environment (Majidifar et al. 2024; Ortiz-Alvarado and Rivera-Marchand 2020), here, we show that the latter could be a common trait across the ants. The ability of the queen caste to adjust its behavior accordingly to the social environment reveals variation beyond a purely reproductive role, challenging the traditional view of ant queens as rigidly specialized germline equivalents (Boomsma and Gawne 2018; Wheeler 1911).

Across the 19 species, where we did not find evidence for queen behavioral flexibility, we revealed that in five species, queens expressed brood care behavior, regardless of worker presence, further highlighting queen behavioral variation past the reproductive role. One reason for the expression could be how changes in social environment influence queen behavior. Previous research showed both that while in some species even a single worker is enough to induce queen behavioral specialization (Majidifar et al. 2022), in other species, workers can still be present and queens can change their behavior upon changes in the social environment (Ortiz-Alvarado and Rivera-Marchand 2020). In some species, queen behavioral specialization might still include brood care behavior, for example in species that rely on nutrients from the larvae, such as *Stigmatomma pallipes* (Traniello 1982; Haskins 1928, Chapter 2).

In 14 species, we could not find evidence for queen behavioral flexibility and no brood care in the presence of workers. Across these species, in most of them (Figure 1), we still see some brood care in the absence of workers, while among three species, we detected very little

brood care overall. These species included *Camponotus lateralis* (Figure 1 D, Total brood care events = 0)*, Linepithema humile* (Figure 1 S, Total brood care events = 1) and *Paratrechina longicornis* (Figure 1 Y, Total brood care events = 7). The latter two share several characteristics, such as being polygynous and globally invasive species, which have lost the ability for queens to perform nuptial flights and instead queens mate inside the nest (Pearcy et al. 2011; Ingram 2002; Krieger and Keller 2000). While this could hint toward a further derived queen state, we did find flexibility in species with similar traits, such as *Monomorium pharaonis* and *Anoplolepis gracilipes.*

We found no evidence that variation in queen behavioral flexibility among the 38 ant species examined is explained by phylogeny. Through three complementary approaches: the first treated brood care as a continuous measure using the maximal log fold change (MLFC); the second treated measures of flexibility as binary (present/absent)'; and a third approach that used the created categories of species based on the presence and absence of brood care based on the social environment. We could show that estimates of Pagel's λ was indistinguishable from zero, indicating an absence of phylogenetic signal. Thus, queen behavioral plasticity is not clustered in a particular clade but rather distributed in a mosaic fashion across the ant phylogeny, similarly to other traits, such as cuticular hydrocarbons (Menzel et al. 2017). Therefore, the trait could have been retained or lost from a common ancestor or evolved independently multiple times.

One such trait, social structure*, i.e.* the number of queens, affects the amount of brood care expressed. Queens from monogynous species express more brood care behavior in the absence of workers than queens from polygynous species. Social structure is strongly intertwined with founding strategies (Tsuji and Tsuji 1996; Keller 1991). In most monogynous species, queens have to express brood care during colony founding. In many polygynous species, queens get readopted after the nuptial flight or never leave the nest in the first place as described above. Therefore, there arises no need for queens to express brood care or even experience changes in the social environment. Consequently, it is likely that queen behavioral plasticity is integrally linked to particular life-history traits.

We were unable to replicate these results in the four investigated social polymorphic species Notably *S. invicta* showed no significant plasticity difference between monogyne and polygyne queens. This is surprising, as the social structure and founding strategy in this species are genetically regulated by a supergene (Ross and Shoemaker 2018; Wurm et al. 2011; Cassill 2002; Ross et al. 1996; Vargo 1993; Vargo and Porter 1989; Ross 1988). This suggests that supergene-regulated social forms may not directly translate to divergent behavioral plasticity in queen brood care.

While we are confident about the instances in which we detect regulation of queen specialization due to the expression of brood care, in cases where we could not detect it, it could be due to several reasons. For example, as mentioned above, the number of workers needed to induce queen behavioral specialization could differ between species. Similarly, the quality of brood and workers, age and status of the queen could further play a role. Furthermore, experimental conditions such as the size of the Petri dish, humidity, time of day, or light could influence queen behavior. Crucially, our within-queen design ensures that when increased brood care is observed, it reflects a real, internally controlled response, and most of the limitations only apply upon the absence of behavior.

These findings of queen behavioral flexibility and its variability across the ants challenge the traditional view of ant queens as rigidly specialized germline equivalents. Instead, queens appear to inhabit a fluid, socially regulated spectrum of specialization, in which behavioral plasticity is common across the phylogeny and varies with ecological context. This flexibility blurs the sharp division of labor between queens and workers, often compared to the separation of soma and germline in multicellular organisms. This suggests that, in many species, colonies are not static superorganisms, but flexible systems in which both castes, queens and workers, can adjust and reallocate tasks as conditions are demanded (Nakata 1995; Starkey and Tamborindeguy 2023; Bernadou et al. 2015; Calabi and Traniello 1989). Perhaps, only in a minority of species, queens have passed the threshold from the duality of being a solitary foundress and a reproductive unit towards being truly “hard-wired” into the singular purpose of reproductive unit. Therefore, the presence or absence of queen behavioral flexibility could become a discriminating marker to discuss the social complexity and superorganismal traits in ants.

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Author contributions

MFB conceived the idea and designed the methodology together with [redacted] and [redacted]. MFB collected the ants in Germany, France and the USA and reached out to colleagues and collaborators for more species. MFB and [redacted] collected the ants and concluded the behavioral experiments in Peru. MFB concluded 90% of the experiments in Mainz, [redacted] and [redacted] respectviely the other 10%. [redacted] and [redacted] concluded the experiments on *Solenopsis invicta* and send the videos to MFB. MFB analysed the behavior. [redacted] and [redacted] spearheaded and supported the analysis of the phylogenetic signal. MFB and [redacted] analyzed the data. MFB, [redacted], and [redacted] led the writing of the manuscript.

Supplementalary Material

Supplement Table 1: All species investigated for queen behavioral flexibility regarding brood care. For each species, the number of colonies investigated, the number of individual queens (replicates), the subfamily, and the country of origin are listed. Sometimes multiple queens from the same polygynous colony were used, therefore explaining the discrepancy between colonies and replicates.

Species	Colonies	Replicates	Subfamily	Origin
Stigmatomma pallipes	19	19	Amblyoponinae	USA
Diacamma rugosum	3	3	Ponerinae	Thailand
Hypoponera ADH0635	2	2	Ponerinae	Peru
Mayaponera arhuaca	1	1	Ponerinae	Peru
Mayaponera constricta	4	5	Ponerinae	Peru
Odontomachus haematodus	3	3	Ponerinae	Peru
Pachycondyla crassinoda	2	2	Ponerinae	Peru
Pseudoponera AGA9688	2	2	Ponerinae	Peru
Rhytidoponera metallica	3	3	Ectatomminae	Australia
Anoplolepis gracilipes	15	15	Formicinae	Thailand
Camponotus ADN8326	2	2	Formicinae	Peru
Camponotus americanus	5	5	Formicinae	USA
Camponotus herculeanus	2	2	Formicinae	USA
Camponotus lateralis	5	5	Formicinae	France
Camponotus nearcticus	2	2	Formicinae	USA
Camponotus novogranadensis	1	1	Formicinae	Peru
Formica fusca	4	4	Formicinae	Germany
Formica rufibarbis	6	6	Formicinae	Germany
Lasius americanus	7	7	Formicinae	USA
Lasius emarginatus	19	19	Formicinae	Germany
Lasius flavus	3	3	Formicinae	Germany
Lasius niger	3	3	Formicinae	Germany
Myrmelachista schumanni	1	1	Formicinae	Peru
Nylanderia ACZ3041	1	1	Formicinae	Peru
Paratrechina cf.	1	1	Formicinae	Peru
Paratrechina longicornis	15	15	Formicinae	Spain
Plagiolepis cf	3	3	Formicinae	France
Gnamptogenys ACJ4176	1	1	Formicinae	Peru
Dolichoderus lutosus	1	1	Dolichoderinae	Peru
Dolichoderus plagiatus	15	15	Dolichoderinae	USA
Linepithema humile	18	18	Dolichoderinae	Germany, France
Tapinoma erraticum	2	2	Dolichoderinae	USA
Tapinoma sessile	1	1	Dolichoderinae	USA
Allomerus octoarticulatus	1	1	Myrmicinae	Peru
Aphaenogaster picea	12	12	Myrmicinae	USA

Aphaenogaster rudis	1	1	Myrmicinae	USA
Aphaenogaster subterranea	1	1	Myrmicinae	France
Cardiocondyla obscurior	5	15	Myrmicinae	Japan
Carebara panamensis	1	5	Myrmicinae	Peru
Cephalotes spinosus	4	17	Myrmicinae	Peru
Crematogaster brasiliensis	3	7	Myrmicinae	Peru
Crematogaster carinata	11	23	Myrmicinae	Peru
Crematogaster msp05	1	1	Myrmicinae	Peru
Crematogaster sotobosque	2	4	Myrmicinae	Peru
Cyphomyrmex major	1	1	Myrmicinae	Peru
Cyphomyrmex minutus	1	1	Myrmicinae	Peru
Cyphomyrmex rimosus	10	12	Myrmicinae	Peru
Messor capitatus	6	6	Myrmicinae	Spain
Monomorium pharaonis	5	17	Myrmicinae	???
Myrmica punctiventris	15	15	Myrmicinae	USA
Pheidole laticornis	2	2	Myrmicinae	Peru
Pheidole pallidula	4	4	Myrmicinae	France
Pheidole sp8	2	2	Myrmicinae	Peru
Pogonomyrmex barbatus	15	15	Myrmicinae	USA
Solenopsis ABA1305	1	1	Myrmicinae	Peru
Solenopsis geminata	3	3	Myrmicinae	Peru
Solenopsis invicta	24	24	Myrmicinae	USA
Solenopsis sp. TD01	3	3	Myrmicinae	Peru
Strumigenys ADH3319	1	1	Myrmicinae	Peru
Strumigenys cordovensis	1	1	Myrmicinae	Peru
Temnothorax americanus	24	24	Myrmicinae	USA
Temnothorax longispinosus	30	30	Myrmicinae	USA
Temnothorax rugatulus	30	30	Myrmicinae	USA
Tetramorium caespitum	5	5	Myrmicinae	Germany
Tetramorium simillimum	2	5	Myrmicinae	Peru
Wasmannia auropunctata	18	19	Myrmicinae	Spain
Pseudomyrmex tenuis	1	1	Pseudomyrmecinae	Peru
Pseudomyrmex termitarius	1	1	Pseudomyrmecinae	Peru

S**upplementary Table 2: Life history traits of species with more than 3 replicates.** The table contains the main founding strategy and social structure of each species and references where applicable for this information. If there were no references, we assumed social structure based on what we encountered in the field. Meanwhile, for founding strategy we stayed conservative and had independent as the main strategy if not mentioned otherwise in the literature.

species	Social structure	Founding strategy	References
Crematogaster brasiliensis	Polygynous	Independent	Longino 2003, Field observation
Crematogaster carinata	Polygynous	Independent	Longino 2003

Cardiocondyla obscurior	Polygynous	Dependent	Heinze and Delabie 2005
Carebara panamensis	Monogynous	Independent	Field observation
Tetramorium caespitum	Monogynous	Independent	Frumhoff and Ward 1992
Tetramorium simillimum	Polygynous	Dependent	Frumhoff and Ward 1992
Temnothorax longispinosus	Polymorphic	Independent/Dependent	Howard 2006
Temnothorax americanus	Monogynous	Dependent	Creighton 1929
Temnothorax rugatulus	Polymorphic	Independent/Dependent[1]	Negroni et al. 2021
Pheidole pallidula	Polygynous	Independent	Frumhoff and Ward 1992
Cephalotes spinosus	Polygynous	Independent	Field observation
Cyphomyrmex rimosus	Polygynous	Independent	Field observation
Wasmannia auropunctata	Polygynous	Dependent	Frumhoff and Ward 1992; Breton et al. 2004
Monomorium pharaonis	Polygynous	Dependent	Frumhoff and Ward 1992; Buczkowski and Bennett 2009
Solenopsis invicta	Polymorphic	Independent/Dependent	Cassill 2002; Helms and Godfrey 2016
Solenopsis geminata	Monogynous	Independent	Frumhoff and Ward 1992
Aphaenogaster picea	Monogynous	Independent	Lubertazzi 2012
Messor capitatus	Monogynous	Independent	Frumhoff and Ward 1992
Pogonomyrmex barbatus	Monogynous	Independent	Johnson 1998
Myrmica punctiventris	Polygynous	Independent	Banschbach and Herbers 1996
Rhytidoponera metallica	Monogynous	Dependent	Ward 1986
Camponotus lateralis	Monogynous	Independent	Field observation
Camponotus americanus	Monogynous	Independent	(Frumhoff and Ward 1992)
Formica fusca	Monogynous	Independent	(Johansson et al. 2018)
Formica rufibarbis	Monogynous	Independent	(Seifert and Schultz 2009)
Plagiolepis cf	Monogynous	Independent	Field observation
Anoplolepis gracilipes	Polygynous	Dependent	(Lenancker et al. 2021)
Paratrechina longicornis	Polygynous	Dependent	(Frumhoff and Ward 1992)
Lasius niger	Monogynous	Independent	(Frumhoff and Ward 1992)
Lasius americanus	Monogynous	Independent	Field observation
Lasius flavus	Monogynous	Independent	(Frumhoff and Ward 1992)
Lasius emarginatus	Monogynous	Independent	(Frumhoff and Ward 1992)
Linepithema humile	Polygynous	Dependent	(Markin 1970)
Dolichoderus plagiatus	Monogynous	Independent	(Frumhoff and Ward 1992)
Odontomachus haematodus	Monogynous	Independent	Field observations
Diacamma rugosum	Monogynous	Dependent	(Fukumoto and Abe 1983)
Mayaponera constricta	Polymorphic	Independent	Field observation
Stigmatomma pallipes	Polymorphic	Independent/Dependent	(Traniello 1982)

1: We only used macrogynes of T.rugatulus, who are capable of independent colony founding while being social polymorphic.

Supplementary Table 3: Statistical analysis queen behavioral flexibility across 38 species of ants. The table included the species, the Model used, the p-value when comparing the brood care in presence of workers compared to either 24h)

Species	Model	Pval 24h	Pval 48h	Flexibility	Brood care in worker presence	Category
Anoplolepis gracilipes	LMM	0.002	0.007	TRUE	/	Evidence for flexibility
Aphaenogaster picea	LMM	0.029	0.463	TRUE	/	Evidence for flexibility
Camponotus americanus	LMM	0.001	0.012	TRUE	/	Evidence for flexibility
Camponotus lateralis	GLM	NA	NA	FALSE	No	No Evidence for flexibility, No Brood Care
Cardiocondyla obscurior	LMM	0.078	0.017	TRUE	/	Evidence for flexibility
Carebara panamensis	LMM	0.383	0.244	FALSE	No	No Evidence for flexibility, No Brood Care
Cephalotes spinosus	LMM	0.188	0.011	TRUE	/	Evidence for flexibility
Crematogaster brasiliensis	LMM	0.143	0.315	FALSE	No	No Evidence for flexibility, No Brood Care
Crematogaster carinata	LMM	0.129	0.116	FALSE	No	No Evidence for flexibility, No Brood Care
Cyphomyrmex rimosus	LMM	0.231	0.927	FALSE	Yes	No Evidence for flexibility, Brood Care
Diacamma rugosum	LMM	0.802	0.074	FALSE	No	No Evidence for flexibility, No Brood Care
Dolichoderus plagiatus	LMM	0.023	0.011	TRUE	/	Evidence for flexibility
Formica fusca	LMM	0.007	0.015	TRUE	/	Evidence for flexibility
Formica rufibarbis	LMM	0.065	0.009	TRUE	/	Evidence for flexibility
Lasius americanus	LMM	0.000	0.000	TRUE	/	Evidence for flexibility
Lasius emarginatus	LMM	0.084	0.001	TRUE	/	Evidence for flexibility
Lasius flavus	LMM	0.323	0.204	FALSE	No	No Evidence for flexibility, No Brood Care
Lasius niger	LMM	0.000	0.000	TRUE	/	Evidence for flexibility
Linepithema humile	GLM	NA	NA	FALSE	No	No Evidence for flexibility, No Brood Care
Mayaponera constricta	LMM	0.364	0.755	FALSE	No	No Evidence for flexibility, No Brood Care
Messor capitatus	LMM	0.000	0.000	TRUE	/	Evidence for flexibility
Monomorium pharaonis	LMM	0.011	0.003	TRUE	/	Evidence for flexibility
Myrmica punctiventris	LMM	0.012	0.000	TRUE	/	Evidence for flexibility
Odontomachus haematodus	LMM	0.166	0.295	FALSE	No	No Evidence for flexibility, No Brood Care

Paratrechina longicornis	GLM	NA	NA	FALSE	No	No Evidence for flexibility, No Brood Care
Pheidole pallidula	LMM	0.332	0.032	TRUE	/	Evidence for flexibility
Plagiolepis cf	LMM	0.843	0.727	FALSE	No	No Evidence for flexibility, No Brood Care
Pogonomyrmex barbatus	LMM	0.101	0.012	TRUE	/	Evidence for flexibility
Rhytidoponera metallica	LMM	0.233	0.376	FALSE	No	No Evidence for flexibility, No Brood Care
Solenopsis geminata	LMM	1.000	0.180	FALSE	No	No Evidence for flexibility, No Brood Care
Solenopsis invicta	LMM	0.005	0.008	TRUE	/	Evidence for flexibility
Stigmatomma pallipes	LMM	0.183	0.890	FALSE	Yes	No Evidence for flexibility, Brood Care
Temnothorax americanus	LMM	0.348	0.348	FALSE	Yes	No Evidence for flexibility, Brood Care
Temnothorax longispinosus	LMM	0.000	0.011	TRUE	/	Evidence for flexibility
Temnothorax rugatulus	LMM	0.131	0.141	FALSE	Yes	No Evidence for flexibility, Brood Care
Tetramorium caespitum	LMM	0.006	0.027	TRUE	/	Evidence for flexibility
Tetramorium simillimum	LMM	0.248	0.209	FALSE	No	No Evidence for flexibility, No Brood Care
Wasmannia auropunctata	LMM	0.266	0.657	FALSE	Yes	No Evidence for flexibility, Brood Care

Unmated queens show worker-like behavior and gene expression in polygynous colonies of the ant Stigmatomma pallipes

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Running title: **Worker-like queens in polygynous ant colonies**

Under Review at Molecular Ecology

Abstract

Insect societies show a reproductive division of labor between egg-laying queens and workers that fulfill all non-reproductive tasks. Polygyny, the coexistence of several queens in a colony, has evolved multiple times in social insects. Although queens in polygynous colonies are often assumed to have similar reproductive outputs, they may actually show variation in terms of physiology and behavior. However, little is known on the mechanistic basis of such variation. Here, we used the ant *Stigmatomma pallipes* to investigate: i) whether nestmate queens from polygynous colonies differed in mobility, behavior, and gene expression; and ii) whether this variation was explained by their mating status. We used individual tracking and behavioral scoring to identify two types of queens: low-mobility (LM) queens that behaved like monogynous queens and high-mobility (HM) queens with worker-like behavior. Dissections of reproductive organs revealed that only one queen per colony was mated and reproductively active, consistently corresponding to the LM category. As a complementary approach, we performed transcriptomic-based analyses of brains and fat bodies, finding that mated LM-polygynous queens had similar transcriptomic profiles to monogynous queens, while unmated HM-polygynous queens resembled workers. Transcriptomic differences between LM- and HM-polygynous queens were primarily associated with processes related to protein synthesis, transcription, and neural activity. Our finding that unmated queens in polygynous *S. pallipes* colonies exhibit worker-like behavior and gene expression challenges traditional views of queen specialization at different biological levels, and provides new insights into reproductive division of labor in insect societies.

Keywords

Social insects, Phenotypic plasticity, Division of labor, Amblyoponinae, Transcriptomics, Functional monogyny

Introduction

The reproductive division of labor is considered a key factor underlying the ecological success of social insects (Wilson 1978; Miura et al. 2022; Muratore et al. 2023). In these societies, a morphologically and behaviorally specialized queen caste monopolizes egg production (Wheeler 1911; Boomsma and Gawne 2018; Hellemans and Hanus 2024) and has often been compared to the highly specialized germline of a multicellular organism, dedicated solely to reproduction (Wheeler 1911; Szathmáry and Smith 1995; Boomsma and Gawne 2018; Miura et al. 2022; Majidifar et al. 2024). Nevertheless, queens can express a broad range of behaviors, both during colony foundation, as well as in established colonies (Lenoir and Dejean 1994; Brown and Bonhoeffer 2003; Evans and Raine 2014; Ortiz-Alvarado and Rivera-Marchand 2020; Mizumoto et al. 2021; Majidifar et al. 2024). In species where multiple queens coexist in a colony (polygyny), this behavioral repertoire may diversify further, as reproductive and social roles may be distributed among queens.

In the ants, polygyny occurs frequently and can take multiple forms, which are not limited to the presence of multiple queens with equal reproductive outputs and similar behavior (Hölldobler and Wilson 1977; Keller 1991; Gadau and Fewell 2009; Boomsma et al. 2014). These differences were observed in colonies with dominance hierarchies (Ito et al. 1996) or with coexisting mated and unmated dealate (wingless) queens (Buschinger 1968). Subordinate and/or unmated queens typically behave like non-reproductive workers, performing actions such as foraging, defense, and brood care (Petersen-Braun 1975; Ito et al. 1996; Peeters 1997; Hora et al. 2005; Nehring et al. 2012; Vieira et al. 2012; Araújo et al. 2016; Murakami 2020). These cases illustrate that the queen caste can encompass multiple behavioral and physiological profiles, nevertheless the molecular background of this variation and how it compares to the worker caste remain poorly understood.

There is no evidence that the observed variation among queens stems from genetic differences, but rather from phenotypic plasticity, whereby individuals with nearly identical genetic backgrounds develop distinct morphological and behavioral profiles due to differences in gene expression (Grozinger et al. 2007; Bonasio et al. 2010; Taylor et al. 2021; Xu and Colgan 2025). Phenotypic plasticity not only produces the morphologically differentiated queen and worker castes (Wilson 1953; Wheeler 1986; Gadagkar 1997; Berens et al. 2015; Corona et al. 2016) but also behavioral variation within the worker caste (Wilson 1953; Wheeler 1986; Toth and Robinson 2007; Smith et al. 2008; Mikheyev and Linksvayer 2015). Such worker behavioral plasticity at the adult stage is associated with distinct transcriptomic profiles, often in specific tissues, such as the brain or fat body (Corona et al. 2013; 2016; Nie et al. 2018; Libbrecht et al. 2018; Kohlmeier et al. 2019; Miyazaki et al.

2021; Qiu et al. 2022; Caminer et al. 2023). Previous transcriptomic studies on the queen caste have provided biological insights into how the molecular phenotype changes after mating (von Wyschetzki et al. 2015; Nagel et al. 2020; Liu et al. 2023), as well as changes associated with age (von Wyschetzki et al. 2015; Lucas et al. 2017; Lucas and Keller 2018; Nagel et al. 2020; Korb et al. 2021). To identify the molecular basis of behavioral variation among queens in polygynous colonies requires investigating the associated transcriptomic variation in relevant tissues. In this regard, the brain reflects differences in behavioral regulation, whereas the fat body provides complementary insights into metabolic and reproductive physiology.

To further our understanding of the functional repertoire of the queen caste in polygynous species, we investigated behavioral and transcriptomic variation within the queen caste in polygynous colonies of the ant *Stigmatomma pallipes*. This species is an exemplar system to study variation among queens because it is socially polymorphic, in that colonies can be monogynous or polygynous, with its polygynous colonies documented to contain queens that express worker-like behaviors (Wheeler 1900; Haskins 1928; Haskins and Enzmann 1937; Traniello 1982), yet the bases and consequences for such variation is unknown. In addition, *S. pallipes* belongs to the Amblyoponinae, an understudied subfamily of ants with limited morphological differences between queens and workers. Using a mobility-based approach to categorize queens, we investigated how queen types differ in mating status, behavior, and transcriptomic profiles. This allowed us to characterize variation among queens across biological scales, ranging from the molecular to the organismal. To facilitate these investigations, we also generated one of the first genome assemblies for this subfamily. Together, these data provide a comprehensive overview of variation within queens in polygynous colonies, contributing to our understanding of the evolution and functional repertoire of the queen caste in social insects.

Methods

Colony collection and maintenance

Colonies of *S. pallipes* were collected under flat rocks at the Huyck Preserve, New York (42°30′56.23″ N, 74°08′21.34″ W) during May 2024. Entire colonies, including queens, workers, eggs, larvae, and pupae were collected and transferred into plastic bags, containing some of the surrounding soil and moist cotton. Upon arrival at the Johannes Gutenberg University of Mainz, Germany, the colonies were moved into plaster nests (14 cm Width x 16 cm Length x 6 cm Height). The nests featured a central depression covered with a glass slip to allow congregation, and the nest was subsequently covered with soil, which was kept moist. Colonies were maintained in a temperature-controlled room at 22 °C in the dark and fed twice weekly with chopped mealworms.

Experimental manipulations and video recording

For our study, we used 25 polygynous (ranging between two to six queens) and five monogynous colonies of *S. pallipes* (Supplementary Table S1). To examine queen mobility variation among queens, the soil and glass slip, under which the ants nested, were removed, and queens were individually marked with dye (blue, green, orange, pink, purple, red, white, and yellow) from a non-toxic marker pen (Edding 750, Edding, Germany) on the thorax and abdomen. In cases where queens lost the marking or were missed during marking, they were assigned the unmarked tag (no colony had more than one unmarked queen). After a one-week acclimation period to allow ants to resettle after the marking, the overlying soil was removed from each nest and remained removed for the duration of the experiment. The 30 colonies were divided into two batches of 15 colonies, which were filmed 24 hours apart. Over two weeks, each colony was filmed five times, with each filming (hereafter, called “observation”) lasting 110 minutes. During each observation, a colony was filmed under an individual camera (Sony FDR-AX33 Handycam, Sony Corporation, Japan) equipped with a ring light (Selfie Ring Light WLD-S1, RealPlus, China). The camera's field of view encompassed the entire nest and immediate surrounding area. To account for possible positional and time-of-day effects, we randomized the order of filming and camera assignments. After the fifth and final observation, the entire colony of each nest was extracted immediately and separated into multiple cryotubes. Cryotubes were flash-frozen using liquid nitrogen and transferred to a –80°C freezer for storage prior to downstream analyses.

Mobility measurement

To test individual differences in queen mobility, the distance traveled by queens between frames was calculated by tracking their positions over time. To do so, we extracted, for each observation, the first frame of every minute using a custom script in FFmpeg v.4.2.7. (FFmpeg developers 2024), omitting the first and last ten minutes of each video to avoid time points where disturbances could have occurred. This resulted in 91 frames per observation. In each frame, we marked the thorax position of each queen manually using a custom macro script in ImageJ v.1.54f (Schindelin et al. 2012) to load in images and save positions. In the first frame of each observation, we also marked the nest entrance and all four nest corners. Pixel distances were transformed into millimeters using a scale factor specific to each recording. This factor was based on the known length (12 cm) or height (8 cm) of the glass slip divided by the respective measured pixel distance. When queens were not visible due to exiting the nest, their location was marked at a single point outside the nest boundary. For consecutive unobservable points, we estimated the distance traveled using the global mean stepwise distance across all queens and observations.

To investigate whether polygynous queens differed in mobility, we used the R package lme4 v.1.1-36 (Bates et al. 2003) to build for each polygynous colony a linear mixed-effects model with the distance traveled between frames as response variable, the queen identity as explanatory variable and the observation as random effect. We ran ANOVAs using the package car v.3.1.3, (Fox et al. 2001) to test for each colony whether there was a main effect of queen identity. From each of the 30 models, we extracted the estimated marginal mean of the distance travelled for each queen (hereafter, referred to as “mobility”) using the emmeans package v.1.11.1 (Lenth 2025). In monogynous colonies, we calculated the mean distance traveled by the queen across all observations to estimate mobility. We used the highest mobility value of monogynous queens, rounded to the nearest whole number, as a threshold to classify queens in polygynous colonies. Queens with a mobility value above this threshold were classified as high-mobility (HM-polygynous queens), and those with a mobility value below the threshold as low-mobility (LM-polygynous queens).

To further investigate how the differences in mobility were associated with behavior, mating status, and gene expression, we selected the polygynous colonies for which we found evidence of mobility variation among queens and that contained at least one HM- and one LM-polygynous queen. Fifteen of the 25 polygynous colonies met these criteria. We also used the queens from the five monogynous colonies. From each polygynous colony, one HM- and one LM-queen were chosen. In the case that multiple queens were on either side of the designated threshold, one queen was selected at random. In total, 30 queens from

polygynous colonies and five queens from monogynous colonies were used for further analyses. Additionally, we added a single worker from each colony as an outgroup for the mating status and gene expression analyses. This resulted in a total of 55 specimens: 15 LM-polygynous queens; 15 HM-polygynous queens; five monogynous queens; and 20 workers.

Tissue sampling for gene expression analyses

To understand how queens differed in terms of gene expression, we focused on two tissues: the brain, important for behavior and locomotion, and the fat body, which plays key roles in metabolism, and reproduction. Dissections were performed in a randomized order and were blind to mobility status. The brain and fat body of each individual were dissected on separate dates.

To dissect the brain, the head was removed and transferred to a precooled 1x PBS solution on an ice-cooled silicone slide. The rest of the body was returned to the cryotube and submerged in liquid nitrogen to prevent possible tissue degradation. During each step, one micro-forceps (No. 11295-10, F.S.T, Canada) was used to hold the head in place by grabbing a piece of cuticle or the mandibles. The antennae were removed at the base. The head was opened by cutting dorsally into the head capsule with a micro-scissor (No 15000-08, F.S.T., Canada), starting from the clypeus and moving up to the ocelli and the vertex along the frontal line. This was followed by two cuts at 90° to the first, in the direction of the eyes, resulting in a cross-like cut. The cuticle encompassing the cut was then removed using two micro-forceps. Once the cuticle towards the eyes had been removed, one micro-forceps was used to rub the inside of the compound eyes on both sides of the head, severing the connection to the retina. This step was skipped for workers, as they lack well-developed eyes. The cuticle was then removed around and above the ocelli and vertex. Once the brain was clearly visible, it was carefully removed by grabbing it from below at the posterior end and lifting it out of the head capsule and into another cooled 1x PBS droplet. It was then transferred to a pre-chilled vial containing 100 µl of TRIzol and incubated on ice. After dissection, all samples were transferred and stored at -80 °C.

To sample the fat body, the gaster was dissected. The gaster was separated at the petiole from the thorax. The gaster was separated into two parts by making an incision with the micro-scissors between the second and third abdominal segments, while holding it in place with the micro-forceps. The lower half of the gaster was removed and placed into a separate 1x PBS droplet for later assessments of mating status. From the upper half, which comprises the first and second abdominal segments, we removed the remaining internal trachea, gastrointestinal tract, and components of the nervous system. In addition, the ventral parts of

the second abdominal segments were removed until only parts of it and the first abdominal segments with subcuticular fat body remained. Fat body with remnants of the first and second abdominal segments were transferred into a vial with 100 µl TRIzol, and stored at -80°C.

Assessment of mating status

The mating status and ovarian development of all queens from colonies with significantly different mobility were assessed, as well as those of the monogynous queens and a single worker from each colony. To reveal the ovaries, we dissected the lower half of the gaster in ice cooled 1x PBS, removing the remaining cuticle, the gastrointestinal tract, and the aculeus (stinger). The ovaries were transferred to a glass slide and imaged with the built-in camera of the microscope (Leica S9i, Leica Microsystems, Germany). To measure ovarian development, ovaries with white ovarioles, often accompanied by yellow bodies, were scored as developed. Ovaries lacking these features were scored as undeveloped. To assess the mating status, we scored the presence of a visible spermatheca, as well as whether it was filled or empty. Individuals with a filled spermatheca were classified as mated, while individuals with either an empty spermatheca or no visible spermatheca were scored as unmated. The scoring of ovarian development and mating status was performed blind to mobility status. We conducted Fisher's exact tests to investigate the association between mobility and mating status, and between mobility and ovarian development.

Behavioral analyses

To investigate whether queens with different mobility express different behavioral patterns, we used the BORIS software v.7.13.9 (Friard & Gamba, 2016) to score a total of 13 behaviors (Table 1).

Table 1**: Summary table of all queen behaviors that were scored in the behavioral analysis.**

Behavior	Description
Resting	Not moving legs / no observable movements.
Walking	Moving around
Digging	Removing soil or plaster
Nest exit	Exiting the nest, not being visible to the observer
Queen-queen interaction	Interaction between queens, such as grooming, aggressive behavior, intense antennation
Queen-worker interaction	Interaction between queens and workers such as grooming, aggressive behavior, intense antennation
Brood care	Actively licking or manipulating the brood (antennation alone was

	not considered as brood care)
Brood carrying	Carrying brood around
Carrying other	Carrying soil or food items around
Feeding on food items	Feeding on food items such as pieces of mealworms
Hemolymph feeding	Squeezing larvae to feed on their hemolymph
Self-grooming	Cleaning and licking own legs, antennae, mandibles and/or gaster
Egg laying	Flexing the gaster and visibly producing an egg

We scored the behavior of each selected queen during the first 10 seconds of every minute across each of the five 90 minute observations, for a total of 91 scans per observation and 455 behavioral scans per individual. Each scan was associated with a single behavior. If multiple behaviors were observed during one scan, the behavior performed the longest was scored.

To analyze behavioral variation among monogynous queens, LM-polygynous queens, and HM-polygynous queens, we first pooled for each queen all scans from the five observations after verifying that queen behavior was consistent across observations by visual inspection (Supplementary Figure S1). We then analyzed behavioral differences among queens by performing a principal component analysis (PCA) on scaled and centered summarized behavioral counts across all observations. We conducted K-means clustering using all principal components (PCs) to identify clusters of queens that shared similar behavioral profiles. Cluster assignments were visualized on the PCA, and their association with mobility categories of the queens (monogynous, LM- and HM-polygynous queens) was evaluated using Fisher's exact tests.

We further analyzed as many PCs as needed to account for 90% of the total variance. We tested the main effect of queen type on PC scores by conducting ANOVAs on the output of linear mixed-effects models that included colony as a random factor. Post-hoc pairwise tests were performed using the emmeans package v.1.11.1 (Lenth 2025) with Tukey HSD adjustment for multiple comparisons. We identified the behaviors that were primarily associated with each PC as those with loading values exceeding 75% of the maximum loading value.

RNA extraction, library preparation, and sequencing

To investigate transcriptomic variation, we used the aforementioned 55 individuals (15 LM-polygynous queens, 15 HM-polygynous queens, five monogynous queens, and 20 workers). From each individual, we extracted total RNA from the brain and fat body separately using a

trizol:chloroform extraction protocol followed by purification using the Qiagen RNeasy extraction kit. This resulted in a total of 110 RNA samples that were shipped on dry ice to a commercial supplier (Novogene, Germany) that conducted quality assessment using an Agilent Bioanalyzer and individual mRNA-enriched library preparations using the NovogeneNGS RNA Library Prep Set (PT042) property kit. The libraries were individually barcoded, pooled, and sequenced on an Illumina Plus X sequencing platform. Sequencing failed for eight samples, six from the brain (two LM-polygynous queens, two HM-polygynous queens, and two workers) and two from the fat body (one LM-polygynous queen and one worker) (Supplementary Table S2), reducing our dataset to 102 samples. For these samples, the amount of sequencing data ranged from 8-76 million paired-end (2*150 bp) reads per sample (Supplementary Table S2).

Sample collection and DNA extraction for genome assembly

To allow for read alignment and associated transcript quantification, a *de novo* genome assembly was generated as a genome assembly for *S. pallipes* was not available at the onset of our analysis. To provide sufficient input material for DNA sequencing, we collected a total of 24 pupae from a single colony that was not part of the behavioral experiment. For the following steps, the 24 pupae were subsampled into sets of two, generating a total of 12 pupal sets. Each pupal set was homogenized in 460 µl of homogenization buffer with 40 µl of 10% Triton X-100. The resulting homogenate was filtered through a 70 µm filter while kept on ice. The retained substrate was resuspended in 400 µl of homogenization buffer and homogenized again before being transferred to a 1.5 ml Eppendorf tube. An additional 500 µl of homogenization buffer was added, and the mixture was centrifuged at 1,000 rpm for 5 minutes at 4 °C. The supernatant was discarded, and the pellet was resuspended in 500 µl of homogenization buffer. This process was repeated once more after subsequent centrifugation. The pellet was then resuspended in 200 µl of homogenization buffer, to which 5 µl of proteinase K, 20 µl of 10% sodium dodecyl sulfate (SDS), and 20 µl of dialysis buffer were added. The mixture was carefully inverted and incubated at 56 °C and 600 rpm for 30 minutes on a heat block, followed by an additional 10 minute incubation at the same temperature.

After incubation, the pupal sets were pooled. To this combined sample, a 0.1x volume of Tris and a 0.25x volume of sodium perchlorate ($NaClO_4$) were added. This was followed by a phenol:chloroform:isoamyl extraction, including one re-extraction of the lower phase with 500 µl homo-buffer. This step was followed by a further chloroform:isoamyl alcohol extraction. The aqueous supernatant was collected and treated with an RNase at room temperature for 30 minutes. The DNA was precipitated by adding a 0.1x volume of 10x

dialysis buffer and two volumes of ethanol. The samples were centrifuged at 15,000 rpm and the supernatant was discarded. The DNA pellet was dissolved in 30 µl HPLC-grade water and incubated at 4 °C for 12 hours. For library preparation, we used Nanopore´s native barcoding kit (SQK-NBD114.24), according to the protocol provided by ONT with minor modifications, which included an extended incubation times for ligation and a bead clean up.

DNA sequencing, genome assembly, and annotation

Genomic libraries were sequenced on an Oxford Nanopore Technologies Promethion flow cell. After sequencing, raw pod5 files were base called using dorado v.0.7.0+71cc744 using model 'dna_r10.4.1_e8.2_400bps_sup@v5.0.0'. The initial number of raw sequences was 6,662,475 reads (mean size: 2606 bp, maximum size: 675,316 bp). We performed quality checks of raw sequences using NanoPlot v.1.44.1 (De Coster and Rademakers 2023) with sequences then filtered using chopper v.0.10.0 (De Coster and Rademakers 2023) to remove sequences with mean base quality less than 10. Using these filtered reads, we next performed a *de novo* assembly using flye in high accuracy mode -nano-hq, v.2.9.1 (Kolmogorov et al. 2019) with a minimum overlap length of 1 kb. Initial quality checks of the resulting genome assembly were performed using QUAST v.5.3.0 (Gurevich et al. 2013), which provides summary statistics on the assembly.

To correct base-level errors and fix mismatches, genome polishing was performed using Racon v.1.5.0 (Vaser et al. 2017), which involved three rounds of polishing and corrects for minor base errors. For each polishing round, reads were remapped against intermediate polished assemblies using minimap2 v.2.28 (Li 2018). This approach was complemented by using Medaka v.2.0.1 (https://github.com/nanoporetech/medaka), a neural network-based polisher that further improves base-level accuracy after initial base corrections. Using the medaka-polished genome assembly, RepeatModeler v.2.0.2 (https://github.com/Dfam-consortium/RepeatModeler) was next used to discover and identify putative repetitive regions in the genome assembly, which were subsequently soft masked using RepeatMasker (https://www.repeatmasker.org).

Lastly, the consensus polished assembly was assessed for coverage by back mapping initial filtered reads against the polished assembly using minimap2. Contigs less than 1,000 bp were removed. This final assembly, which consisted of 822 contigs (total length: 232.4 Mb), was assessed for completeness through comparisons with both the latest Insecta and Hymenoptera BUSCOs (Benchmarking Universal Single-Copy Orthologs).

To annotate the genome assembly, we generated transcriptome-informed gene model predictions. First, we performed data quality evaluations for all 102 samples. For each

sample, the general quality metrics were generated and examined using FastQC v.0.12.1 (Andrews 2010) providing information on base quality and potential adapter contamination. As a complementary approach, the proportion of raw reads mapping to the generated genome assembly was investigated using HISAT v2.2.1 (Kim et al. 2019), finding high mapping rates (mean: 91.55%). The outputs were combined and visualized for both sets of quality assessments using MultiQC v.1.29 (Ewels et al. 2016). Based on the results of the quality assessment, adapter sequences were removed, which can affect mapping rates and quality, and reads filtered by quality (PHRED score ≥ 15) and length (minimum length ≥ 50 bp) using fastp v.0.20.1 (Chen et al. 2018). Due to low read count, two samples were removed prior to subsequent gene model prediction.

The filtered reads were then used in conjunction with a protein database comprised of arthropod-derived proteins from OrthoDB v12 (Kuznetsov et al. 2023), published hymenopteran proteins (Kapheim et al. 2015), and all proteins from the curated Swiss-Prot database (The UniProt Consortium 2023) to predict gene models within the soft-masked genome assembly using BRAKER3 v3.0.8 (Gabriel et al. 2024). Initial predictions were further quality filtered and processed to identify and correct putative incorrect gene models. To this end, predicted genes with no overlap among their transcripts were split into separate genes, and gene predictions with missing start or stop codons, or premature stop codons were removed to retain only the highest quality predictions. As predicted gene models occasionally included very short introns of 1-3 bp, which are highly likely to represent technical errors, we joined adjacent exons if they were separated by less than 4 bp. Finally, quality-filtered gene model predictions were then translated to predicted proteins and used as input to Compleasm v.0.2.7, mode: “protein” (Huang and Li 2023; Manni et al. 2021), resulting in a BUSCO completeness of 97.06% (database: hymenoptera_odb12, n = 5,912). This level of completeness is comparable to the assembly’s BUSCO completeness of 97.89% (mode: “assembly”, database: hymenoptera_odb12, n = 5,912), indicating that most genes that were correctly assembled were also annotated with gene model predictions.

Differential expression and GO term enrichment analyses

For all RNA-seq samples, raw paired-end reads were filtered using fastp v.0.20.1 (Chen et al. 2018) to remove reads containing more than 15 ambiguous bases (“N”s) and reads shorter than 120 bp, with filtered reads saved separately for forward and reverse pairs. Afterwards, a quality assessment of filtered reads was performed via FastQC v.0.12.1 (Andrews 2010), with output files summarized and visualized using MultiQC v.1.29 (Ewels et al. 2016). Transcript abundances were next quantified per sample by pseudo-aligning filtered RNA-seq data against a predicted transcriptome informed from our *de novo*-assembled genome using

Kallisto v.0.50 (Bray et al. 2016). This resulted in transcript abundance estimates for all 102 sample (49 brain samples: five monogynous queens; 13 HM-polygynous queens; 13 LM-polygynous queens; and 18 workers; 53 fat body samples: five monogynous queens;15 HM-polygynous queens; 14 LM-polygynous queens; and 19 workers), which were loaded into R using tximport v.1.37.2 (Soneson et al. 2016), summarized to gene-level counts and analyzed using the R package DESeq2 v.1.49.4 (Love et al. 2014). Prior to differential expression analysis, we filtered out genes with less than five reads in five samples.

To investigate transcriptomic variation among the four individual types, we used variance-stabilized transformed counts to perform two separate PCAs: one for the brain samples; and one for the fat body samples. Next, we applied K-means clustering to all PCs to identify clusters of samples that share relatively similar transcriptomic profiles. We subsetted polygynous queen samples to test the association between cluster membership and polygynous queen mobility status types using a Fisher's exact test, and quantified the strength of the association with Cramer's V.

To assess whether gene expression differed among individual types, we used likelihood ratio tests (LRT) from DESeq2. We tested the main effect of individual type on gene expression by comparing a full model that included individual type to a reduced model that did not. Genes with a Benjamini-Hochberg adjusted p–values of less than 0.05 were considered differentially expressed genes (DEGs), as the analysis provided evidence that their expression differed between at least two of the four individual types. We clustered the DEGs using the DEGreport package v.1.42.0 (Pantano 2025), to produce gene clusters that contain DEGs with similar expression differences across individual types.

We investigated whether DEGs in gene clusters that encompassed more than 5% of all DEGs were enriched for biological processes using Gene Ontology (GO) term enrichment analyses with the topGO package v.2.52.0, (Alexa 2019). GO annotations for *Stigmatomma pallipes* genes were inferred through homology to *Drosophila melanogaster* using OrthoFinder v.2.5.4 (Emms and Kelly 2015). Predicted protein sequences, generated from our assembled and annotated *S. pallipes* genome, were used as input for our homology-based inference analysis along with predicted proteins from the latest *D. melanogaster* genome assembly. The resulting homologous mappings were used to assign GO terms to *S. pallipes* genes based on annotations assigned to the homologue in the fruit fly. The GO annotations were imported as a gene-to-term mapping table in R and converted into a list structure compatible with topGO. In total, 7,323 genes were annotated with a non-redundant list of 8,572 GO-terms. Genes, either without GO annotations or not expressed in the dataset, as well as genes with GO-terms with less than five annotated genes (node size = 5),

were excluded from our analysis, with the remaining annotated genes defined as the background gene universe.

For the enrichment analysis, a separate background gene universe was generated for each tissue, consisting of all expressed genes with GO annotation (brain: 2,951 genes; fat body: 1,863 genes). These numbers represented the subset of the 7,323 annotated genes that were expressed in each respective tissue. Of these, 2,736 and 1,715 genes, respectively, could be mapped to GO terms in the Directed Acyclic Graphs (DAGs) used by topGO, The GO DAGs used contained 5,794 terms with 12,206 relations (*i.e.*, directional links between GO terms indicating hierarchical relationships) for the brain and 2,771 terms with 10,592 relations for the fat body. A binary gene list indicating the presence (1) or absence (0) of genes in each cluster, relative to the full gene universe, was created. Functional enrichment was tested separately for each cluster using the “weight01” algorithm, and Fisher’s exact tests applied to terms from the Biological Process (BP) ontology. Resulting *p*-values were adjusted for multiple testing using the Benjamini–Hochberg correction (via p.adjust() in R) before applying a significance threshold of adjusted $p < 0.05$. Enrichment results were retrieved using the GenTable() function and GO term descriptions were appended using the GO.db R package v.3.15.0 (Carlson, 2023).

Results

Queens differed in mobility in polygynous colonies

To investigate whether queens in polygynous colonies of the ant *S. pallipes* differed in their mobility, we assessed the distance travelled between frames (one frame per minute) from 455 minutes of video recording per colony. In total, we analyzed the mobility of 89 queens: 84 were from 25 polygynous colonies; and five queens were from five monogynous colonies (Supplementary Table S3). One queen (C5_Green) died during the experiment and, thus, was excluded from the analysis. We found differences in mobility among queens in 88% (22/25) of the polygynous colonies (Figure 1, Supplementary Table S4).

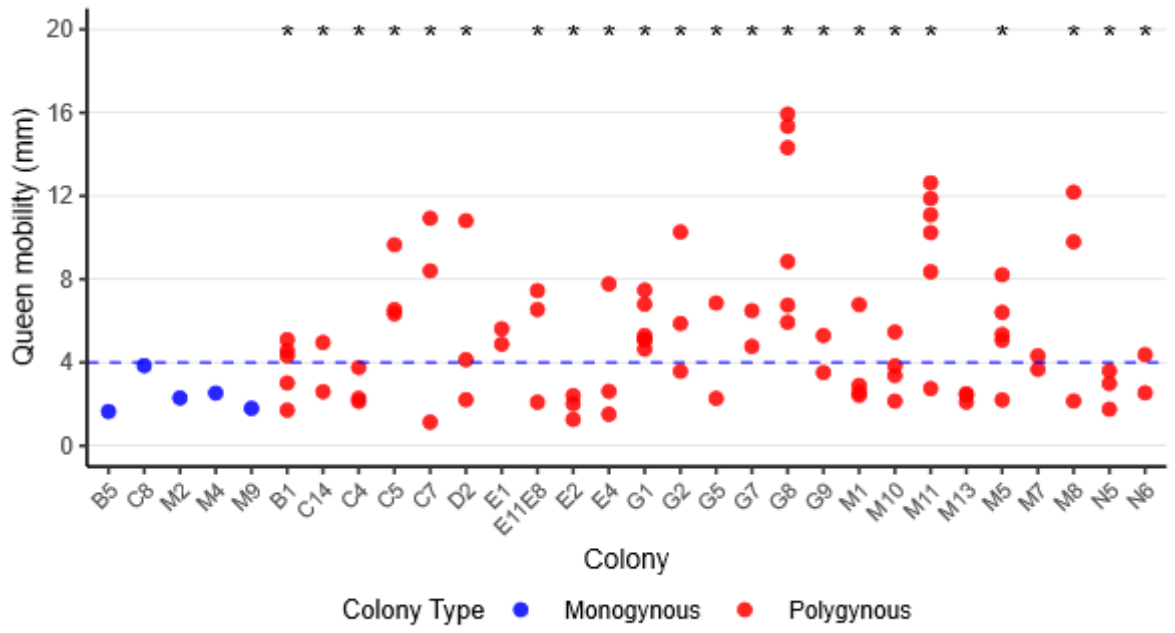

Figure 1: Mobility of *Stigmatomma pallipes* queens differs in polygynous colonies, with some having higher values and some similar values to monogynous queens. A dot plot highlighting evidence for variation among queens in mobility in most polygynous colonies (marked with an asterisk). Many polygynous queens (red dots) expressed higher mobility than monogynous queens (blue dots). The dashed blue line at 4 mm indicates the threshold used to differentiate and classify low-mobility (LM) and high-mobility (HM) polygynous queens. Colony identity is provided on the x-axis while queen mobility (estimated marginal mean for polygynous colonies, mean for monogynous queens) is provided on the y-axis.

The estimated marginal means for polygynous queen mobility ranged from 1.1 mm to 15.9 mm. In addition, the average mobility of monogynous queens ranged from 1.6 mm to 3.8 mm. We used this information to determine a threshold of 4 mm for the estimated

marginal mean to classify polygynous queens as either low-mobility queens (LM-polygynous queens) or high-mobility queens (HM-polygynous queens). Among the 22 polygynous colonies with significant differences in queen mobility, 15 colonies had at least one LM-polygynous queen and one HM-polygynous queen (Figure 1).

Prior to the assessment of mating status and ovarian development, one queen of each mobility category was selected from each polygynous colony, resulting in 30 queens from 15 polygynous colonies (Supplementary Figure S2). The selected queens were further analyzed for mating status, ovarian development, behavior, and gene expression in the brain and fat body. Mating status and ovarian development were assessed for the non-selected queens of these colonies as well, resulting in a total of 21 LM-polygynous queens, 29 HM-polygynous queens, and five monogynous queens examined.

Low-mobility queens were more likely to be mated than high-mobility queens

To investigate whether mobility was associated with reproductive activity, we assessed the mating status and ovarian development of all analyzed queens (Supplementary Table S5). For comparison, we included a single worker from each colony as a non-mated control. Out of the 15 polygynous colonies examined, which included 21 LM-queens and 29 HM-queens, all but two colonies (M1 and M10) contained one queen with a filled spermatheca, indicative of the queen being mated. All monogynous queens were mated. We also observed variation among queens in ovarian development: some presented well-developed ovaries with yellow bodies, indicative of recent egg laying activity, while other queens had underdeveloped ovaries that resembled those of workers (Figure 2).

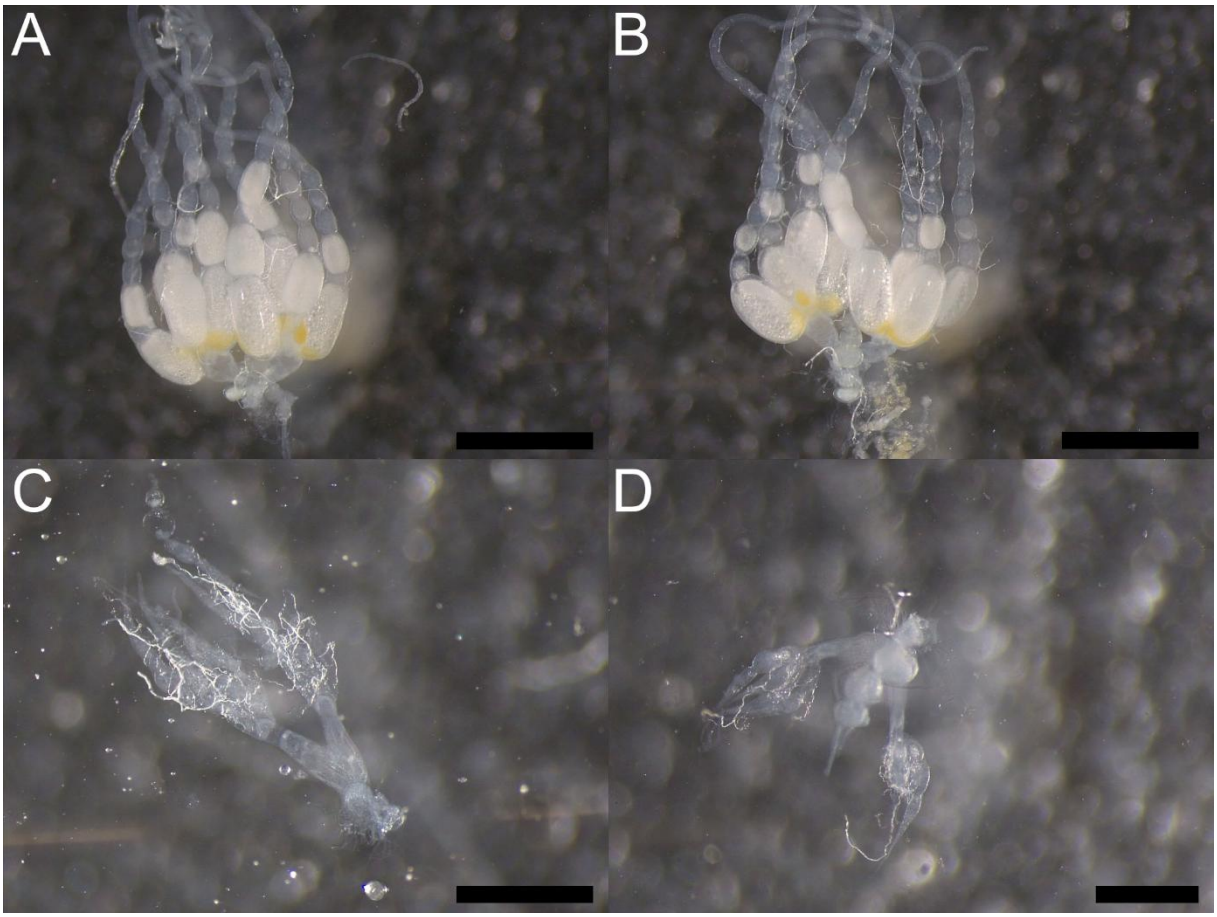

Figure 2: Ovarian development differs between *queen types as well as* workers in *Stigmatomma pallipes*. Representative images of the dissected ovaries of: A) monogynous queens; B) low-mobility (LM) polygynous queens; C) high-mobility (HM)polygynous queens; and D) workers. Black bars represent 1 mm (A-C) or 500 µm (D).

To elucidate whether mating status differed between LM- and HM-queens in polygynous colonies, we tested the association between mobility category and mating status. We found very strong evidence for an association between mobility and mating status (Fisher's exact test, $p < 0.0001$). More specifically, 13 out of the 21 LM-polygynous queens were mated (61%), whereas none of the 29 HM-polygynous queens were. We also found that the level of mobility was associated with the degree of ovarian development (Fisher's exact test, $p < 0.0001$). Mated LM-queens also had developed ovaries (69%), while only two (7%) of the HM-queens had developed ovaries. LM-queens were substantially more likely to be mated and have developed ovaries compared to HM-queens in polygynous colonies. Of the 30 queens from polygynous colonies chosen for further behavioral and molecular investigations, all 15 HM-polygynous queens were unmated, and all but two (13%) had undeveloped ovaries. Of the 15 LM-polygynous queens chosen, 13 (87%) were mated and had developed ovaries.

Behavioral clustering of queens revealed two clusters associated with mating and mobility

To investigate whether queen types differed in general terms of their overall behavioral phenotype, we scored 13 distinct behaviors (Table 1) every minute across five observation periods, for a total of 455 minutes. To examine general behavioral trends across queens, we applied a principal component analysis (PCA) using all observations and then performed K-means clustering based on all principal components, identifying two clusters, which largely separated polygynous queens based on mobility (Fisher's exact test, $p < 0.0001$; Figure 3).

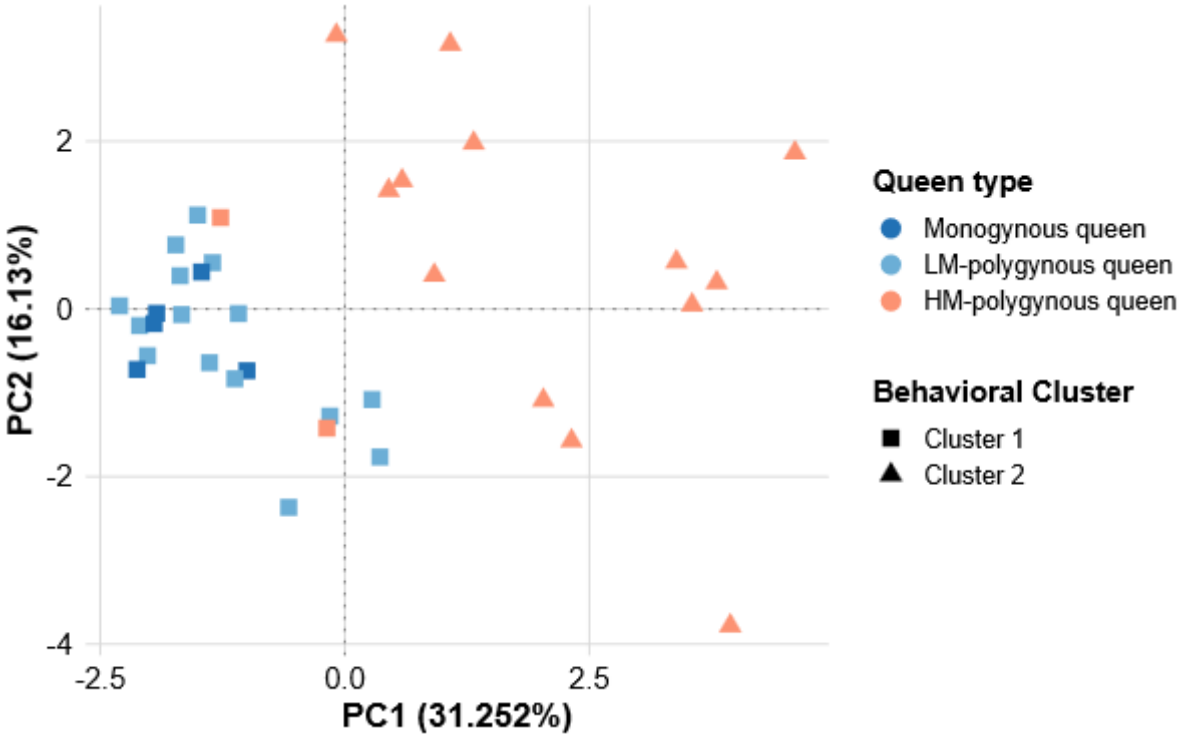

Figure 3: Principal Component Analysis reveals behavioral phenotypes of polygynous and monogynous queens associated with differences in mobility and mating status. A scatterplot displaying the first two principal components (PCs) from a principal component analysis (PCA) based on 13 scored behaviors for 35 queens. K-means clustering based on all principal components revealed two clusters. The percentage of variance explained by each PC is provided in parentheses. Each point represents an individual queen with colors indicating individual queen types, while shapes denote behavioral cluster membership.

Cluster 1 consisted of all five monogynous queens (100%), all 15 LM-polygynous queens (100%), and two out of the 15 HM-polygynous queens (13%). These two queens had developed ovaries but were unmated (Supplementary Table S5). Cluster 2 contained the remaining 13 out of 15 HM-polygynous queens (87%).

Behavioral differences among queens were driven by resting, self-grooming, and walking

We next examined which behaviors differed the most between queen types, by investigating the PCA loading of each behavior (Figure 4).

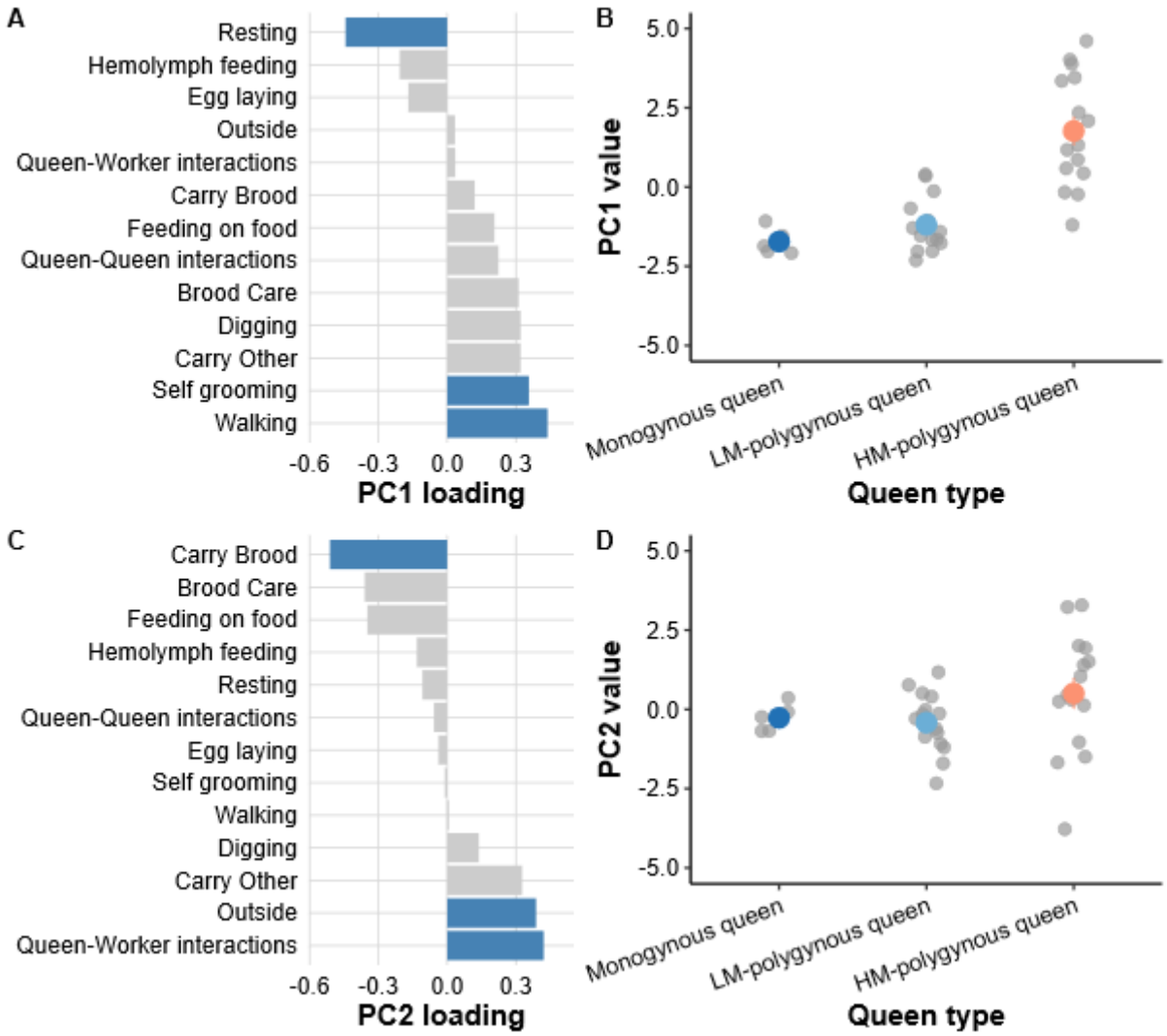

Figure 4: Loading of principal components reveal differences in behaviors associated with mobility in queens of *S.pallipes* . A) Principal component (PC) 1 captures variation in resting, self-grooming, and walking (highlighted in blue). B) There was strong evidence that PC1 values differed among queen types ($\chi^2 = 54.23$, $p < 0.0001$). C) PC2 captures variation in brood carrying, being outside, and interacting with workers (highlighted in blue). D) There was weak evidence that PC2 values differed among queen types ($\chi^2 = 5.40$, $p = 0.067$). Grey dots represent individual queens; colored dots and error bars indicate mean ± SE for each queen type.

Collectively, eight PCs explained 90% of the variance. Based on loadings, PC1 was primarily associated with resting, self-grooming, and walking behaviors (Figure 4A). PC1 values

differed among queen types (LMM, $\chi^2 = 54.23$, $p < 0.0001$; Figure 4B). Pairwise comparisons revealed that HM-polygynous queens had higher PC1 values than monogynous ($t = -5.16$, $p < 0.0001$) and LM-polygynous queens ($t = 6.66$, $p < 0.0001$), indicating they express more walking and self-grooming behavior, and less resting. LM-polygynous and monogynous queens did not differ based on PC1 ($t = -0.78$, $p = 0.72$). PC2 was positively associated with brood carrying behavior, and negatively correlated to being outside and expressing queen-worker interactions (Figure 4C). We found weak evidence that queen types differed based on PC2 (LMM, $\chi^2 = 5.40$, $p = 0.067$; Figure 4D). For PC3 to PC8, we found no evidence that queen types differed (all $p > 0.1$; Supplementary Table S6).

Clustering of RNAseq samples revealed that brain and fat body transcriptomic variation was associated with mating and mobility

We examined whether the brain and fat body transcriptomes were sufficient to group queens based on their mobility status. To do so, we conducted for each tissue a PCA on the full transcriptomes and performed K-means clustering based on the principal components (Figure 5).

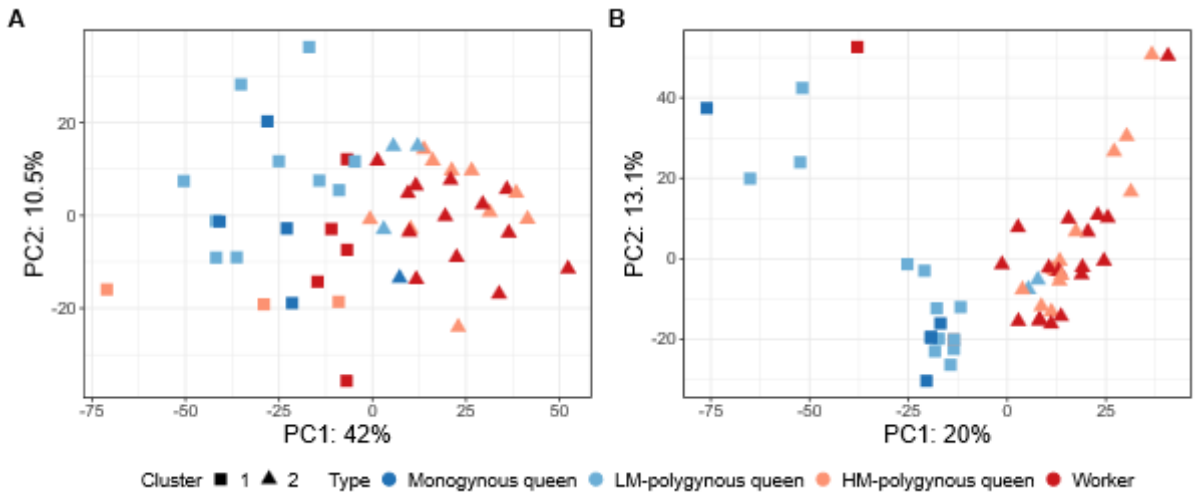

Figure 5: Brain and fat body transcriptomes show variation associated with differences in mobility and mating status. Scatterplots displaying the first two principal components (PCs) from principal component analyses (PCA) based on: A) brain; and B) fat body transcriptomes for queens and workers. K-means clustering analyses based on all PCs revealed two clusters for each tissue. Each point represents an individual with colors indicating individual types and shapes denoting cluster membership. The percentage of variance explained by each PC is provided.

In the brain, the analysis identified two clusters (Figure 5A). Cluster 1 included four out of five (80%) monogynous queens and 10 out of 13 (77%) LM-polygynous queens, as well as three out of 13 (23%) HM-polygynous queens and five out of 18 (28%) workers. Cluster 2 contained one out of five monogynous queen (20%), three out of 13 LM-polygynous queens (23%), 10 out of 13 HM-polygynous queens (77%), and 13 out of 18 workers (72%). Cluster membership for polygynous queens was significantly associated with queen mobility (Fisher's exact test, $p = 0.0169$, Cramer's $V = 0.538$).

In the fat body, our analysis also revealed two clusters (Figure 5B). Cluster 1 contained all five monogynous queens, 12 out of 14 (86%) LM-polygynous queens, one out of 15 (7%) HM-polygynous queens, and one out of 19 (5%) workers. The one HM-polygynous queen in Cluster 1 had developed ovaries. Cluster 2 comprised 13 out of 14 (93%) HM-polygynous queens (93%) and 18 out of 19 workers (95%). In polygynous colonies, cluster membership was significantly associated with mobility (Fisher's exact test, $p < 0.001$, Cramer's $V = 0.794$).

Gene expression patterns of monogynous and LM-polygynous queens differed from those of HM-polygynous queens and workers

To investigate whether the individual types differed in gene expression in brain and fat body, we quantified the number of differentially expressed genes (DEGs) between types. In the brain, we found 4,456 DEGs among individual types (adjusted $p < 0.05$; 37% of the 11,871 tested genes). To identify general transcriptional patterns, we clustered DEGs based on expression profile yielding six distinct expression groups (Figure 6)

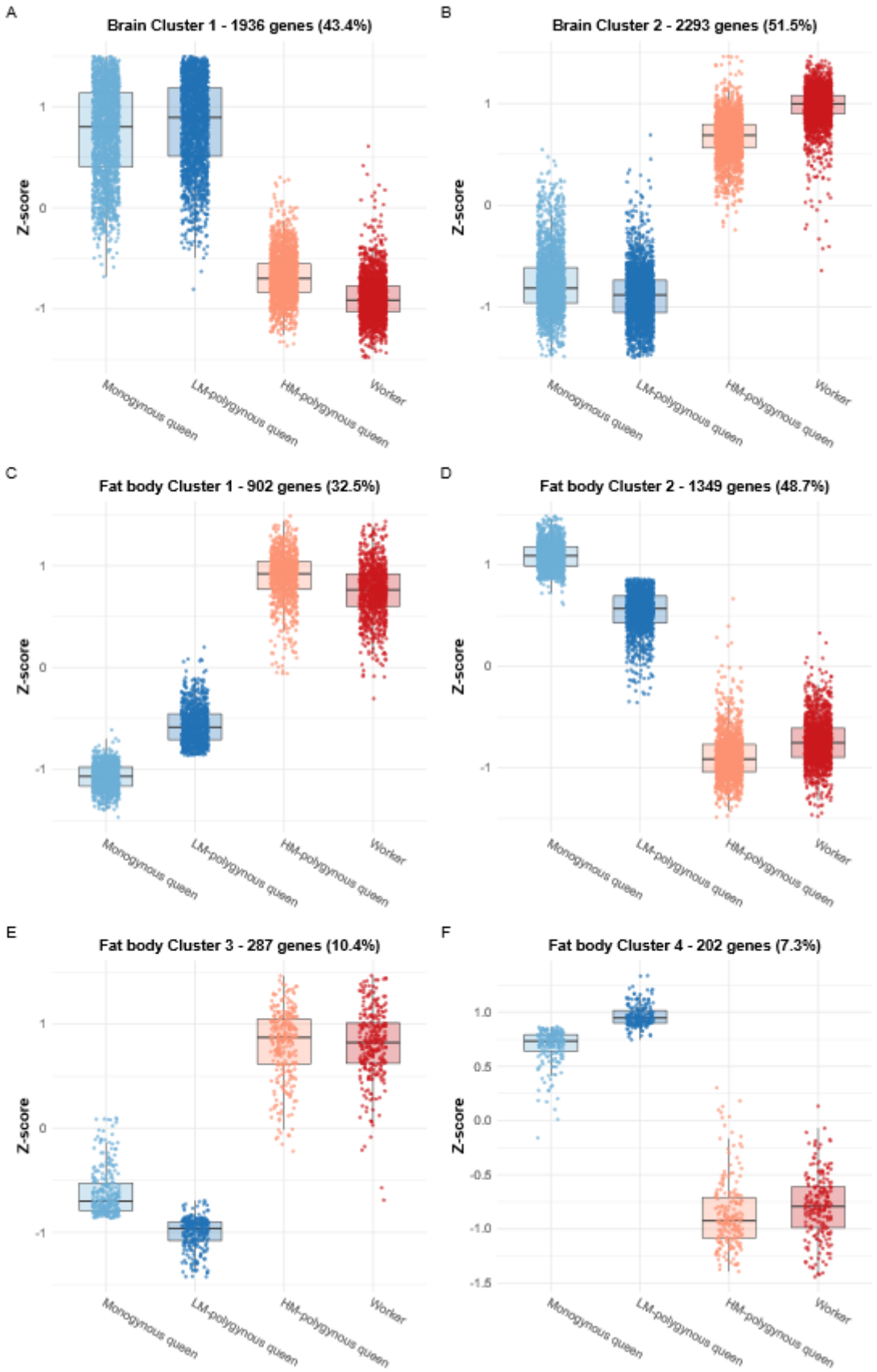

Figure 6: Clusters of differentially expressed genes (DEGs) separate mated from unmated individuals across A-B) the brain and C-F) the fat body. Each dot represents the z-score-transformed mean value of one gene, calculated from all individuals of that type. Colored boxes and error bars indicate mean ± SE for each type.

The majority of DEGs (94%) were assigned to the first two clusters (Figure 6A-B): Cluster 1 (n = 1,936, 43% of DEGs) contained genes with higher relative expression in monogynous queens and LM-polygynous queens compared to HM-polygynous queens and workers; and cluster 2 (n = 2,293, 51% of DEGs) showed the opposite pattern. The remaining four clusters contained less than 5% of all DEGs (Supplementary Figure S3A-D).

In the fat body, we found 2,788 DEGs (adjusted $p < 0.05$, 23% of the 12,084 tested genes). Clustering of these DEGs based on expression produced five clusters, of which the first four accounted for 98% of all genes (Figure 6C-E). All clusters showed relative similarities between HM-polygynous queens and workers, with both differing from monogynous and LM-polygynous queens. Clusters differed in whether and how expression levels varied between monogynous and LM-polygynous queens. Gene clusters 1 (n = 902, 32% of DEGs) and 4 (n = 202, 7% of DEGs) included genes with higher expression in LM-polygynous queens relative to monogynous queens, whereas clusters 2 (n = 1,349, 48% of DEGs) and 3 (n = 287, 10% of DEGs) showed the opposite pattern. Cluster 5 only contained 1.2% of the DEGs (Supplementary Figure S3E).

Genes that differed in expression between LM- and HM-polygynous queens were enriched for translational and neuronal processes

To investigate the functional profiles of subsets of DEGs that shared similar expression differences among individual types, we performed Gene Ontology (GO) term enrichment analyses per cluster (Table 2).

Table 2: Functional enrichment based on gene ontology terms identified gene clusters in the brain and fat body to be enriched for translational and neurological terms. For each tissue, we provide the cluster number, the GO term ID, the GO term description, and the adjusted p-value.

Tissue	Cluster	GO.ID	Description	Adjusted p-value
Brain	1	GO:0006418	tRNA aminoacylation for protein translation	0.046
		GO:0006364	rRNA processing	0.002
		GO:0032543	mitochondrial translation	2.31e- 3
		GO:0002181	cytoplasmic translation	1.08e-15
	2	GO:0007268	chemical synaptic transmission	0.039
		GO:0048010	vascular endothelial growth factor receptor signaling pathway	0.037
		GO:0072659	protein localization to plasma membrane	0.037
		GO:0008286	insulin receptor signaling pathway	0.037
		GO:0045944	positive regulation of transcription by RNA polymerase II	0.035
		GO:0007616	long-term memory	0.035
		GO:0008355	olfactory learning	0.029
		GO:0050803	regulation of synapse structure or activity	0.029
		GO:0045880	positive regulation of smoothened signaling pathway	0.020
		GO:0032482	Rab protein signal transduction	0.014
		GO:0016319	mushroom body development	0.013
		GO:0048190	wing disc dorsal/ventral pattern formation	0.013
		GO:0007391	dorsal closure	0.005
		GO:0006468	protein phosphorylation	0.005
		GO:0090263	positive regulation of canonical Wnt signaling pathway	0.005
		GO:0007173	epidermal growth factor receptor signaling pathway	0.004
		GO:0008543	fibroblast growth factor receptor signaling pathway	0.003
		GO:0007298	border follicle cell migration	0.002
		GO:0007411	axon guidance	0.002
		GO:0008587	imaginal disc-derived wing margin morphogenesis	0.002
Fat body	1	GO:0006120	mitochondrial electron transport, NADH to ubiquinone	3.74e-05
		GO:0032981	mitochondrial respiratory chain complex I assembly	6.61e-03
	2	GO:0006357	regulation of transcription by RNA polymerase II	1.77e-03

GO term enrichment analysis of cluster-associated DEGs identified in the brain revealed significant enrichment for DEGs in clusters 1 and 2 (Table 2). DEGs in cluster 1, which had relatively higher expression in monogynous queens and LM-polygynous queens, were enriched for four terms, including cytoplasmic translation (GO:0002181), mitochondrial translation (GO:0032543), rRNA processing (GO:0006364), and RNA aminoacylation for protein translation (GO:0006418). For DEGs within cluster 2, which were elevated in workers and HM-polygynous queens (Figure 6), 20 enriched terms were identified, including axon guidance (GO:0007411), epidermal growth factor receptor signaling pathway (GO:0007173), mushroom body development (GO:0016319), and insulin receptor signaling pathway (GO:0008286).

In the fat body, similar to the brain, we detected enriched GO terms for DEGs in clusters 1 and 2 (Table 2). DEGs in cluster 1, which encompassed genes with higher expression in HM-polygynous queens and workers, were enriched for two terms: mitochondrial electron transport, NADH to ubiquinone (GO:0006120) and mitochondrial respiratory chain complex I assembly (GO:0032981). DEGs in cluster 2, characterized by genes with higher expression in monogynous queens and LM-polygynous queens, showed enrichment for a single term: Regulation of transcription by RNA polymerase II (GO:0006357).

Discussion

To better understand behavioral and physiological variation within the queen caste in polygynous colonies, we investigated the mobility, mating status, behavior, and gene expression of queens in polygynous colonies of the ant *S. pallipes* and compared them to monogynous queens and workers. Across our analyses, two distinct polygynous queen types emerged: low-mobility (LM) mated queens that shared characteristics with monogynous mated queens; and high-mobility (HM) unmated queens that grouped with workers on different biological scales ranging from the molecular to the organismal (Figure 7). These findings reveal that some queens in polygynous *S. pallipes* colonies resemble workers in both their behavioral phenotype, as well as the underlying molecular mechanisms.

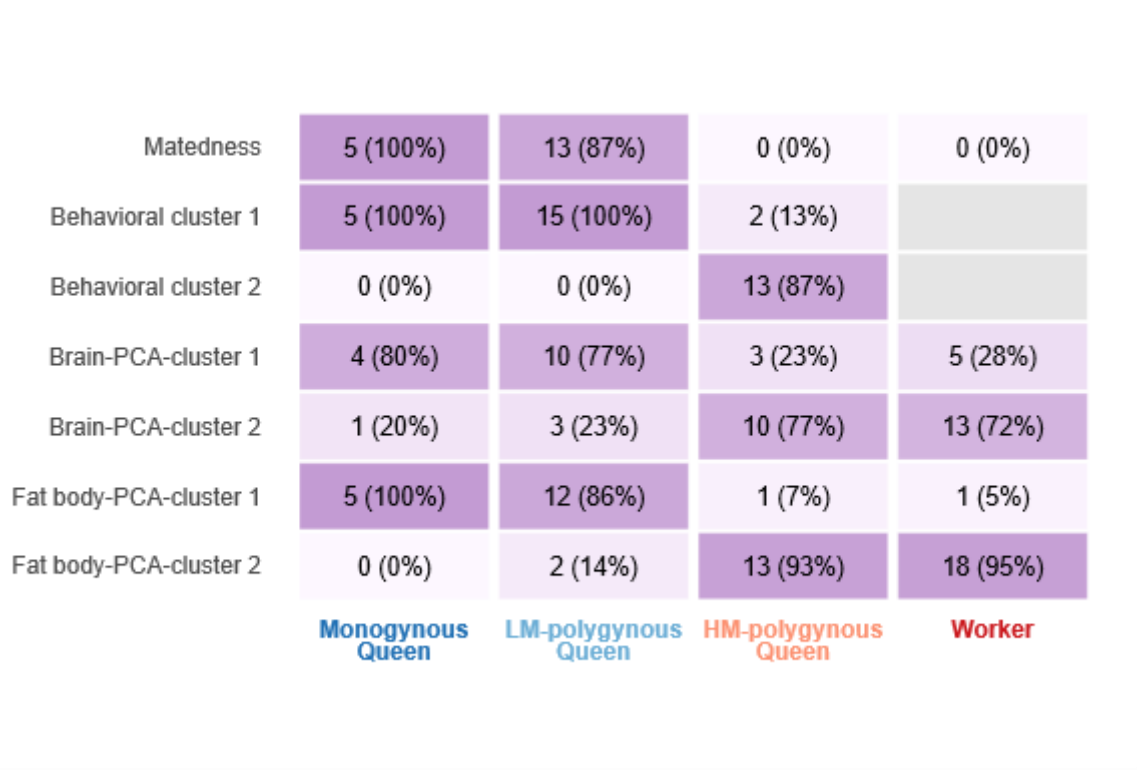

Figure 7: The relative distributions of mating status, behavior-based clusters, and transcriptome-based clusters illustrate that low-mobility polygynous queens resembled monogynous queens, while high-mobility polygynous queens were similar to workers. A heatmap illustrating the proportion of individuals assigned to each cluster category for monogynous queens, low-mobility (LM) polygynous queens, high-mobility (HM) polygynous queens, and workers. Numbers denote the number of individuals in the respective category with the percentage compared to the overall number of individuals in that group provided in parenthesis. The color gradient indicates the percentage of individuals within each group assigned to the respective category.

The behavioral differences between the mated LM- and unmated HM-queens were primarily driven by mobility-related behaviors, such as resting, walking, and foraging outside the nest. LM-polygynous and monogynous queens, which were mated and reproductively active, showed behavioral similarities to established queens in other social insect species, as the focus on reproduction is usually associated with reduced mobility (Free et al. 1992; Nagel et al. 2020). For example, *Harpegnathos saltator* mated workers move less and are more likely to stay inside the nest compared to unmated workers (Penick et al. 2021; Haight and Liebig 2025). Interestingly, within our analysis, LM-polygynous and monogynous queens also engaged in interactions with the brood, which stands in contrast to the usual specialization of ant queens in egg laying when in the presence of workers (Majidifar et al. 2024). Previous behavioral observations of two *S. pallipes* queens from a single colony revealed a comparable behavioral repertoire (Traniello 1982) and queens have been reported to partake in upkeep of the colonies (Haskins 1928). The interactions with the brood may be explained by *S. pallipes* queens feeding on the regurgitated liquids of larvae (Haskins 1928; Traniello 1982b).

The transcriptomic analyses were consistent with the behavioral analyses in that they revealed that LM-polygynous queens and monogynous queens had similar brain and fat body gene expression patterns, which differed from HM-polygynous queens and workers. This finding was supported by both the clustering of samples based on overall gene expression and the targeted analysis of the genes that showed expression differences among individual types. In both tissues, we found a large number of differentially expressed genes (DEGs) between LM- and HM-polygynous queens. Previous transcriptomic studies that compared queens in different conditions, such as age and mating status, reported fewer differentially expressed genes (Lucas and Keller 2018; Nagel et al. 2020; Ferreira et al. 2025). Finding thousands of differentially expressed genes is usually restricted to comparisons between anatomically and morphologically differentiated castes (Grozinger et al. 2007; Harrison et al. 2015; Morandin et al. 2016).

The transcriptomic variation between LM- and HM-polygynous queens may be partly explained by differences in mating status. Although this was not conducted in the context of polygyny, previous studies that compared unmated queens before colony foundation and established mated queens also reported large transcriptomic variation associated with the mating status (von Wyschetzki et al. 2015; Nagel et al. 2020; Liu et al. 2023). The age of the queens was also found to be associated with variation in gene expression in multiple ant species (von Wyschetzki et al. 2015; Lucas et al. 2017; Lucas and Keller 2018; Negroni et al. 2019; Nagel et al. 2020; Harrison et al. 2021). While the exact age of the queens used in our

study remains unknown, the unmated queens may have been younger because they were observed to be adopted by established colonies with older, mated queens (Traniello 1982).

Differentially expressed genes between HM-polygynous queens and workers compared to LM-polygynous queens and monogynous queens showed tissue-specific functional enrichment. In the brain, genes that were more highly expressed in HM-polygynous queens and workers relative to LM-polygynous queens and monogynous queens were enriched for axon guidance, insulin signaling, mushroom-body development and synaptic signaling, which are expected to support navigation, sensory processing, and task flexibility (Ament et al. 2008; Alleman et al. 2019; Nagel et al. 2020). This is consistent with reports in other ant species, where unmated queens upregulated genes involved in mushroom body functioning and axon guidance (Nagel et al. 2020), and reproductive individuals showed reduced expression of genes involved in neural investment (Julian and Gronenberg 2002; Groh et al. 2006; Penick et al. 2021). Meanwhile, genes overexpressed in the brain of LM-polygynous queens and monogynous queens showed the highest confidence for enrichment for processes related to cytoplasmic translation, which was also enriched in genes upregulated in the brain of mated queens compared to unmated queens in another ant species (Nagel et al. 2020). In the fat body, genes with higher expression in HM-polygynous queens and workers were enriched for mitochondrial processes, which aligns with previous reports that insect fat bodies adjust mitochondrial content and activity in response to physiological demands (Arrese and Soulages 2010; Treidel et al. 2023). Finally, our finding of enhanced protein synthesis in the fat body of LM-polygynous and monogynous queens may reflect physiological processes associated with egg production (Price 1973; Jensen and Børgesen 2000; Arrese and Soulages 2010) as both sets of queens were mostly reproductive active.

Our results provide strong evidence that our *S. pallipes* polygynous colonies were actually functionally monogynous, as they contained only one inseminated and reproductively active queen with the other queens being unmated. This stands in contrast with a previous report of multiple reproductively active queens within *S. pallipes* colonies (Traniello 1982). However, this earlier finding was merely supported by behavioral observations and only concerned a subset of the observed colonies (Traniello 1982). As the colonies from that study were collected in Westford, Massachusetts in spring and summer 1977-1978 (Traniello 1982), and the colonies in our study were collected in the Huyck Preserve, New York in May 2024, it may also be that the social structure of *S. pallipes* differs among populations, as found in other ants (Gill et al. 2009), or that colonies with multiple reproductively active queens are rare. Given our focus on colonies with variation among queens in mobility values, it may also be that colonies with several, mated and, thus, LM-queens were not analyzed further, potentially limiting the representation of this social structure.

Several lines of evidence show that unmated HM-queens in polygynous *S. pallipes* colonies were analogous to workers at behavioral and molecular levels. First, they exhibited high mobility and more routinely expressed behaviors, such as foraging outside, digging, and transporting nest material, which represent typical worker behaviors. Such expression of non-reproductive behaviors by queens was also reported in several species of ants, including *S. pallipes*, where unmated queens do not mate or disperse, and instead shed their wings and remain in their natal colony to perform tasks usually carried out by workers (Haskins 1928; Johnson et al. 2007; Nehring et al. 2012). Our finding also echoes the description of a worker-like reproductive caste expressing worker-like behaviors in the closely related polygynous species *Mystrium oberthueri* (Molet et al. 2007).

While previous reports of queens resembling workers were generally limited to behavioral description and the analysis of ovarian development (Buschinger 1968; Ito et al. 1996; Hora et al. 2005; Holbrook et al. 2007; Vieira et al. 2012; Nehring et al. 2012; Araújo et al. 2016; Murakami 2020), we demonstrate that unmated HM-queens in polygynous *S. pallipes* colonies had very similar transcriptomic profiles to workers, both in the brain and in the fat body. In contrast, they showed large differences in gene expression compared to mated queens from either monogynous or polygynous colonies. Interestingly, while queens and workers have very distinct transcriptomic profiles (Grozinger et al. 2007; Ferreira et al. 2013; Feldmeyer et al. 2014; Morandin et al. 2017; Das and de Bekker 2022), our analyses did not detect any brain nor fat body gene expression differences when collectively comparing the queen and worker castes, presumably because of the worker-like gene expression patterns observed within HM-polygynous queens.

Our study establishes measurements of mobility as a non-invasive method to predict reproductive variation between queens in polygynous *S. pallipes* colonies, although future work will be needed to validate it in other ant species. This method opens the possibility for experimental manipulations of colony composition through the targeted removal of specific individuals to assess their influence on colony organization and reproductive dynamics. Such studies will contribute to better understanding how reproductive roles are regulated across polygynous systems.

The behavior of social insect queens is traditionally viewed as more homogeneous than that of the workers because the role of queens in mature colonies is restricted to producing eggs while workers fulfill all non-reproductive tasks. The behavioral heterogeneity of the worker caste is best illustrated by age polyethism, whereby younger workers tend to perform intranidal tasks, such as brood care, while older workers tend to leave the nest and forage for food (Tripet and Nonacs 2004; Winston 1991; Camargo et al. 2007; Wilson 1976; Seeley

1982; Beshers and Traniello 1996). This behavioral transition is associated with transcriptomic and physiological changes (Kohlmeier et al. 2019; Dolezal et al. 2013; Kühbandner et al. 2014). Here, we report large behavioral and transcriptomic variation within the queen caste, with the coexistence in polygynous *S. pallipes* colonies of mated queens that monopolize egg production and unmated queens that are characterized by worker-like behavior and gene expression. Our results, together with recent reports of context-dependent queen specialization (Majidifar et al. 2024; Woodard et al. 2013) raise questions on the constitutive specialization in egg production of social insect queens and call for further studies of variation within the queen caste at multiple phenotypic levels to better understand division of labor in insect societies.

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Conflicts of interest

The authors declare no conflicts of interest.

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Data accessibility statement

All raw data used in this study are available as part of the Supporting Information or will be made publicly available from the NCBI Sequence Read Archive (BioProject: PRJNA1371108) upon acceptance. The code and input data for all analyses is provided as Supporting Information.

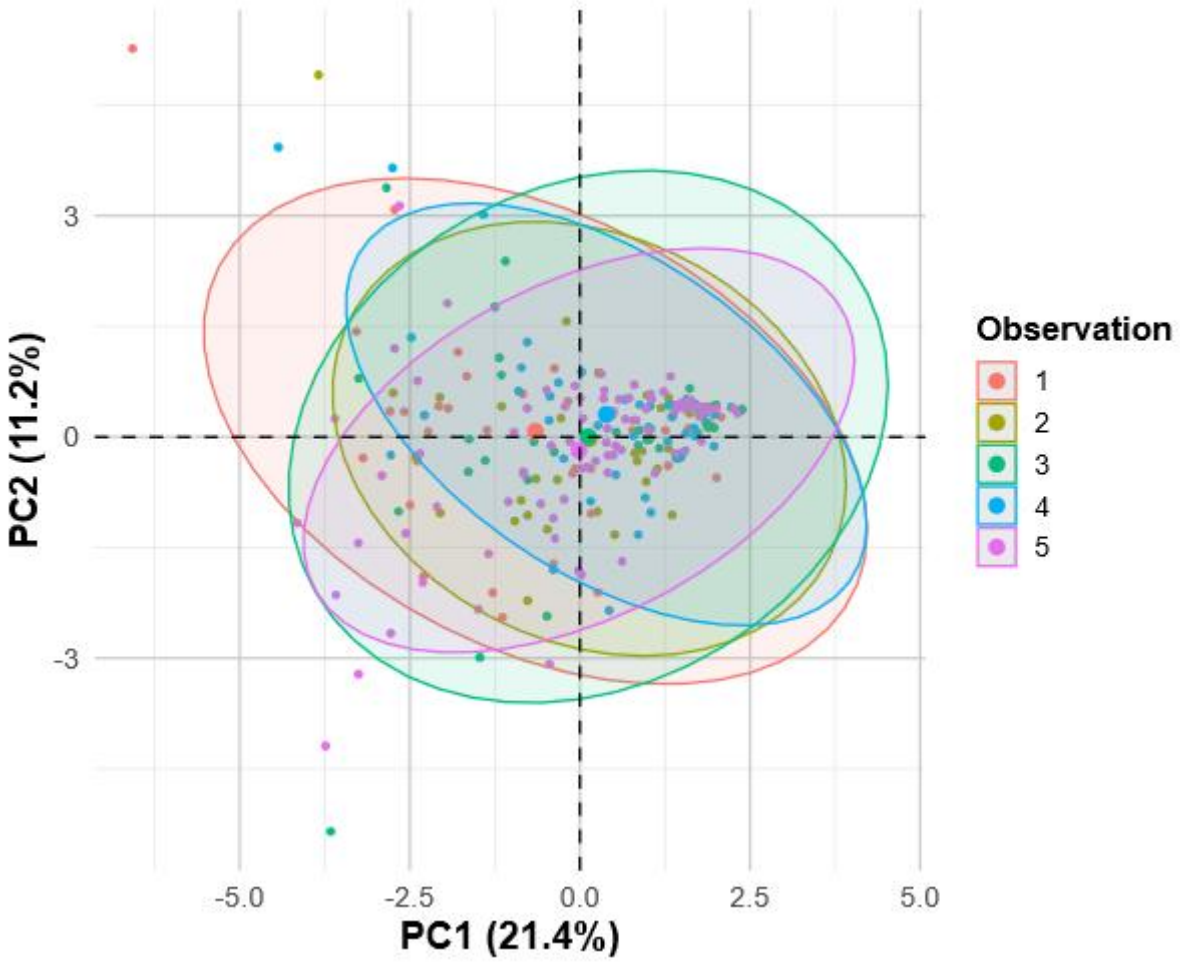
Benefit sharing statement

Raw sequence data have been uploaded to the NCBI Sequence Read Archive (BioProject: PRJNA1371108). The assembled genome and annotations will be hosted on a publicly accessible GitHub repository and will be released upon acceptance: https://github.com/Joscolgan/stigmatomma_rnaseq.

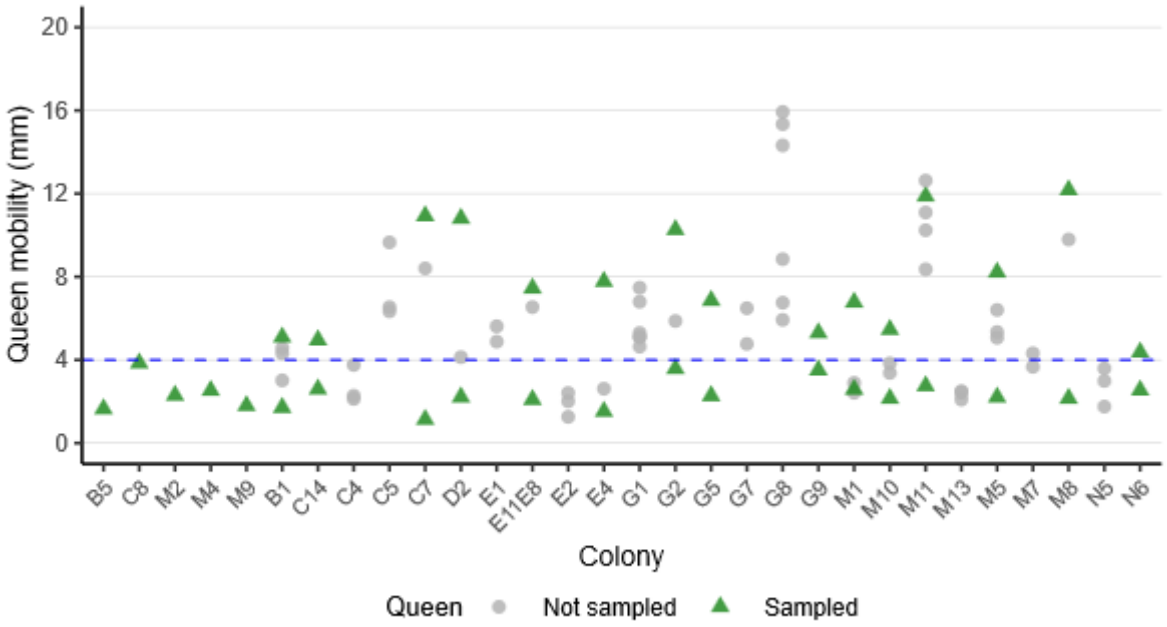
Author contributions

MFB conceived the idea and designed the methodology together with [redacted] and [redacted]. MFB collected the ants and conducted the experiments. MFB and [redacted] analyzed the data. [redacted] and [redacted] assembled and annotated the genome. MFB, [redacted], and [redacted] led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

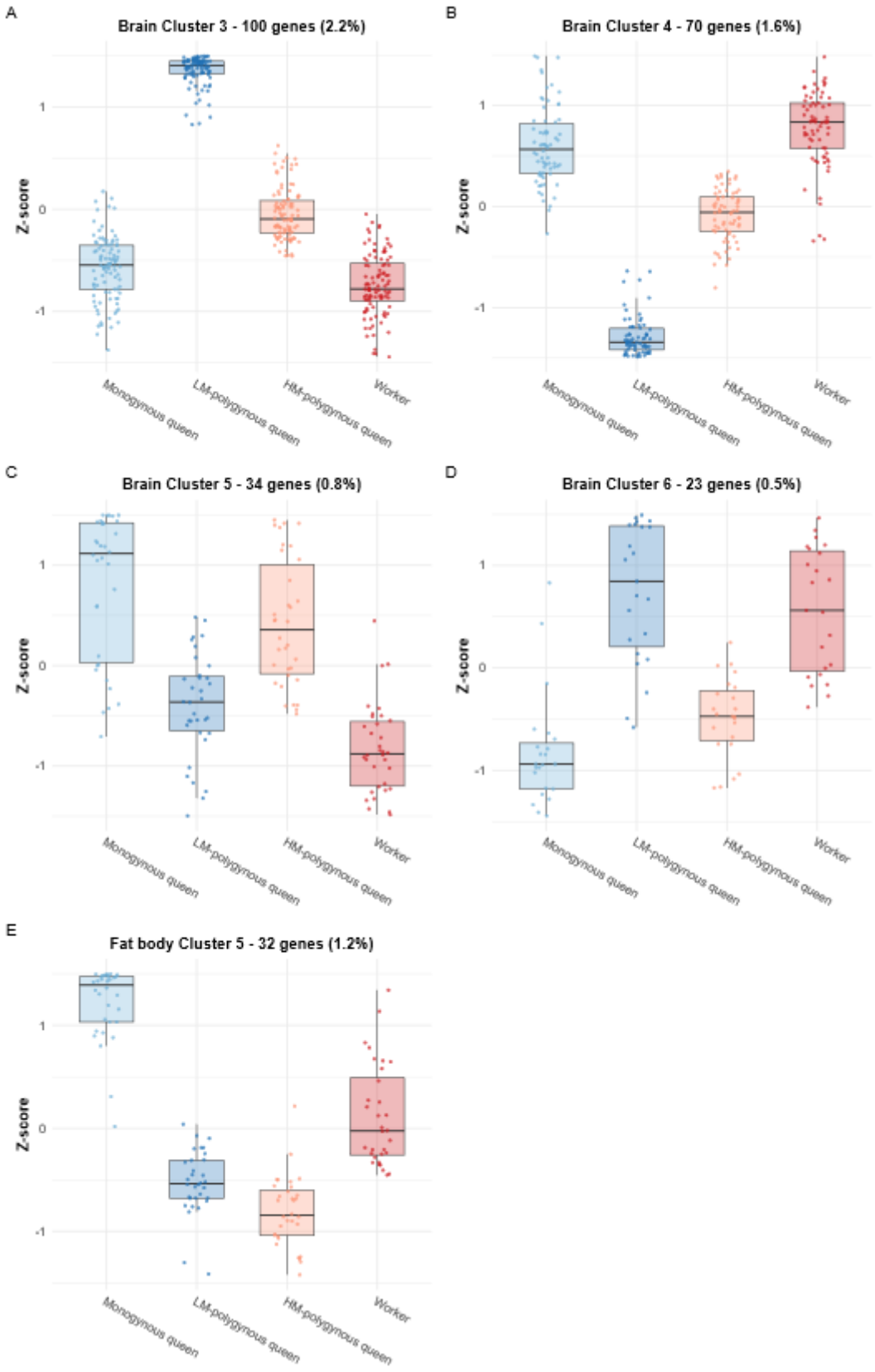
Supplementary Material



Supplementary Figure S1: Limited variation across observations in queen behavior. The scatterplot displays the first two principal components from a principal component analysis (PCA) based on 13 scored behaviors for 35 queens across the five observations. The percentage of variance explained by each PC is provided in parentheses. Ellipses represent 95% confidence intervals around group centroids, colors indicate different observations, small dots correspond an individual queen, big dots the group centroid.



Supplementary Figure S2. Mobility variation among *Stigmatomma pallipes* queens from monogynous and polygynous colonies allowed the selection of samples for further analyses. The dashed blue line at 4 mm indicates the threshold used to differentiate low mobility (LM) and high mobility (HM) polygynous queens. Indicated as green triangles are queens that were selected for mating status, ovarian development, behavior, and gene expression in the brain and fat body. Colony identity is provided on the x-axis while queen mobility (estimated marginal mean for polygynous, mean for monogynous queens) is provided on the y-axis.



Supplementary Figure 3: Clusters of differentially expressed genes (DEGs) that show similar expression differences across individual types for A-D) the brain and E) the fat body. Each cluster represented less than 5% of the number of DEGs. Each dot represents the z-score-transformed mean value of one gene, calculated from all individuals of that category.

Supplementary Table 1: Summary table of the colonies used in the study, providing the number of queens and workers per colony.

Colony ID	Number of queens	Number of workers
B1	5	23
B5	1	31
C14	2	25
C4	3	25
C5	3	3
C7	3	6
C8	1	22
D2	3	12
E1	2	26
E11/E8	3	15
E2	3	10
E4	3	23
G1	6	7
G2	3	17
G5	2	17
G7	2	7
G8	6	12
G9	2	22
M1	4	5
M10	4	17
M11	6	8
M13	3	21
M2	1	15
M4	1	19
M5	5	13
M7	2	12
M8	3	7
M9	1	17
N5	4	11
N6	2	7

Supplementary Table 2: Reads of RNA sequences from fat body and brain from RNAseq.

Sample ID	Colony	Color	Category	Fat body sequences (million reads)	Brain sequences (million reads)
B1_G	B1	Green	LM-polgynous queens	13.7	14.8
B1_P	B1	Pink	HM-polgynous queens	17.9	22.3
B1_W	B1	/	Worker	19.3	46.4
B5_B	B5	Blue	Monogynous queen	16.0	14.5

B5_W	B5	/	Worker	3.7	14.4
C14_B	C14	Blue	HM-polgynous queens	12.2	13.8
C14_G	C14	Green	LM-polgynous queens	14.5	14.7
C14_W	C14	/	Worker	19.4	14.4
C7_B	C7	Blue	LM-polgynous queens	24.4	14.7
C7_G	C7	Green	HM-polgynous queens	20.6	/
C7_W	C7	/	Worker	11.0	11.2
C8_B	C8	Blue	Monogynous queen	28.5	12.8
C8_W	C8	/	Worker	14.8	/
D2_B	D2	Blue	LM-polgynous queens	22.1	24
D2_G	D2	Green	HM-polgynous queens	23.3	15
D2_W	D2	/	Worker	21.7	23.3
E11E8_B	E11E8	Blue	LM-polgynous queens	21.3	13.6
E11E8_O	E11E8	Orange	HM-polgynous queens	9.9	35.2
E11E8_W	E11E8	/	Worker	21.2	34.8
E4_G	E4	Green	LM-polgynous queens	18.9	26.2
E4_U	E4	Unmarked	HM-polgynous queens	17.0	21
E4_W	E4	/	Worker	14.0	41.9
G2_B	G2	Blue	HM-polgynous queens	20.1	14
G2_G	G2	Green	LM-polgynous queens	/	32.5
G2_W	G2	/	Worker	23.1	8.3
G5_B	G5	Blue	HM-polgynous queens	22.8	28.6
G5_O	G5	Orange	LM-polgynous queens	33.6	/
G5_W	G5	/	Worker	16.2	26.8
G9_G	G9	Green	LM-polgynous queens	19.0	46.4
G9_W	G9	/	Worker	14.9	25.9
G9_Y	G9	Yellow	HM-polgynous queens	18.6	/
M1_O	M1	Orange	HM-polgynous queens	16.0	27
M1_P	M1	Pink	LM-polgynous queens	15.0	/
M1_W	M1	/	Worker	79.5	14.7
M10_B	M10	Blue	HM-polgynous queens	17.1	9.9
M10_O	M10	Orange	LM-polgynous queens	22.1	27.3
M10_W	M10	/	Worker	29.6	/
M11_G	M11	Green	HM-polgynous queens	32.2	74.9
M11_O	M11	Orange	LM-polgynous queens	14.2	19.4
M11_W	M11	/	Worker	16.2	16.3
M2_P	M2	Pink	Monogynous queen	20.4	14.1
M2_W	M2	/	Worker	14.3	43
M4_B	M4	Blue	Monogynous queen	18.6	12.6
M4_W	M4	/	Worker	21.6	41.2
M5_B	M5	Blue	LM-polgynous queens	22.3	14.8
M5_G	M5	Green	HM-polgynous queens	18.3	13.9
M5_W	M5	/	Worker	17.2	76.4
M8_B	M8	Blue	HM-polgynous queens	12.4	24.5

M8_G	M8	Green	LM-polgynous queens	20.8	17.3
M8_W	M8	/	Worker	/	10.4
M9_B	M9	Blue	Monogynous queen	19.5	20.3
M9_W	M9	/	Worker	20.8	29
N6_B	N6	Blue	LM-polgynous queens	23.8	13.6
N6_O	N6	Orange	HM-polgynous queens	21.7	16.3
N6_W	N6	/	Worker	17.6	14.2

Supplementary Table 3: Mobility values of *Stigmatomma pallipes* queens from 30 different colonies. The table include the colony, the subject, mobility (emmean) value and colony type.

Unique_Subject	Colony	Subject	mobility	Colony Type
B5_B	B5	Blue	1.644894	Monogynous
C8_B	C8	Blue	3.847729	Monogynous
M2_P	M2	Pink	2.293884	Monogynous
M4_B	M4	Blue	2.528135	Monogynous
M9_B	M9	Blue	1.794306	Monogynous
B1_B	B1	Blue	4.559646	Polygynous
B1_G	B1	Green	1.707689	Polygynous
B1_O	B1	Orange	4.316558	Polygynous
B1_P	B1	Pink	3.014856	Polygynous
B1_Pu	B1	Purple	5.064608	Polygynous
C14_B	C14	Blue	4.957165	Polygynous
C14_G	C14	Green	2.594513	Polygynous
C4_B	C4	Blue	2.124176	Polygynous
C4_G	C4	Green	3.75847	Polygynous
C4_U	C4	Unmarked	2.278928	Polygynous
C5_B	C5	Blue	6.540678	Polygynous
C5_Y	C5	Yellow	9.653923	Polygynous
C7_B	C7	Blue	1.133714	Polygynous
C7_G	C7	Green	10.92631	Polygynous
C7_O	C7	Orange	8.401123	Polygynous
D2_B	D2	Blue	2.21216	Polygynous
D2_G	D2	Green	10.80424	Polygynous
D2_O	D2	Orange	4.132391	Polygynous
E1_B	E1	Blue	4.881672	Polygynous
E1_G	E1	Green	5.616775	Polygynous
E11E8_B	E11E8	Blue	2.090738	Polygynous
E11E8_G	E11E8	Green	6.540061	Polygynous
E11E8_O	E11E8	Orange	7.447583	Polygynous
E2_B	E2	Blue	2.409262	Polygynous
E2_G	E2	Green	2.011197	Polygynous
E2_O	E2	Orange	1.262286	Polygynous

E4_B	E4	Blue	2.613408	Polygynous
E4_G	E4	Green	1.51563	Polygynous
E4_Un	E4	Unmarked	7.765669	Polygynous
G1_B	G1	Blue	4.636591	Polygynous
G1_G	G1	Green	5.301307	Polygynous
G1_O	G1	Orange	5.100007	Polygynous
G1_P	G1	Pink	6.796147	Polygynous
G1_Pu	G1	Purple	7.471597	Polygynous
G1_Y	G1	Yellow	5.095319	Polygynous
G2_B	G2	Blue	10.26099	Polygynous
G2_G	G2	Green	3.575617	Polygynous
G2_O	G2	Orange	5.872779	Polygynous
G5_B	G5	Blue	6.856189	Polygynous
G5_O	G5	Orange	2.272784	Polygynous
G7_B	G7	Blue	6.484336	Polygynous
G7_Y	G7	Yellow	4.77091	Polygynous
G8_B	G8	Blue	6.751972	Polygynous
G8_G	G8	Green	15.92481	Polygynous
G8_O	G8	Orange	15.33514	Polygynous
G8_Pu	G8	Purple	14.31486	Polygynous
G8_W	G8	White	8.84341	Polygynous
G8_Y	G8	Yellow	5.92344	Polygynous
G9_G	G9	Green	3.512788	Polygynous
G9_Y	G9	Yellow	5.296591	Polygynous
M1_B	M1	Blue	2.416721	Polygynous
M1_G	M1	Green	2.897273	Polygynous
M1_O	M1	Orange	6.772417	Polygynous
M1_P	M1	Pink	2.582998	Polygynous
M10_B	M10	Blue	5.460928	Polygynous
M10_G	M10	Green	3.375489	Polygynous
M10_O	M10	Orange	2.144744	Polygynous
M10_P	M10	Pink	3.835166	Polygynous
M11_B	M11	Blue	10.23686	Polygynous
M11_G	M11	Green	11.87246	Polygynous
M11_O	M11	Orange	2.745354	Polygynous
M11_P	M11	Pink	8.344872	Polygynous
M11_Un	M11	Unmarked	12.62707	Polygynous
M11_W	M11	White	11.08944	Polygynous
M13_P	M13	Purple	2.499494	Polygynous
M13_R	M13	Red	2.575501	Polygynous
M13_Y	M13	Yellow	2.094679	Polygynous
M5_B	M5	Blue	2.203201	Polygynous
M5_G	M5	Green	8.202179	Polygynous
M5_O	M5	Orange	5.332778	Polygynous

M5_P	M5	Pink	6.404881	Polygynous
M5_Y	M5	Yellow	5.063879	Polygynous
M7_G	M7	Green	4.317933	Polygynous
M7_P	M7	Pink	3.666536	Polygynous
M8_B	M8	Blue	12.17611	Polygynous
M8_G	M8	Green	2.148315	Polygynous
M8_O	M8	Orange	9.793806	Polygynous
N5_B	N5	Blue	2.991087	Polygynous
N5_G	N5	Green	3.593383	Polygynous
N5_R	N5	Red	2.569293	Polygynous
N5_Y	N5	Yellow	1.755652	Polygynous
N6_B	N6	Blue	2.539885	Polygynous
N6_O	N6	Orange	4.372279	Polygynous

Supplementary Table 4: **Summary outputs of ANOVAs to test mobility differences among queens within each polygynous colony**. P values below the 5% threshold are indicated in bold.

Colony	Number of queens	χ^2 value	P value
B1	5	105.335	**7.185E-22**
C14	2	38.337	**5.952E-10**
C4	3	34.695	**2.924E-08**
C5	3	22.272	**1.458E-05**
C7	3	313.247	**9.532E-69**
D2	3	293.912	**1.506E-64**
E1	2	2.418	1.199E-01
E11E8	3	132.248	**1.917E-29**
E2	3	16.948	**2.088E-04**
E4	3	274.037	**3.117E-60**
G1	6	46.903	**5.946E-09**
G2	3	142.220	**1.310E-31**
G5	2	106.347	**6.188E-25**
G7	2	11.613	**6.548E-04**
G8	6	294.260	**1.716E-61**
G9	2	14.965	**1.095E-04**
M1	4	149.238	**3.847E-32**
M10	4	52.953	**1.877E-11**
M11	6	310.905	**4.527E-65**
M13	3	2.324	3.128E-01
M5	5	144.431	**3.175E-30**
M7	2	1.490	2.222E-01
M8	3	327.770	**6.695E-72**
N5	3	39.756	**2.329E-09**
N6	2	32.369	**1.275E-08**

Supplementary Table 5: Output of the examination of reproductive activity in *S.pallipes* queens and workers.

Subject ID	Colony	Detection of the spermatheca	Status of the spermatheca	Mated	Ovarian development	Colony Type	Mobility
B5_B	B5	Yes	filled	Mated	Developed	monogynous	LM
B5_W	B5	No	/	Unmated	underdeveloped	monogynous	/
C8_B	C8	Yes	filled	Mated	Developed	monogynous	LM
C8_W	C8	No	/	Unmated	underdeveloped	monogynous	/
M2_P	M2	Yes	filled	Mated	Developed	monogynous	LM
M2_W	M2	No	/	Unmated	underdeveloped	monogynous	/
M4_B	M4	Yes	filled	Mated	Developed	monogynous	LM
M4_W	M4	No	/	Unmated	underdeveloped	monogynous	/
M9_B	M9	Yes	filled	Mated	Developed	monogynous	LM
M9_W	M9	No	/	Unmated	underdeveloped	monogynous	/
B1_B	B1	Yes	empty	Unmated	underdeveloped	polygynous	HM
B1_G	B1	Yes	filled	Mated	Developed	polygynous	LM
B1_O	B1	No	/	Unmated	underdeveloped	polygynous	HM
B1_P	B1	No	/	Unmated	underdeveloped	polygynous	LM
B1_Pu	B1	No	/	Unmated	underdeveloped	polygynous	HM
B1_W	B1	No	/	Unmated	underdeveloped	polygynous	/
C14_B	C14	Yes	empty	Unmated	underdeveloped	polygynous	HM
C14_G	C14	Yes	filled	Mated	Developed	polygynous	LM
C14_W	C14	No	/	Unmated	underdeveloped	polygynous	/
C7_B	C7	Yes	filled	Mated	Developed	polygynous	LM
C7_G	C7	Yes	empty	Unmated	underdeveloped	polygynous	HM
C7_O	C7	No	/	Unmated	underdeveloped	polygynous	HM
C7_W	C7	No	/	Unmated	underdeveloped	polygynous	/
D2_B	D2	Yes	filled	Mated	Developed	polygynous	LM
D2_G	D2	Yes	empty	Unmated	underdeveloped	polygynous	HM
D2_O	D2	No	/	Unmated	underdeveloped	polygynous	HM
D2_W	D2	No	/	Unmated	underdeveloped	polygynous	/
E11E8_B	E11E8	Yes	filled	Mated	Developed	polygynous	LM
E11E8_G	E11E8	No	/	Unmated	underdeveloped	polygynous	HM
E11E8_O	E11E8	No	/	Unmated	underdeveloped	polygynous	HM
E11E8_W	E11E8	No	/	Unmated	underdeveloped	polygynous	/
E4_B	E4	No	/	Unmated	underdeveloped	polygynous	LM
E4_G	E4	Yes	filled	Mated	Developed	polygynous	LM
E4_Un	E4	Yes	empty	Unmated	underdeveloped	polygynous	HM
E4_W	E4	No	/	Unmated	underdeveloped	polygynous	/
G2_B	G2	Yes	empty	Unmated	underdeveloped	polygynous	HM
G2_G	G2	Yes	filled	Mated	Developed	polygynous	LM
G2_O	G2	Yes	/	Unmated	underdeveloped	polygynous	HM

G2_W	G2	No	/	Unmated	underdeveloped	polygynous	/
G5_B	G5	Yes	empty	Unmated	underdeveloped	polygynous	HM
G5_O	G5	Yes	filled	Mated	Developed	polygynous	LM
G5_W	G5	No	/	Unmated	underdeveloped	polygynous	/
G9_G	G9	Yes	filled	Mated	Developed	polygynous	LM
G9_W	G9	No	/	Unmated	underdeveloped	polygynous	/
G9_Y	G9	No	/	Unmated	Developed	polygynous	HM
M1_B	M1	No	/	Unmated	underdeveloped	polygynous	LM
M1_G	M1	No	/	Unmated	underdeveloped	polygynous	LM
M1_O	M1	Yes	empty	Unmated	underdeveloped	polygynous	HM
M1_P	M1	Yes	empty	Unmated	underdeveloped	polygynous	LM
M1_W	M1	No	/	Unmated	underdeveloped	polygynous	/
M10_B	M10	No	/	Unmated	Developed	polygynous	HM
M10_G	M10	No	/	Unmated	underdeveloped	polygynous	LM
M10_O	M10	No	/	Unmated	underdeveloped	polygynous	LM
M10_P	M10	No	/	Unmated	underdeveloped	polygynous	LM
M10_W	M10	No	/	Unmated	underdeveloped	polygynous	/
M11_B	M11	Yes	empty	Unmated	underdeveloped	polygynous	HM
M11_G	M11	Yes	empty	Unmated	underdeveloped	polygynous	HM
M11_O	M11	Yes	filled	Mated	Developed	polygynous	LM
M11_P	M11	No	/	Unmated	underdeveloped	polygynous	HM
M11_Un	M11	No	/	Unmated	underdeveloped	polygynous	HM
M11_W	M11	No	/	Unmated	underdeveloped	polygynous	HM
M11_W	M11	No	/	Unmated	underdeveloped	polygynous	/
M5_B	M5	Yes	filled	Mated	Developed	polygynous	LM
M5_G	M5	No	/	Unmated	underdeveloped	polygynous	HM
M5_O	M5	No	/	Unmated	underdeveloped	polygynous	HM
M5_P	M5	No	/	Unmated	underdeveloped	polygynous	HM
M5_W	M5	No	/	Unmated	underdeveloped	polygynous	/
M5_Y	M5	No	/	Unmated	underdeveloped	polygynous	HM
M8_B	M8	No	/	Unmated	underdeveloped	polygynous	HM
M8_G	M8	Yes	filled	Mated	Developed	polygynous	LM
M8_O	M8	No	/	Unmated	underdeveloped	polygynous	HM
M8_W	M8	No	/	Unmated	underdeveloped	polygynous	/
N6_B	N6	Yes	filled	Mated	Developed	polygynous	LM
N6_O	N6	No	/	Unmated	underdeveloped	polygynous	HM
N6_W	N6	No	/	Unmated	underdeveloped	polygynous	/

Supplementary Table 6: Outputs of ANOVAs to test the effect of queen type on the principal components extracted from the PCA based on the full behavioral dataset. P values below the 5% threshold are indicated in bold.

Principal Component	χ^2 value	P value
PC1	54.226	**1.679e-12**
PC2	5.397	6.730e-02
PC3	0.587	7.457e-01
PC4	1.683	4.311e-01
PC5	4.321	1.153e-01
PC6	4.086	1.297e-01
PC7	0.277	8.707e-01
PC8	2.455	2.930e-01

Transcriptomic changes associated with the socially regulated behavioral specialization of ant queens

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Abstract

Reproductive division of labor is one of the key aspects of the ecological success of eusocial insects. Similar to the differentiation of soma and germline in a multicellular organism, these societies are composed of a specialized reproductive, and non-reproductive worker caste. But while cell differentiation, the transition from totipotent to specialized cells, was widely studied in multicellular organisms, the ontogeny of insect societies, the transition from a pluripotent founding queen to a specialized established queen, is still poorly understood. Previous studies have shown that the social environment is key to induce and maintain this behavioral specialization in queens, as they can switch between the two states depending on the presence of workers. However, the molecular changes that translate worker presence in queen behavioral specialization remain unknown. To investigate whether queens experience changes in their gene expression according to their social environment, we generated transcriptomic data from *Lasius niger* queens experimentally exposed to various manipulations of the social environment. Our analysis shows that 123 genes were differentially expressed between queens with and without workers. Among these, we identified 13 genes of interest, known to influence insect behavior, including genes coding for the transcription factor *Krueppel-homolog 1*, a *juvenile hormone esterase* and i*nsulin*. Moreover, we revealed that the expression of some genes of interest mirrors the behavioral changes in response to further manipulations of the social environment. Overall, this study identifies candidate genes and pathways for the regulation of queen behavioral specialization in ants.

Introduction

Social insect colonies are characterized by a reproductive division of labor between queens that specialize in egg laying and workers that fulfill non-reproductive tasks, such as brood care, foraging or cleaning the nest(Andersson 1984; Robinson et al. 2009; Lin and Michener 1972). Queens and workers have distinct phenotypes, as their morphology(Divieso et al. 2020; Miyazaki et al. 2005), physiology(Barchuk et al. 2007; Kramer and Schaible 2013) and gene expression (Friedman and Gordon 2016; Lockett et al. 2016; Graeff et al. 2007) underlie their behavioral specialization. Most studies of reproductive division of labor focused on the process of caste determination that occurs during larval development (Villalta et al. 2016; Beetsma 1979; de Azevedo and Hartfelder 2008; Reginato and Cruz-Landim 2003), thus assuming that the queen specialization in egg laying is fixed at the adult stage.

However, in most social insect species, the queens are not yet specialized in egg production at the beginning of their adult life (Brown and Bonhoeffer 2003). Young, newly mated queens that found their colony independently (without the help of workers) express a wide behavioral repertoire to produce the first workers, including non-reproductive behaviors, such as digging, cleaning, foraging and caring for the brood (Augustin et al. 2011; Cassill 2002; Chouvenc 2022; Majidifar et al. 2024). Only upon the emergence of the first workers do queens specialize in egg laying, transitioning from a pluripotent to a specialized state (Augustin et al. 2011; Cassill 2002; Majidifar et al. 2024; Woodard et al. 2013).

Experimental studies revealed that the presence of workers induces the queen specialization in egg production, but also maintains it in established colonies (Majidifar et al. 2024; Woodard et al. 2013). The experimental removal of workers caused queens to revert to express brood care behavior, and thus, to resemble founding queens. Such a flexible specialization, and its regulation by the social environment, stand in contrast with the commonly accepted constitute specialization of the queen caste. It also differs from other social insect species such as the termite *Coptotermes gestroi,* where the reproductive individuals irreversibly lose the ability to ensure the proper development of their offspring once the colony is established (Chouvenc 2022; Chouvenc and Su 2017)

Further investigations of the effect of workers on queen specialization in the ant *Lasius niger* revealed that queens exposed to the mere presence of worker cues did not become specialized in egg production (Majidifar et al. 2024). For example, the behavior of founding queens was not affected by the presence of workers that were separated from the queen and brood by a wire mesh, suggesting that close contacts between workers and the queen, and/or between workers and the brood are required to produce the queen specialization (Majidifar et al. 2024).Although the behavioral response of ant queens to the presence of

workers has been well characterized (Bourke 1991; Hölldobler and Wilson 1990), the molecular mechanisms that translate the presence of workers into queen behavioral specialization remain to be investigated.

In social insects, other cases of behavioral variation are regulated by changes in gene expression, and are often correlated with modifications of the social environment. First, removing workers performing a specific task results in others adjusting their behavior to fulfill that task via changes in gene expression (Fussnecker and Grozinger 2008; Kohlmeier et al. 2019; Manfredini et al. 2022). Several studies show that worker gene expression across tissues, such as brain, fat body, and ovaries, changes in response to alterations in their social environment (Hoover et al. 2003; Lenhart et al. 2024; Kay et al. 2023), sometimes causing changes in their behaviors toward the brood (Kohlmeier et al. 2018; 2019). Thus, variation in gene expression underlies changes in worker behavior in response to various modifications of their social environment.

We hypothesized that the behavioral specialization of queens into egg laying is regulated by similar candidate genes and pathways as polyethism in the worker caste. Changes in the expression of genes in the vitellogenin (Vg), juvenile hormone (JH) and insulin/insulin-like signaling (IIS) pathways regulate the transition of honey bee and ant workers from inside to outside tasks as they age in the brain and whole body (Ben-Shahar 2005; Gospocic et al. 2017; Kohlmeier et al. 2019). The gene *Krüppel homolog 1* (*Kr-h1*), coding for a JH-responsive transcription factor, regulates behavioral transitions in bee workers when expressed in their brain (Fussnecker and Grozinger 2008; Shpigler et al. 2010), suppresses worker reproduction when expressed in their ovaries (Fussnecker and Grozinger 2008; Kilaso et al. 2017) or activates the reproduction (and associated behavioral changes) when expressed in the queenless ant brain (Gospocic et al. 2017; Opachaloemphan et al. 2023). Genes linked to pheromonal perception may also be involved in the queen response to worker presence, as variation in the brain expression of genes coding for gustatory or odorant receptors affect worker behavioral transitions (Simcock et al. 2017) and the activation of worker reproduction (Opachaloemphan et al. 2023) .Furthermore, the presence of workers affects the expression in the brain of bumble bee queens of the *cycle* gene, which is a molecular component of the circadian clock and is also involved in learning and memory (Woodard et al. 2013). As a consequence, we expect similar pathways to play a role in the regulation of queen behavioral specialization.

To investigate the transcriptomic changes that translate the presence of workers into the behavioral specialization of ant queens, we first identified candidate genes that showed differential expression in the brain of founding queens experimentally maintained with or without workers (Majidifar et al. 2024).We then investigated whether the expression of the

candidate genes mirrored the behavioral response to further manipulations of the social environment (Majidifar et al. 2024). This produced a robust set of candidate genes and pathways for the molecular regulation of queen specialization in ants.

Methods

To investigate the transcriptomic response of *Lasius niger* founding queens to experimental manipulations of the presence of workers and/or larvae, we used samples that were collected after behavioral analyses reported in Majidifar et al (2024) and samples that were specifically produced for this study. All experiments were conducted using *L. niger* queens that were collected in Mainz (Germany) after the nuptial flight in July 2019 and July 2020, and then kept in a climate chamber at 21°C and 80% humidity in the dark.

Sample collection

To investigate the effect on queen brain gene expression of experimentally adding and/or removing workers, we used queens from experiment 2.2.4 in Majidifar et al (2024). In this experiment, founding queens that had never produced workers were separated into three treatments: (i) queens kept with five larvae and no workers; (ii) queens kept with five larvae and five workers; and (iii) queens kept with five larvae that formerly had workers for three days. The behavioral analyses revealed that queens that had their workers removed performed more brood care than queens with workers, but as much brood care as queens without workers. More details on the experimental methods, statistical analyses and results can be found in Majidifar et al (2024). Founding queens were maintained in these experimental conditions for 48 h before they were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

To investigate the effect on queen brain gene expression of experimentally adding workers together with the queen or separated from the queen by a wire mesh, we used queens from experiment 2.2.12 in Majidifar et al. (2024). In this experiment, founding queens that had never produced workers were separated in three treatments: (i) queens kept with five larvae and no workers: (ii) queens kept with five larvae and three workers in the same arena: and (iii) queens kept with five larvae and three workers that were separated from the queens and the larvae by a wire mesh. The behavioral analyses revealed that, contrary to queens kept in the same arena as workers, queens provided with separated workers did not show a reduction in brood care behavior compared to queens kept without workers. More details on the experimental methods, statistical analyses and results can be found in Majidifar et al (2024). Founding queens were maintained in these experimental conditions for 24 h before they were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

To verify that the wire mesh enabled antennal contacts between the queens and the separated workers, we reanalyzed the 1 h-long videos that were recorded two h, six h and 24 h after the experimental set-up (Majidifar et al, 2024). For each queen, we used focus animal sampling (Altmann 1974) with the BORIS software (version 8.0.5) (Friard and Gamba

2016) to measure the number of times that queens passed their antennae through the wire mesh to make antennal contacts with the antennae of the separated workers.

To study the effect on queen brain gene expression of expressing brood care and being in contact with larvae, we used *L. niger* founding queens that were collected after the nuptial flight in Mainz in July 2020. The experiment started 70 days after collecting. The founding queens, which had not produced workers yet, were distributed in two treatments: (i) queens kept with six larvae; and (ii) queens kept without larvae. The larvae were sourced from field-collected colonies that also served as sources of workers in Majidifar et al (2024). During the experiment, the queens were kept in the same conditions as in experiment 2.2.4 in Majidifar et al. (2024). After 72 h, the queens were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Brain dissection, RNA extraction and sequencing

To select representative samples for each treatment, we used the scored brood care behavior as a proxy for the overall distribution of the trait. For every experiment, we first determined the full range of brood care scores (minimum to maximum) across all individuals in the treatment group. This range was then divided into four equal intervals (quartiles). From each quartile we selected three queens, yielding a total of 12 samples per treatment. In Experiment 2.2.1., two samples were removed as workers entered the arena of queens that should have been separated from workers. This resulted in a total of 94 samples (Table 1).

Table 1. Summary of experiments and samples used to investigate the effects of worker presence/absence. The table included the social environments experienced by the queens.

Description of the experiments	Experimental addition and removal of workers	Experimental addition of workers and separated workers	Experimental manipulation of the presence of larvae
Experiment number in Majidifar et al (2024)	Experiment 2.2.4	Experiment 2.2.12	Not from Majidifar et al (2024)
Number of RNAseq samples	Queens without workers (12 samples) Queens with workers (12 samples) Queens with removed workers (12 samples)	Queens without workers (12 samples) Queens with workers (12 samples) Queens with separated workers (10 samples)	Queens without larvae (12 samples) Queens with larvae (12 samples)

To dissect the brain, the order in which samples were dissected across experiments was randomized. Samples were dissected across several days. On a given day, the samples were transferred from the -80°C storage to dry ice. From there, the samples were removed from the cryotube and transferred to a silicon slip, set in ice, under a microscope (Leica S9i, Leica Microsystems, Germany). To manipulate the samples, we used two micro-forceps (No. 11295-10, F.S.T, Canada), one being used to keep the head in place. The head was removed and placed in a cooled 1x PBS droplet. The body was returned to the cryotube, which was placed again inside the box filled with dry ice. The antennae were removed at the base and returned to the cryotube as well. The head capsule was opened via cutting dorsally from the clypeus along the frontal line up to the hind margin using a micro-scissor (No 15000-08, F.S.T., Canada). This was followed by two cuts, 90° from the first, resulting in a T-shaped cut. Afterwards, the cuticle encompassing the cut was removed using the micro-forceps. To sever the connection to the retina, one micro-forceps was used to scrape along the inside of the compound eyes. Any additional cuticle obscuring the brain above the ocelli and hind margin was removed. The brain, once visible, was carefully lifted out of the head capsule, by grabbing it from below. It was then placed into another precooled PBS droplet. From there, it was transferred to a pre-chilled vial containing 100 µl of TRIzol and kept on ice. After dissection, all samples were transferred and stored at -80°C.

To investigate transcriptomic variation across queens in the brain, we used 94 samples (Table 1). From each sample, the total RNA was extracted using a TRIzol:chloroform extraction protocol followed by purification using a Qiagen RNeasy extraction kit and a modified version of its protocol. Total RNA was shipped on dry ice to a commercial supplier

(Novogene, UK) where quality assessment was performed using an Agilent Bioanalyzer, before an individual mRNA-enriched library preparation was performed for each sample. The libraries were individually barcoded, pooled, and sequencing on an Illumina Plus X sequencing platform generating on a minimum of 20 million paired-end reads per sample.

Gene expression analyses

Raw paired-end reads were filtered using fastp v.0.20.1 (Chen et al. 2018) to remove reads with more than 15 ambiguous bases and reads shorter than 120 bp. Filtered reads were saved separately for forward and reverse pairs. Quality assessment was performed via FASTQC v.0.12.1 (Andrews 2010) with output files summarized and visualized using MultiQC v.129 (Ewels et al. 2016). Abundance of transcripts was quantified per sample by pseudo-aligning filtered RNA-seq data against the predicted transcriptome informed from the assembled genome, downloaded from the Electronic research Data archive of the Copenhagen university (Masson et al. 2024; Vizueta et al. 2025), using Kallisto v0.50 (Bray et al. 2016). The resulting 94 abundance files were loaded into R, summarized to gene-level counts and analyzed using the R package DESeq2 (Love et al. 2014). Of the 13.903 genes annotated in the *L. niger* genome, we found 11.268 genes that were present at least once in 50% of our samples. For all analyses, genes with read counts equal to zero were removed. All differential expression analyses were performed using R (version 4.4.2) and RStudio (version 2024.04.2).

To investigate the transcriptomic response to the experimental manipulation of the presence of workers, we compared brain gene expression between founding queens kept without workers (n = 24) and founding queens kept with workers (n = 24). We used 24 queens from Experiment 2.2.4 (12 without workers; 12 with workers) and 24 queens from Experiment 2.2.12 (12 without workers; 12 with workers) in Majidifar et al. (2024) (Table 1). To implement the differential expression analysis while controlling for batch effects driven by differences between the experiments that produced the samples, we performed a likelihood ratio test (LRT) using the DESeq2 package (Love et al., 2014) to compare a full model that included worker presence and batch as explanatory variables to a reduced model that only included batch.

To investigate the biological functions of the differentially expressed genes, we conducted a Gene Ontology (GO) term enrichment analysis. We identified biological process- and molecular function-related GO terms that were overrepresented in the list of differentially expressed genes, using the GO terms data file downloaded from the Electronic research Data archive of the Copenhagen university (Masson et al. 2024; Vizueta et al. 2025), using Fisher's exact tests implemented with the topGO package (Alexa 2017).

To identify genes of interest, we manually inspected the functional annotations of the differentially expressed genes from the *L. niger* annotation files (Masson et al. 2024; Vizueta et al. 2025)) for the regulation of queen behavioral specialization in response to changes in the social environment. We defined genes of interest as those with reported behavioral functions associated with their expression in the brain of insects.

We investigated whether changes in the expression of the genes of interest in the brain of founding queens in response to the experimental removal of workers mirrored the behavioral response (Majidifar et al. 2024). To do so, we used 36 queens from Experiment 2.2.4 in Majidifar et al. (2024) (Table 1) to compare the expression of the genes of interest among treatments: queens without workers (n = 12); queens with workers (n = 12); and queens with workers removed (n = 12). We extracted the normalized counts for the genes of interest using the 'counts()' function of the DESeq2 package (Love et al. 2014), and conducted ANOVAs using the car package (Fox et al. 2001) on linear models that explained gene counts (log-transformed when necessary) by the treatment. When the analyses detected an effect of the experimental treatments, we used the 'emmeans()' function from the emmeans package (Lenth 2025) to perform pairwise comparisons. Our criteria to conclude that a gene of interest mirrored the behavioral response was that its expression should be affected by a main effect of treatment, differ between queens with workers and queens with workers removed, and go in the same direction for queens without workers and queens with workers removed when compared to queens with workers (Majidifar et al. 2024).

We tested whether changes in the expression of the genes of interest in response to the presence of workers separated by a wire mesh mirrored the behavioral response (Majidifar et al. 2024). To do so, we used 34 queens from Experiment 2.2.12 in Majidifar et al. (2024) (Table 1) to compare the expression of the genes of interest among treatments: queens without workers (n = 12); queens with workers (n = 12); and queens with separated workers (n = 10). We then used the same method for the effect of worker removal to test differences among treatments in the expression of the genes of interest. Similarly, our criteria to conclude that a gene of interest mirrored the behavioral response was that its expression should be affected by a main effect of treatment, differ between queens with workers and queens with separated workers, and go in the same direction for queens without workers and queens with separated workers when compared to queens with workers (Majidifar et al. 2024).

We studied the effect of interacting with larvae on the expression of the genes of interest by comparing queens kept with six larvae (n = 12) and queens kept without larvae (Table 1). To do so, we used the same method as for the effect of worker removal to extract normalized

counts for the genes of interest and perform ANOVAs on linear models explaining the read counts by the presence of larvae.

Results

To better understand the molecular mechanisms that translate the presence of workers into behavioral specialization in *L. niger* founding queens, we used 94 brain RNAseq samples to investigate the transcriptomic changes associated with a series of experimental modifications of the social environment that produced queen behavioral variation (Majidifar et al., 2024).

Effect of the presence of workers on brain gene expression in queens

We hypothesized that the expression of genes that translate changes in the social environment into queen behavioral specialization would be affected by the experimental addition of workers, which was found to drastically reduce the brood care behavior of founding queens (Majidifar et al., 2024). To test this hypothesis, we conducted a differential brain gene expression analysis between same-age founding queens that were either kept without workers (n = 24) or experimentally provided with workers (n = 24). We identified 123 genes that differed in expression between the two experimental conditions (adjusted $p < 0.05$): 63 genes (51.2%) were upregulated in the brain of queens kept without workers, and 60 genes (48.8%) were upregulated in the brain of queens provided with workers (Figure 1, Supplementary Table 1).

To assess whether specific functions were overrepresented in the list of differentially expressed genes, we conducted a GO term enrichment analysis. We found 16 GO terms to be enriched ($p < 0.01$): 11 GO terms for biological processes and five GO terms for molecular functions (Figure 1, Supplementary Table 2). Many enriched GO terms were related to functions and processes associated with brain energy metabolism, such as carbohydrate metabolism and transport (GO:0015771, trehalose transport; GO:0005978, glycogen biosynthetic process; GO:0005536, D-glucose binding), and lipid metabolism (GO:0010898, positive regulation of triglyceride catabolic process; GO:0019432, triglyceride biosynthetic process; GO:0034375, high-density lipoprotein particle remodeling; GO:0006658, phosphatidylserine metabolic process; GO:0046471, phosphatidylglycerol metabolic process). Other enriched GO terms related to neurotransmission and signaling regulation, such as the transport of ions and metabolites across membranes (GO:0015293, symporter activity; GO:0015318, inorganic molecular entity transmembrane transport; GO:0055085, transmembrane transport; GO:0097164, ammonium ion metabolic process), and the response to peptides (GO:1901653; cellular response to peptide).

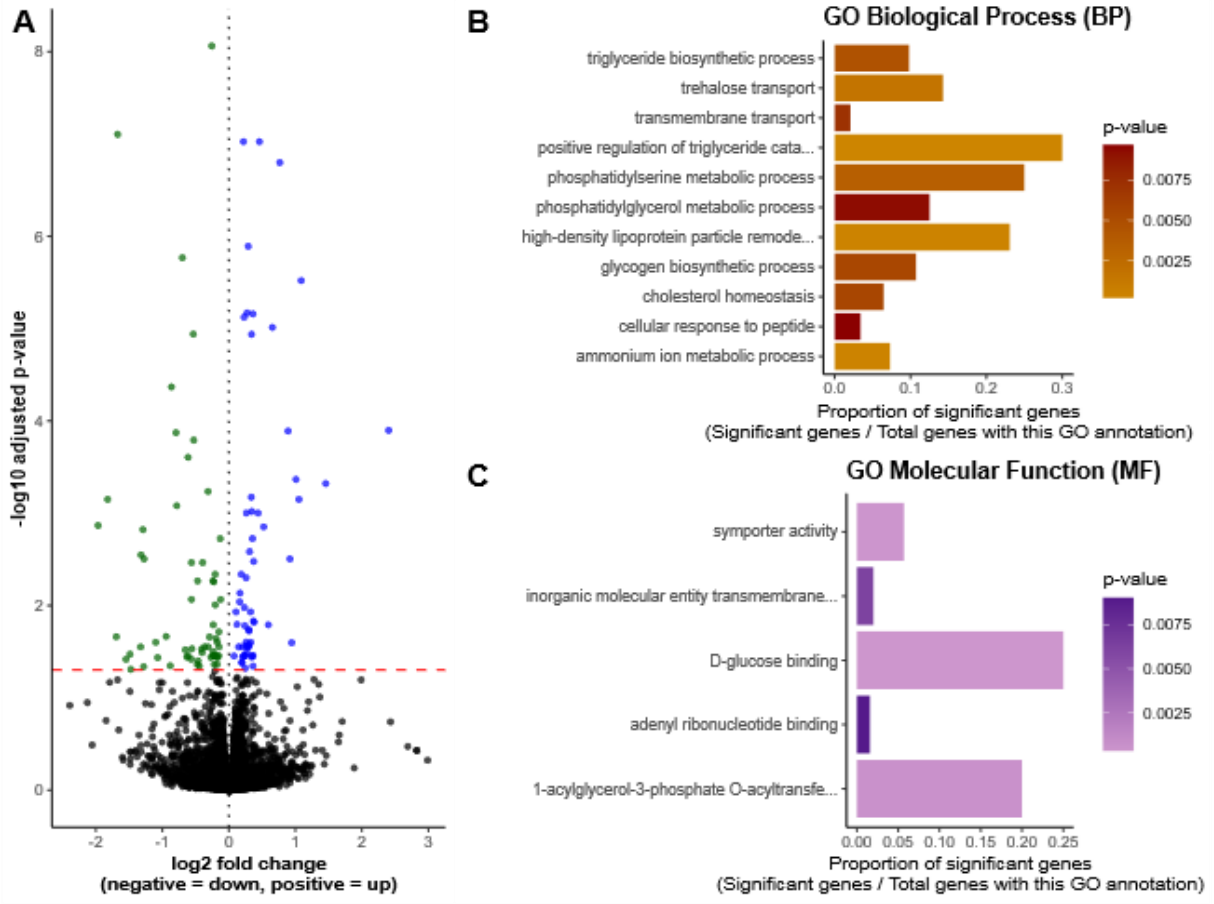

Figure 1. The experimental addition of workers affected the brain gene expression of *L. niger* founding queens. A. Volcano plot summarizing the differential expression analysis. Colored dots represent the 123 genes that were differentially expressed between queens without (n = 24) and with (n = 24) workers (adjusted $p < 0.05$; above the red line). Green dots are genes that were overexpressed in the brain of queens without workers. Blue dots are genes that were overexpressed in the brains of queens that were experimentally provided with workers. B-C. The GO term enrichment analysis revealed that B. 11 GO terms for biological processes and C. 5 GO terms for molecular function were enriched in the list of differentially expressed genes. Bar length represents the proportion of GO terms found.

Identification of genes of interest

We inspected the list of 123 differentially expressed genes manually to identify genes of particular interest, which we defined as genes expressed in the brain with reported behavioral functions in insects. Using these criteria, we identified 13 genes of interest (Table 2): 7 genes (53.8%) were overexpressed in the brain of queens kept without workers; and 6 genes (46.2%) were overexpressed in the brain of queens kept with workers (Figure 2).

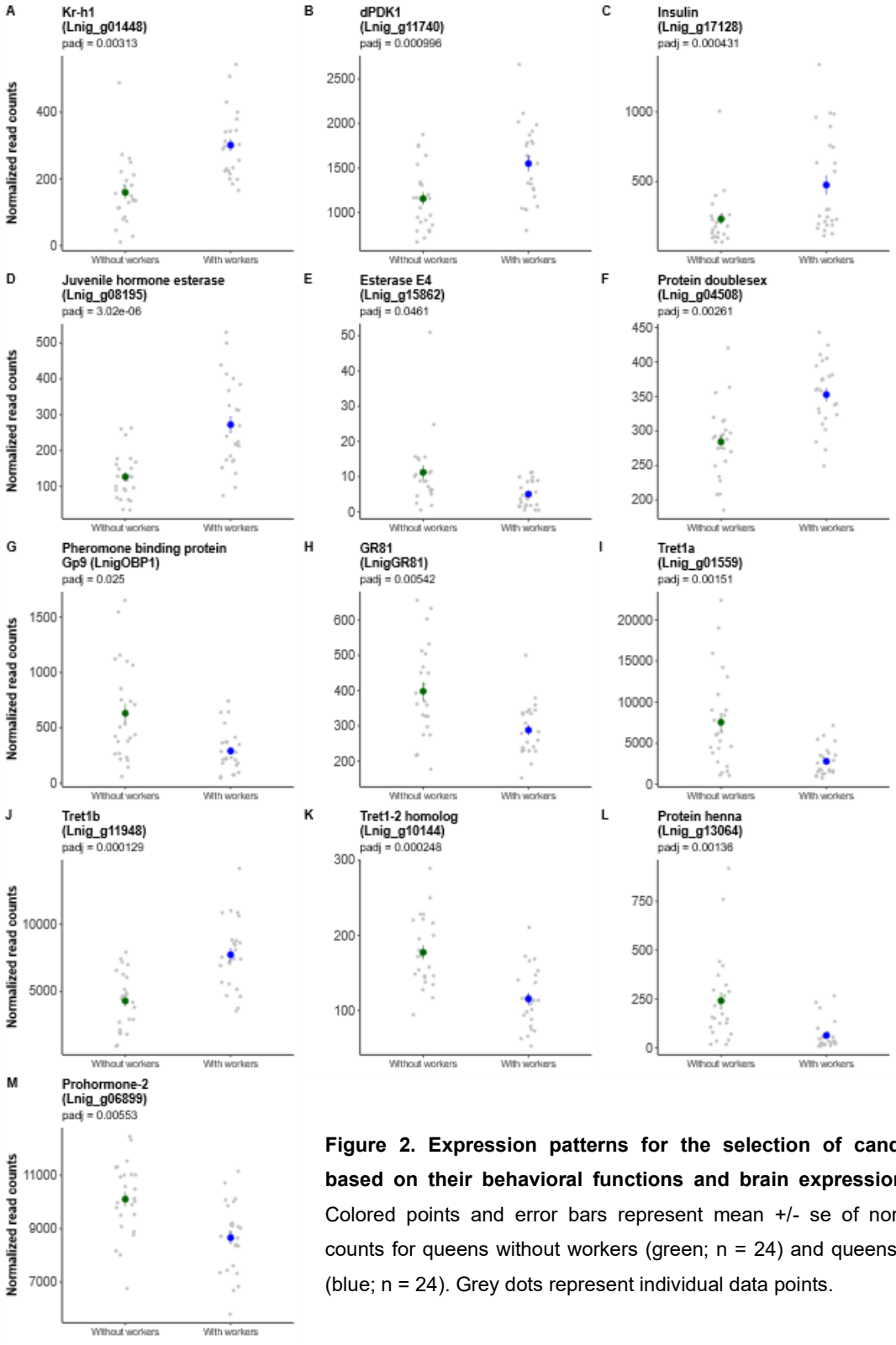

Figure 2. Expression patterns for the selection of candidate genes based on their behavioral functions and brain expression in insects. Colored points and error bars represent mean +/- se of normalized read counts for queens without workers (green; n = 24) and queens with workers (blue; n = 24). Grey dots represent individual data points.

Table 2. Selection of candidate genes based on their behavioral functions and brain expression in insects. Gene annotation and ID from the genome annotation files (Masson et al., 2024; Vizueta et al., 2025), behavioral function in insects, and supporting scientific references are provided.

Gene annotation	Gene ID	Behavioral function in insects	References
Krueppel homolog 1 (Kr-h1)	Lnig_g01448	Kr-h1 is a JH-responsive transcription factor that regulates task specialization in bees and the behavioral changes associated with reproductive activation in queenless ants.	Kayukawa et al., 2012 Minakuchi et al., 2009 Fussnecker & Grozinger, 2008 He & Zhang, 2022 Kilaso et al., 2017 Opachaloemphan et al., 2021
3-phosphoinositide-dependent protein kinase (dPDk1)	Lnig_g11740	dPDK1, a component of the insulin signaling pathway, regulates task specialization in termites with its expression correlating with the transition of workers from nurses to foragers.	Haroon et al., 2020 Vinayagam et al., 2016 Weger & Rittschof, 2024
Insulin	Lnig_g17128	**Expression of the insulin-like peptide gene** correlates with the transition of workers from nurses to foragers and with vitellogenin production, which promotes maternal behavior, increases egg-laying, and extends queen lifespan in ants. While in Ooceraea biroi, brain production of the peptide is induced by larvae presence and regulates brood care behavior.	Ament et al., 2008 Badisco et al., 2013 Chandra et al., 2018 Chen et al., n.d Libbrecht et al., 2013 Okada et al., 2010
Juvenile hormone esterase	Lnig_g08195	Juvenile hormone esterase and Esterase E4 are coding for JH esterase enzymes JH esterase enzymes degrade JH and regulate its concentration in the brain JH is known for influencing social behaviors in insects by regulating vg expression level Brain JH also participate in the nurse/forager transition and the	Amdam et al., 2003 Amsalem et al., 2014 Brito et al., 2021 Ju et al., 2023 Libbrecht et al., 2013 Mackert et al., 2008 Miyazaki et al., 2021 Pandey & Bloch, 2015 Starkey & Tamborindeguy, 2023 Wheeler et al., 2013
Esterase E4	Lnig_g15862		

		brood care regulation in eusocial insects	
Protein doublesex (dsx)	Lnig_g04508	The doublesex gene encodes a protein initially studied for sex determination that mediates JH-dependent polyphenism within sexes, potentially regulating reproductive versus non-reproductive behavior, It is necessary for male-specific neuron development in Drosophila, which regulates sexual behavior.	Johnson & Jasper, 2016 cKlein et al., 2016 Rideout et al., 2010
Protein henna	Lnig_g13064	The henna gene encodes a dopamine-synthesis protein involved in gregarious behaviors, with its expression influencing locust gregarization through epigenetic processes.	Burrows et al., 2011 Chen et al., n.d. Li et al., 2025 Zayed et al., 2012
Prohormone-2	Lnig_g06899	Prohormone genes enable neuropeptide synthesis linked to honeybee social behavior variation, with their brain expression associated with division of labor in bees.	Han et al., 2015 Predel & Neupert, 2007
Pheromone-binding protein Gp-9	LnigOBP1	The Gp-9 gene encodes a chemoreceptive pheromone-binding protein whose expression affects red fire ant social form and decreases in the brain during Harpegnathos saltator worker-to-gamergate transition, coinciding with egg-laying specialization.	Opachaloemphan et al., 2021 Zhang et al., 2024
Gustatory receptor which mediates acceptance or avoidance behavior, depending on its substrates (GR81)	LnigGR81	GR81 encodes a chemoreceptive gustatory receptor whose brain knockdown in honeybee workers accelerates the nurse-to-forager transition.	Paerhati et al., 2015 Simcock et al., 2017 Nishimura et al., 2012
Facilitated trehalose transporter Tret1 (Tret1a)	Lnig_g01559	Tret1 genes encode trehalase enzymes regulating trehalose hydrolysis and tissue-specific uptake in insects, and are overexpressed in forager brains (vs. nurses) in Lasius niger and dominant Euglossa dilemma individuals, correlating with foraging	H. Zhang et al., 2024 Quque et al., 2023 Saleh et al., 2021
Facilitated trehalose transporter Tret1 (Tret1b)	Lnig_g11948		
Facilitated trehalose transporter Tret1-2 homolog (Tret1-2 homolog)	Lnig_g10144		

		behavior and social dominance.	

Effect of worker removal on the expression of the genes of interest

We made the prediction that, if the genes of interest translated modifications in the social environment into behavioral specialization, the changes in expression in response to further manipulations of the presence of workers and/or larvae should mirror the behavioral responses to these manipulations (Majidifar et al. 2024).

First, we studied the effect of the experimental removal of workers on the expression of genes of interest. Same-age *L. niger* founding queens were exposed to one of the following conditions: without workers; experimentally provided with workers; and provided with workers that were then removed after 3 days. The behavioral analyses revealed that after worker removal, queens expressed more brood care behavior than queens kept with workers, and similar levels of brood care as queens that never had any workers (Majidifar et al. 2024). These results showed that the behavioral specialization of *L. niger* queens is reversible. We hypothesized that genes regulating behavioral specialization would also show reversible changes in expression upon manipulations of the presence of workers.

To investigate this, we used 36 brain RNAseq samples from same-age founding queens kept without workers (n = 12), with workers (n = 12), or after worker removal (n=12) (Majidifar et al. 2024). We focused our analysis of differential expressions of the 13 genes of interest. We argued that for genes to mirror the reversible behavioral response, their expression should be affected by the experimental manipulation of workers (main effect of treatment), differ between queens with workers and queens with workers removed, and go in the same direction for queens without workers and queens with workers removed when compared to queens with workers. We found that six out of the 13 genes of interest showed such reversible expression patterns (Supplementary Table 3), thus, mirroring the behavioral response to worker removal. These genes were annotated as *Kr-h1* (Lnig_g01448), *Juvenile hormone esterase* (Carboxylesterase_type_B_Lnig_g08195), *Insulin* (Insulin-like_Lnig_g17128), *Pheromone-binding protein Gp-9* (LnigOBP1), *Tret1-2 homolog* (Lnig_g10144), and *Prohormone-2* (Lnig_g06899) (Figure 3,A-E;G-H;J-M). The brain expression of the other genes of interest did not differ between queens with workers and queens with workers removed (Figure 3), indicating that the expression changes in response to the addition of workers were not reversible, and thus differed from the behavioral response (Majidifar et al. 2024).

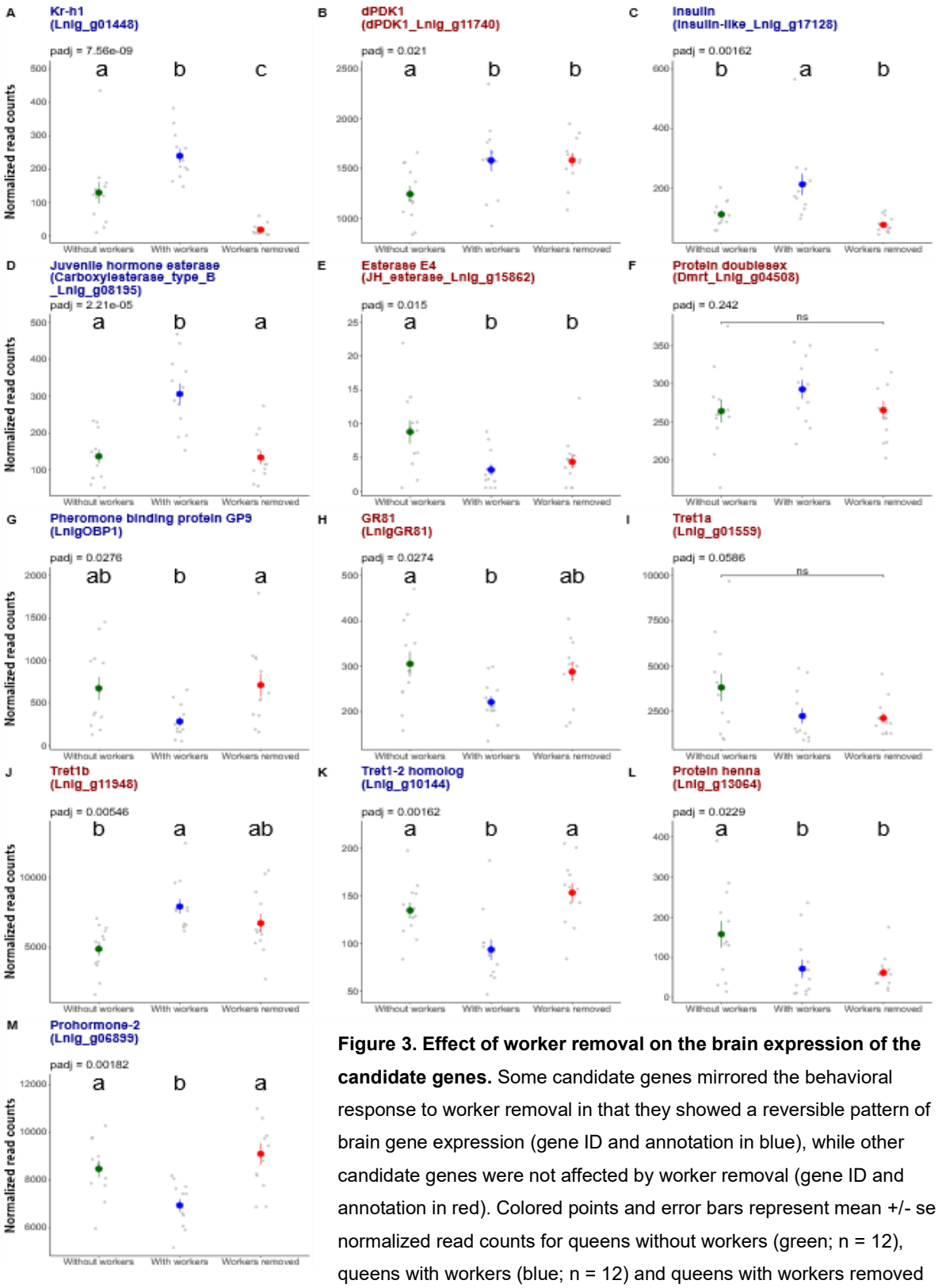

Figure 3. Effect of worker removal on the brain expression of the candidate genes. Some candidate genes mirrored the behavioral response to worker removal in that they showed a reversible pattern of brain gene expression (gene ID and annotation in blue), while other candidate genes were not affected by worker removal (gene ID and annotation in red). Colored points and error bars represent mean +/- se of normalized read counts for queens without workers (green; n = 12), queens with workers (blue; n = 12) and queens with workers removed (red; n = 12). Letters indicate statistically supported differences between groups. “ns” indicates that we could not detect a main effect of the experimental manipulations (adjusted p > 0.05).

Effect of separated workers on the expression of the genes of interest

We next investigated the effect of providing workers that were separated from queens by a wire mesh. We verified that queens could make antennal contacts with workers through the wire mesh, therefore, they had the ability to detect their presence, by measuring the number of times that queens passed their antennae through the wire mesh to make antennal contacts with the antennae of the separated workers (Supplementary Figure 1). We found that contrary to queens that were placed in the same area as workers, queens provided with separated workers did not respond with changes in their brood care behavior (Majidifar et al. 2024). We hypothesized that genes regulating the socially driven behavioral specialization of queens would similarly show changes in expression in presence of workers but not in presence of workers that were separated by a wire mesh.

To study this question, we used 36 brain RNAseq samples from same-age founding queens kept without workers (n = 12), with workers (n = 12), or with workers separated by a wire mesh (n=10) (Majidifar et al. 2024). We focused on analyzing the effect of these manipulations on the expression of the 13 genes of interest. We argued that if genes did mirror the behavioral response, their expression should be affected by the experimental manipulation of workers (main effect of treatment), differ between queens with workers and queens with separated workers, and go in the same direction for queens without workers and queens with separated workers when compared to queens with workers. We found that nine of the 13 genes of interest showed such expression patterns (Supplementary Figure 5): *Kr-h1* (Lnig_g01448), *Juvenile hormone esterase* (Carboxylesterase_type_B_Lnig_g08195), *Insulin* (Insulin-like_Lnig_g17128), *Protein doublesex* (Dmrt_Lnig_g04508), *dPDK1* (dPDK1_Lnig_g11740), *Protein henna* (Lnig_g13064), *Tret1a* (Lnig_g01559), *Tret1b* (Lnig_g11948), and *Tret1-2 homolog* (Lnig_g10144) (Figure 4, A-D;F,H-M). The other genes of interest did not differ between queens with workers and queens with separated workers (Figure 4,E;G), indicating that their expression changes differed from the behavioral response (Majidifar et al. 2024).

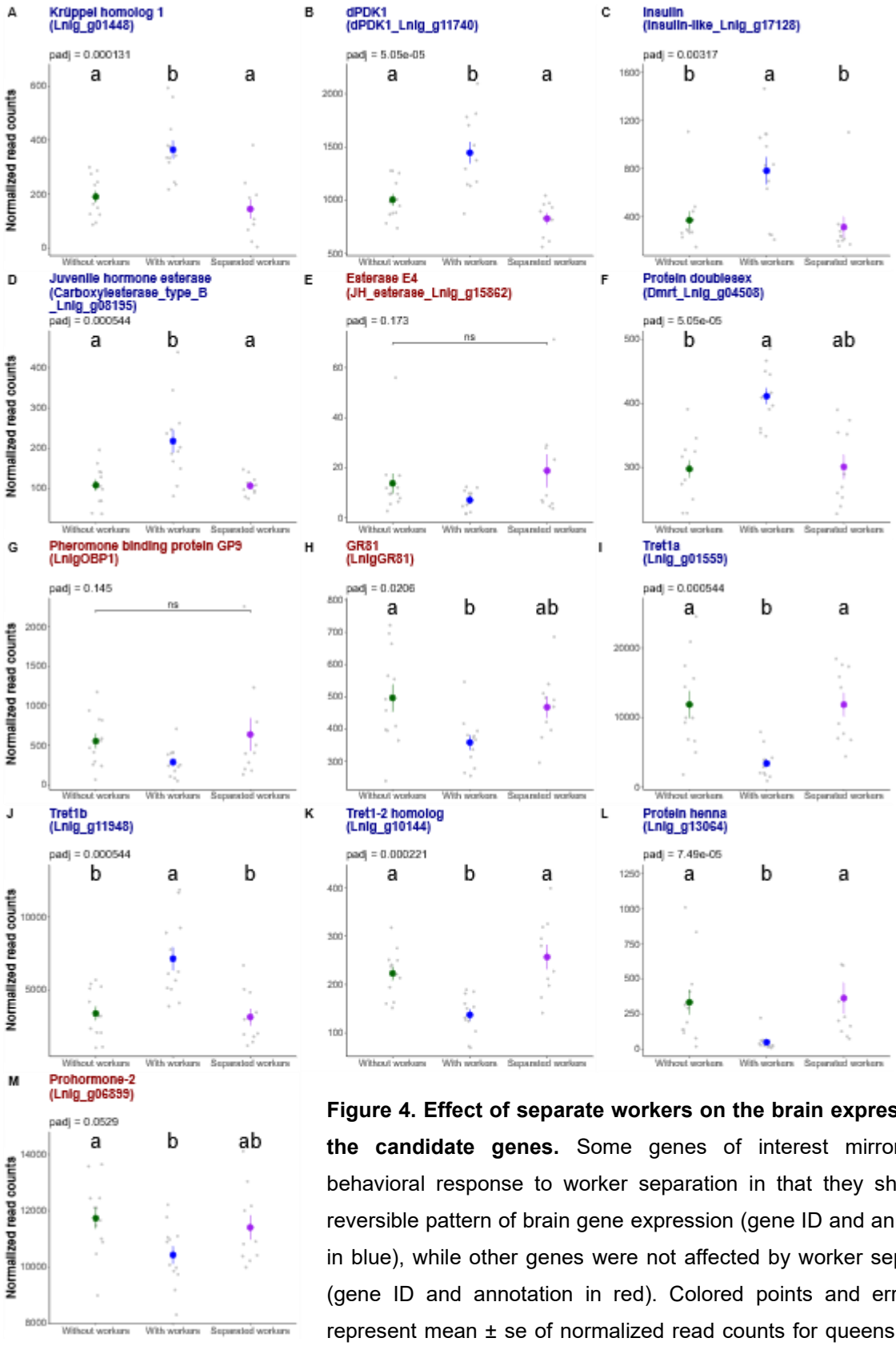

Figure 4. Effect of separate workers on the brain expression of the candidate genes. Some genes of interest mirrored the behavioral response to worker separation in that they showed a reversible pattern of brain gene expression (gene ID and annotation in blue), while other genes were not affected by worker separation (gene ID and annotation in red). Colored points and error bars represent mean ± se of normalized read counts for queens without workers (green; n = 12), queens with workers (blue; n = 12), and queens with workers separated by wire mesh (purple; n = 10). Letters indicate statistically supported differences between groups. “ns” indicates that we could not detect a main effect of the experimental manipulations (adjusted p > 0.05).

Effect of the presence of larvae on the expression of the genes of interest

The experimental addition of workers affects the brood care behavior of queens (Majidifar et al. 2024) and is, thus, confounded with whether or not queens interact with larvae: queens without workers have many interactions with larvae, while queens with workers have few or no interactions with larvae. Thus, our differential expression analysis may have captured expression changes that were the consequence of interacting with larvae rather than the response to the experimental manipulation of the presence of workers. To verify that changes in the expression of the genes of interest were not the mere consequence of interacting with larvae, we used 24 brain RNAseq samples from same-age funding queens that were kept with 6 larvae (n = 12) or without larvae (n = 12). None of those queens were provided with workers, thus, the queens with larvae expressed brood care and interacted with larvae, and the queens without larvae did not.

We predicted that if the effect of the presence of workers on the expression of the genes of interest was the mere consequence of interacting with larvae, we would detect expression differences between queens with and without larvae in a specific direction: genes overexpressed in queens kept with workers (that had few or no interactions with brood) would be overexpressed in queens without larvae, and genes overexpressed if queens kept without workers (that had many interactions with brood) would be overexpressed in queens with larvae. We found that nine of the 13 genes of interest did not differ between queens with and without larvae (Supplementary Table 5): Insulin (Insulin-like_Lnig_g17128), Juvenile hormone esterase (Carbocylesterase_type_B_Lnig_g08195), *Esterase E4* (JH_esterase_Lnig_g15862), Protein doublesex (Dmrt_Lnig_g04508), GR81 (LnigGR81), *Tret1a* (Lnig_g01559), Tret1b (Lnig_g11948), *Protein henna* (Lnig_g13064) and Prohormone-2 (Lnig_g06899) (Figure 5,C-F;H-J;L-M). The other four genes were affected by the presence of larvae, but in the opposite direction to what would be expected if the effect of the presence of workers was explained by an effect of interacting with larvae: *Kr-h1* (Lnig_g01448) and *dPDK1* (dPDK1_Lnig_g11740) were more expressed with larvae (Figure 5,A-B), but less expressed in absence of workers (Figure 2;A-B), while Pheromone binding protein *GP9* (LnigOBP1), and *Tret1-2 homolog* (Lnig_g10144) were more expressed without larvae (Figure 5, G;K), but less expressed in presence of workers (Figure 2,G;K).

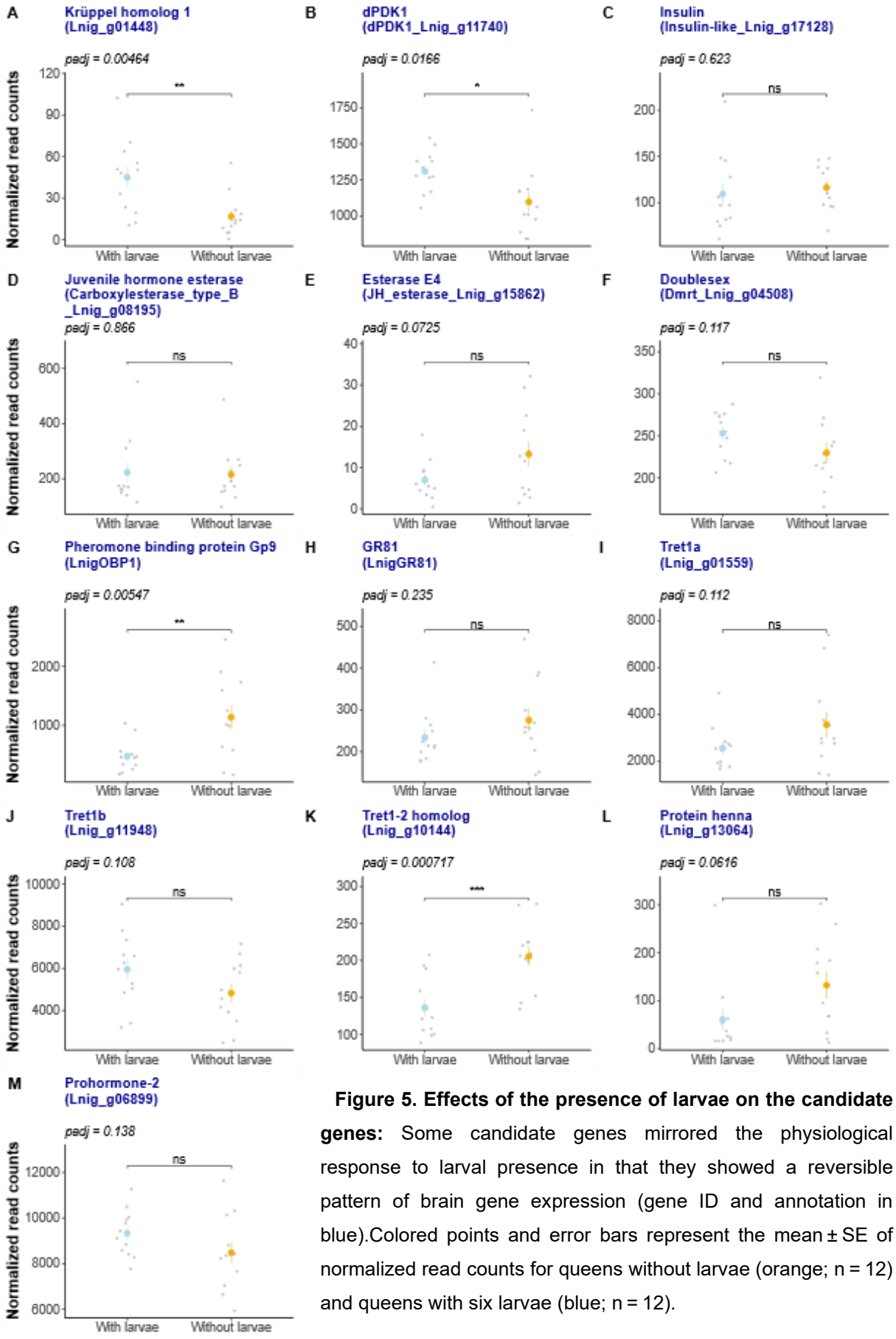

Figure 5. Effects of the presence of larvae on the candidate genes: Some candidate genes mirrored the physiological response to larval presence in that they showed a reversible pattern of brain gene expression (gene ID and annotation in blue).Colored points and error bars represent the mean ± SE of normalized read counts for queens without larvae (orange; n = 12) and queens with six larvae (blue; n = 12).

Discussion

Ant queens transition from pluripotent behavior during colony foundation to specialized egg-laying after worker emergence. Recent research revealed that this specialization is reversible, but little was known about the molecular mechanisms behind this reversibility. To address this gap, we leveraged RNA-seq data from 94 age- and nutritionally-matched queens of *Lasius niger* in diverse social environments (without workers, with workers, formerly with workers, with separated workers, with larvae, and without larvae). The comparison of queens with and without workers revealed 123 differential expressed genes (DEGs), including 13 genes of interest, with reported roles in regulating social insect behavior (Table 2). Crucially, further manipulations of the social environment revealed that four of these genes fully mirrored the behavioral responses, and thus, represent candidate genes for the regulation of queen specialization (Table 3).

Table 3: Classification of genes according to their expression patterns observed in our analysis. The genes in bold are those whose expression patterns matched the brood care behavioral pattern described in Majidifar et al. (2024): when workers were removed or separated by a wire mesh, these genes showed expression levels similar to queens without workers, and distinct from queens kept with workers. In addition, these genes were either not differently expressed between queens kept with and without larvae, or they were affected by the presence of larvae, in the opposite direction to what would be expected if the effect of the presence of workers was explained by an effect of interaction with the larvae.

Gene name	Evidence that gene expression changes in response to worker presence are reversible (yes or no)	Evidence that antennations with workers through a wire mesh is not sufficient to produce gene expression changes (yes or no)	Evidence that interactions with larvae are sufficient to explain the effect of workers on gene expression (yes or no)
Krueppel homolog 1 (Kr-h1)	**YES**	**YES**	**NO**
3-phosphoinositide-dependent protein kinase (dPDk1)	NO	YES	NO
Insulin	**YES**	**YES**	**NO**
Juvenile hormone esterase	**YES**	**YES**	**NO**
Esterase E4	NO	NO	NO
Protein doublesex	NO	YES	NO
Protein henna	NO	YES	NO
Prohormone-2	YES	NO	NO
Pheromone-binding protein Gp-9	YES	NO	NO

Gustatory receptor which mediates acceptance or avoidance behavior, depending on its substrates (GR81)	NO	NO	NO
Facilitated trehalose transporter Tret1a	NO	YES	NO
Facilitated trehalose transporter Tret1b	NO	YES	NO
Facilitated trehalose transporter Tret1-2 homolog	**YES**	**YES**	**NO**

Gene Ontology (GO) term analysis across the 123 DEGs revealed two distinct functional clusters. First, brain-related signaling processes (e.g., transmembrane transport, ion movement, and peptide responsiveness) suggest that social cues remodeled neural communication, potentially altering neurotransmission or neuropeptide sensitivity, to facilitate behavioral plasticity. Second, metabolic regulation pathways (carbohydrate/lipid metabolism, e.g., trehalose transport, triglyceride catabolism) indicate social environment-driven energy reallocation *within the brain*, likely to support synaptic remodeling or neuromodulator synthesis rather than nutritional differences (as food access was controlled). Critically, these metabolic shifts occur despite identical diets, implicating social cues in brain-specific energy management for behavioral transitions. GO term analysis of the comparison between mated and unmated queens in *Monomorium pharaonis* revealed similar processes (Nagel et al. 2020), with ion-transmembrane being unmated queen biased for example, therefore, hinting at non-reproductive behavioral expression.

Among the 13 genes of interest, we identified four candidate genes based on their expression patterns. First, the candidate genes mirrored the reversibility of queen specialization: they showed the same direction of expression before workers were added and after workers were removed. Second, they did not respond to mere worker cues, as demonstrated in the wire mesh separation experiment. Third, their expression patterns were not explained by larval interactions alone, as they showed either no pattern or a reverse pattern compared to the one with/without workers. These genes, *Kr-h1*, *Juvenile hormone esterase*, *Insulin*, and *Tret1-2 homolog* represent robust molecular signals of the queen's social environment-driven specialization, directly linking worker presence to brain gene expression changes underlying behavioral plasticity.

The transcription factor Kr-h1 was upregulated in queens brains with workers and downregulated in queen brains without workers. Kr-h1 functions as a juvenile hormone (JH)-responsive transcription factor across social insects. In the honey bee (*Apis mellifera*), Kr-h1

expression correlates with the nurse-to-forager transition and is suppressed by queen pheromones (Fussnecker and Grozinger 2008; Shpigler et al. 2010). In the bumble bee *Bombus terrestris*, higher Kr-h1 expression is associated with the reproductive worker status in queenless colonies (Shpigler et al. 2010). Critically, in the ant *Harpegnathos saltator*, Kr-h1 regulates behavioral transitions between worker and reproductive roles without correlating to caste identity; its knockdown alters expression of JH- and ecdysone-responsive genes through chromatin-based mechanisms, inducing caste-specific behavioral changes (Opachaloemphan et al. 2023). The reversible expression pattern in queens suggests Kr-h1 mediates suppression of brood care behavior in response to worker contact, likely through JH-dependent transcriptional regulation of social behavior pathways.

Juvenile hormone esterase (JHE) is reversibly downregulated in queens with workers and upregulated upon worker removal. JHE encodes an enzyme that hydrolyzes JH, directly regulating its concentration in the brain and hemolymph (Riddiford 1982). In social insects, JH influences behavioral transitions by modulating vitellogenin (vg) expression, which regulates nurse/forager division of labor and brood care (Amsalem et al. 2014; Libbrecht et al. 2013). Elevated brain JH levels can induce neural reorganization, such as increased mushroom body neuropil volume in honey bee foragers (Fahrbach and Robinson 1996) The reversible suppression of JHE in queens with workers suggests reduced JH degradation elevates brain JH titers, potentially driving vg-mediated reproductive specialization while suppressing brood care behavior.

Insulin-like peptide expression is reversibly increased in queens with workers and decreased without workers. Insulin-like peptides are ligands for the insulin/insulin-like growth factor signaling (IIS) pathway, which interacts with JH signaling to regulate fecundity, metabolism, and behavior in ants (Chandra et al. 2018; Libbrecht et al. 2013). In social insects, insulin-like-peptide expression correlates with vitellogenin production, which promotes egg-laying and extends queen lifespan, and with nurse-to-forager transitions in honey bees (*A. mellifera*) and red fire ants (*Solenopsis invicta*) (Ament et al. 2008, 20; Liu et al. 2023). The reversible upregulation in queens suggests worker contact acutely enhances IIS pathway activity, potentially linking social cues to reproductive physiology through vg-mediated mechanisms. While insulin expression correlates with food intake in insects (Badisco et al. 2013), the absence of nutritional differences in our design indicates this response is directly tied to social environment rather than trophallaxis.

The trehalose transporter Tret1-2 homolog shows reversible upregulation in queens with workers and downregulation upon worker removal or separation. Tret1-2 regulates tissue-specific uptake of trehalose, the primary circulatory sugar in insects (Saleh et al. 2021; Shukla et al. 2015). In *Lasius niger*, Tret1 is overexpressed in foragers compared to nurses,

potentially reflecting energy demands of foraging behavior (Quque et al. 2023). Similarly, in orchid bees (*Euglossa dilemma*), Tret1-2 overexpression correlates with dominance status, ovary size, and reproductive behavior(Saleh et al. 2021). Despite identical nutritional conditions in our study, the reversible expression pattern suggests worker contact triggers acute reallocation of energy resources in the queen brain to support reproductive specialization. This metabolic shift likely interacts with the JH/IIS pathway, as trehalose homeostasis is regulated by insulin signaling and influences vitellogenin production.

The nine other genes of interest reveal a distinct, longer-term imprint of the social environment. One gene, dPDK1, a component of the insulin-signalling cascade (Storz and Toker 2002), changes only when workers are in direct contact and its altered transcription persists after workers are removed, pointing to a role in stabilizing metabolic set-points established during prolonged social exposure. Esterase E4 shows no response to either workers or larvae, consistent with a permanent shift in pheromone or neuro-modulator catabolism that could lock queens into a new chemical profile (Shpigler et al. 2017). Likewise, dsx and the pigment synthesis enzyme henna are sensitive solely to direct contact, while the trehalose transporters Tret1a/b, the pheromone-binding protein Gp-9, and the gustatory receptor GR81 retain altered expression irrespective of worker presence, suggesting durable adjustments in carbohydrate allocation, sensory tuning, and neural circuitry (Ross and Keller 1998; Kikawada et al. 2007). Together, these patterns underscore a dichotomy: a subset of genes drives rapid, reversible behavioral switches, whereas this broader set underlies lasting physiological and neural remodeling after social cues subside.

While recent work established that queen behavioral specialization reversibly depends on worker presence (Majidifar et al. 2024), the molecular mechanisms translating social cues into brain gene expression changes remained unknown. Here, through a well-replicated, well-controlled transcriptomic approach combined with targeted social manipulations, we identify four candidate genes whose reversible expression patterns directly track queen behavioral plasticity: *Kr-h1*, *Juvenile hormone esterase*, and *Insulin* from the JH/IIS pathway, and the metabolic regulator *Tret1-2 homolog*. These findings reveal how worker contact modulates conserved hormonal and metabolic pathways to suppress brood care while promoting reproductive specialization. Future studies should functionally validate these candidates via RNAi across social insect lineages and characterize their spatial expression in brain regions governing social behavior. Such work will determine whether this regulatory axis represents a conserved mechanism for queen behavioral plasticity, addressing a critical gap in understanding how "egg-laying specialists" dynamically adjust their roles within the colony.

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Author contributions

[redacted] conceived the idea and designed the methodology together with MFB. MFB dissected the ants and extracted the RNA. [redacted] and [redacted] analyzed the data. [redacted], MFB and [redacted] led the writing of the manuscript.

Supplementary material

Supplementary Table 1: Significant differential expressed genes between queens with and without workers. For each gene, we provide the gene identifier, log2 fold change, adjusted p-value (padj), and expression direction.

gene	log2FoldChange	padj	Expression direction
gLnigGR81	-0.469	0.00542	Underexpressed with workers
gLnigOBP1	-1.1	0.025	Underexpressed with workers
Lnig_g00051	0.108	0.0117	Overexpressed with workers
Lnig_g00060	-0.63	0.0362	Underexpressed with workers
Lnig_g00605	-0.363	0.0281	Underexpressed with workers
Lnig_g00658	0.351	0.035	Overexpressed with workers
Lnig_g00662	0.344	0.0362	Overexpressed with workers
Lnig_g00696	0.23	7.52E-06	Overexpressed with workers
Lnig_g00732	-0.174	0.0435	Underexpressed with workers
Lnig_g00791	-1.55	0.0383	Underexpressed with workers

Lnig_g00794	-0.21	0.035	Underexpressed with workers
Lnig_g00926	0.327	0.0117	Overexpressed with workers
Lnig_g01448	0.919	0.00313	Overexpressed with workers
Lnig_g01559	-1.29	0.00151	Underexpressed with workers
Lnig_g01860	-0.475	0.0435	Underexpressed with workers
Lnig_g01886	0.526	0.00141	Overexpressed with workers
Lnig_g01908	0.261	0.025	Overexpressed with workers
Lnig_g01972	-0.174	0.0369	Underexpressed with workers
Lnig_g01993	-0.123	0.0086	Underexpressed with workers
Lnig_g02018	0.46	9.42E-08	Overexpressed with workers
Lnig_g02155	-0.256	8.67E-09	Underexpressed with workers
Lnig_g03026	0.259	0.00501	Overexpressed with workers
Lnig_g03330	-0.238	0.0315	Underexpressed with workers
Lnig_g03526	-1.67	7.88E-08	Underexpressed with workers
Lnig_g03596	-0.795	0.000134	Underexpressed with workers
Lnig_g03713	0.122	0.016	Overexpressed with workers
Lnig_g04065	-0.291	0.0219	Underexpressed with workers
Lnig_g04415	0.221	9.42E-08	Overexpressed with workers
Lnig_g04419	0.342	1.15E-05	Overexpressed with workers
Lnig_g04508	0.313	0.00261	Overexpressed with workers
Lnig_g04689	-0.532	1.15E-05	Underexpressed with workers
Lnig_g04954	0.23	0.035	Overexpressed with workers
Lnig_g04977	2.4	0.000127	Overexpressed with workers
Lnig_g05184	0.946	0.0253	Overexpressed with workers
Lnig_g05264	-1.82	0.000709	Underexpressed with workers
Lnig_g05796	0.163	0.00914	Overexpressed with workers
Lnig_g05955	-1.33	0.0281	Underexpressed with workers
Lnig_g05988	-0.183	0.035	Underexpressed with workers
Lnig_g06021	0.306	0.0265	Overexpressed with workers
Lnig_g06130	-0.463	0.0397	Underexpressed with workers
Lnig_g06161	-0.698	1.71E-06	Underexpressed with workers
Lnig_g06163	0.231	0.035	Overexpressed with workers
Lnig_g06188	0.29	0.0281	Overexpressed with workers
Lnig_g06214	0.378	0.0152	Overexpressed with workers
Lnig_g06276	-0.174	0.025	Underexpressed with workers
Lnig_g06690	-0.232	0.035	Underexpressed with workers
Lnig_g06814	0.346	0.000956	Overexpressed with workers
Lnig_g06899	-0.227	0.00553	Underexpressed with workers
Lnig_g08195	1.09	3.02E-06	Overexpressed with workers
Lnig_g08546	0.233	0.0105	Overexpressed with workers
Lnig_g08552	-0.205	0.0046	Underexpressed with workers
Lnig_g08641	-1.48	0.0337	Underexpressed with workers
Lnig_g08990	-0.653	0.0302	Underexpressed with workers
Lnig_g09976	-0.153	0.035	Underexpressed with workers

Lnig_g10029	0.241	0.0166	Overexpressed with workers
Lnig_g10062	-0.941	0.0216	Underexpressed with workers
Lnig_g10144	-0.612	0.000248	Underexpressed with workers
Lnig_g10294	0.363	6.94E-06	Overexpressed with workers
Lnig_g10297	0.356	0.00188	Overexpressed with workers
Lnig_g10488	0.277	6.75E-06	Overexpressed with workers
Lnig_g10627	-1.47	0.0492	Underexpressed with workers
Lnig_g11692	-0.61	0.035	Underexpressed with workers
Lnig_g11740	0.44	0.000996	Overexpressed with workers
Lnig_g11759	-0.784	0.000831	Underexpressed with workers
Lnig_g11767	0.205	0.0362	Overexpressed with workers
Lnig_g11817	0.305	0.0188	Overexpressed with workers
Lnig_g11880	-0.409	0.0296	Underexpressed with workers
Lnig_g11948	0.891	0.000129	Overexpressed with workers
Lnig_g12074	0.195	0.0416	Overexpressed with workers
Lnig_g12336	0.331	0.025	Overexpressed with workers
Lnig_g12344	-0.556	0.0294	Underexpressed with workers
Lnig_g12345	-0.393	0.00343	Underexpressed with workers
Lnig_g12492	0.249	0.0479	Overexpressed with workers
Lnig_g12530	0.365	0.035	Overexpressed with workers
Lnig_g12556	-0.13	0.00188	Underexpressed with workers
Lnig_g12669	-0.311	0.000581	Underexpressed with workers
Lnig_g12819	-0.309	0.0281	Underexpressed with workers
Lnig_g12940	0.206	0.0281	Overexpressed with workers
Lnig_g12988	0.37	0.0147	Overexpressed with workers
Lnig_g13001	0.2	0.0416	Overexpressed with workers
Lnig_g13064	-1.96	0.00136	Underexpressed with workers
Lnig_g13106	0.269	0.0324	Overexpressed with workers
Lnig_g13335	0.766	1.59E-07	Overexpressed with workers
Lnig_g14172	-0.271	0.0364	Underexpressed with workers
Lnig_g14309	-0.561	0.0086	Underexpressed with workers
Lnig_g14382	-0.231	0.035	Underexpressed with workers
Lnig_g14507	-1.28	0.00313	Underexpressed with workers
Lnig_g14626	-0.191	0.0229	Underexpressed with workers
Lnig_g14760	-0.238	0.00542	Underexpressed with workers
Lnig_g15014	-0.527	0.000161	Underexpressed with workers
Lnig_g15270	-1.07	0.0367	Underexpressed with workers
Lnig_g15343	-1.32	0.00283	Underexpressed with workers
Lnig_g15352	0.146	0.0281	Overexpressed with workers
Lnig_g15415	-1.69	0.0218	Underexpressed with workers
Lnig_g15424	0.299	0.0183	Overexpressed with workers
Lnig_g15443	0.595	0.0162	Overexpressed with workers
Lnig_g15671	0.0785	0.0352	Overexpressed with workers
Lnig_g15686	0.257	0.0274	Overexpressed with workers

Lnig_g15688	-0.205	0.00985	Underexpressed with workers
Lnig_g15860	-0.56	0.00343	Underexpressed with workers
Lnig_g15862	-1.28	0.0461	Underexpressed with workers
Lnig_g16060	0.374	0.00333	Overexpressed with workers
Lnig_g16062	0.29	0.0281	Overexpressed with workers
Lnig_g16183	-0.443	0.0453	Underexpressed with workers
Lnig_g16441	-0.862	4.29E-05	Underexpressed with workers
Lnig_g16489	0.655	9.70E-06	Overexpressed with workers
Lnig_g16550	1.46	0.000477	Overexpressed with workers
Lnig_g16580	0.364	0.0453	Overexpressed with workers
Lnig_g16712	-0.881	0.0449	Underexpressed with workers
Lnig_g16769	-0.15	0.0193	Underexpressed with workers
Lnig_g16840	0.264	0.000996	Overexpressed with workers
Lnig_g17025	-0.221	0.0435	Underexpressed with workers
Lnig_g17105	0.245	0.035	Overexpressed with workers
Lnig_g17108	0.339	0.00067	Overexpressed with workers
Lnig_g17127	1.05	0.000709	Overexpressed with workers
Lnig_g17128	1.01	0.000431	Overexpressed with workers
Lnig_g17199	0.291	1.28E-06	Overexpressed with workers
Lnig_g17282	-0.564	0.0383	Underexpressed with workers
Lnig_g18209	-0.403	0.0324	Underexpressed with workers
Lnig_g18399	0.187	0.0046	Overexpressed with workers
Lnig_g18434	-0.149	0.0281	Underexpressed with workers
Lnig_g18468	-0.235	0.0162	Underexpressed with workers
Lnig_g18623	0.168	0.00733	Overexpressed with workers

Supplementary Table 2: Significant Go-Terms found.

GO.ID	Term	Annotated	Significant	Expected	pvalue	Ontology
GO:0010898	positive regulation of triglyceride catabolic process	10	3	0.12	0.00017	Biological process
GO:0097164	ammonium ion metabolic process	96	7	1.11	0.00036	Biological process
GO:0034375	high-density lipoprotein particle remodeling	13	3	0.15	0.00039	Biological process
GO:0015771	trehalose transport	21	3	0.24	0.00171	Biological process
GO:0006658	phosphatidylserine metabolic process	12	3	0.14	0.00347	Biological process
GO:0019432	triglyceride biosynthetic process	51	5	0.59	0.00462	Biological process
GO:0005978	glycogen biosynthetic process	28	3	0.32	0.00552	Biological process
GO:0042632	cholesterol homeostasis	62	4	0.72	0.00562	Biological process
GO:0055085	transmembrane transport	915	19	10.57	0.00718	Biological process
GO:0046471	phosphatidylglycerol metabolic process	24	3	0.28	0.0093l4	Biological process
GO:1901653	cellular response to peptide	206	7	2.38	0.00971	Biological process
GO:0005536	D-glucose binding	12	3	0.14	0.00031	Molecular Function
GO:0015293	symporter activity	105	6	1.22	0.00036	Molecular Function
GO:0003841	1-acylglycerol-3-phosphate O-acyltransferase activity	15	3	0.17	0.00063	Molecular Function
GO:0015318	inorganic molecular entity transmembrane transporter activity	501	10	5.84	0.00624	Molecular Function
GO:0032559	adenyl ribonucleotide binding	1074	17	12.51	0.00905	Molecular Function

Supplementary Table 3: Statistical results of the genes of interest's expression comparison between queens with workers, with no workers, and with workers removed. The text in bold indicates genes showing a main effect between the three categories, with a difference in expression between queens with and with removed workers, and with a t-value indicating that the effect is in the same direction as that observed in queens without workers. Genes meeting these conditions were considered to follow the same expression pattern as the queen's behaviors in Majidifar et al (2024)

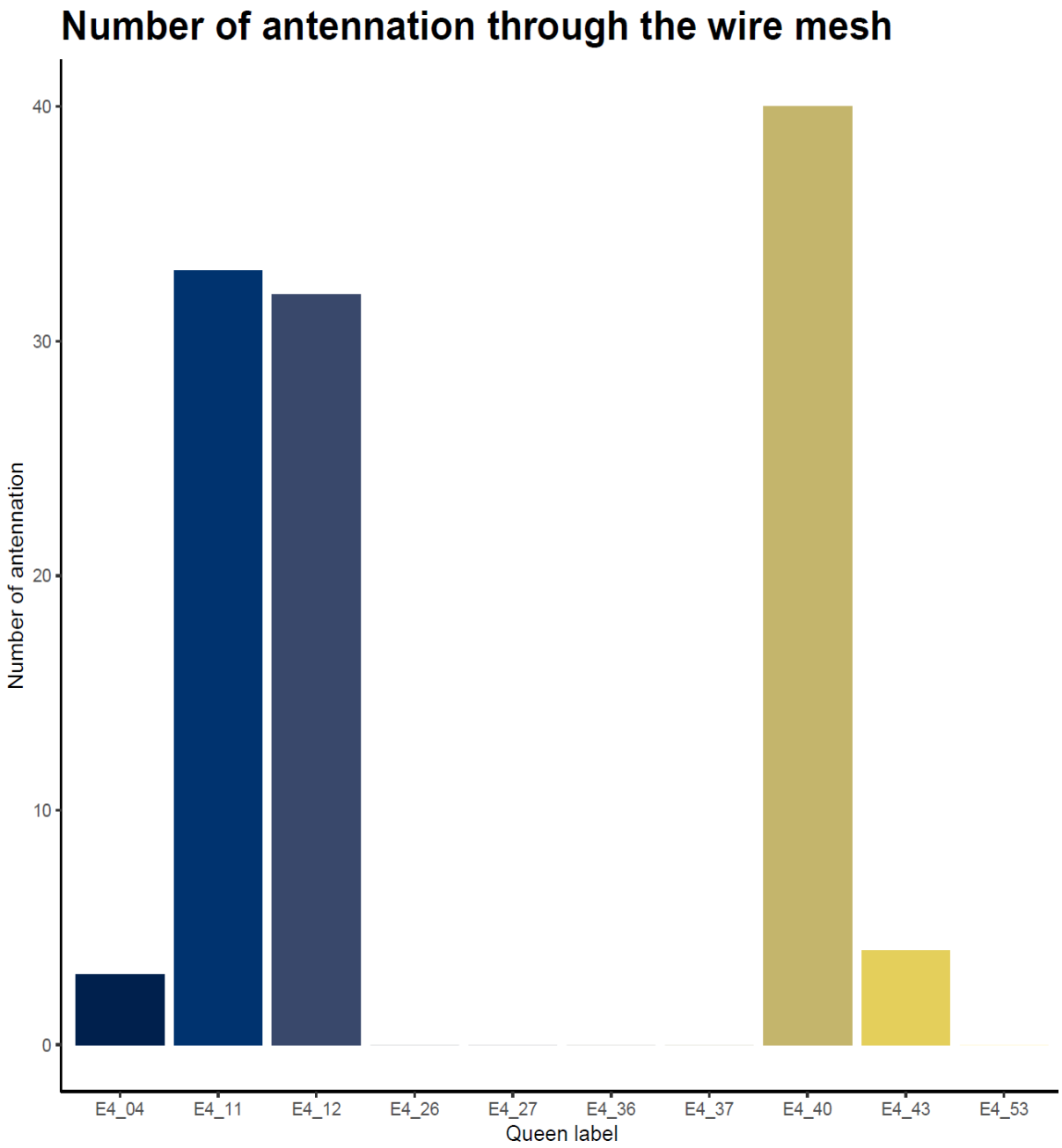
Genes of interest	Main effect (Pvalue)	Two-by-two comparison: No workers / Presence of workers (Pvalue & t test)	Two-by-two comparison: No workers / Workers removed (Pvalue & t test)	Two-by-two comparison: Presence of workers / Workers removed (Pvalue & t test)
Krueppel homolog 1 (Kr-h1)	**5.815e-10**	**Pvalue: 0.0151 t value: -2.962**	**Pvalue: <.0001 t value: 6.166**	**Pvalue: <.0001 t value: 9.128**
3-phosphoinositide-dependent protein kinase (dPDk1)	**0.01294**	**Pvalue: 0.0270 t value: -2.723**	**Pvalue: 0.0259 t value: -2.740**	**Pvalue: 0.9998 t value: -0.017**
Insulin	**0.00043**	**Pvalue: 0.0089 t value: -3.174**	**Pvalue: 0.5131 t value: 1.113**	**Pvalue: 0.0004 t value: 4.287**
Juvenile hormone esterase	**3.399e-06**	**Pvalue: <.0001 t value: -5.277**	**Pvalue: 0.9955 t value: 0.091**	**Pvalue: <.0001 t value: 5.368**
Esterase E4	**0.00810**	**Pvalue: 0.0089 t value: 3.172**	**Pvalue: 0.0448 t value: 2.503**	**Pvalue: 0.7830 t value: -0.669**
Protein doublesex	0.2416	Pvalue: NA t value: NA	Pvalue: NA t value: NA	Pvalue: NA t value: NA
Protein henna	**0.01582**	**Pvalue: 0.0446 t value: 2.505**	**Pvalue: 0.0230 t value: 2.790**	**Pvalue: 0.9564 t value: 0.285**
Prohormone-2	**0.0007**	**Pvalue: 0.0160 t value: 2.939**	**Pvalue: 0.4523 t value: -1.216**	**Pvalue: 0.0006 t value: -4.155**
Pheromone-binding protein Gp-9	**0.02335**	**Pvalue: 0.0576 t value: 2.389**	**Pvalue: 0.9696 t value: -0.237**	**Pvalue: 0.0339 t value: -2.626**
Gustatory receptor which mediates acceptance or avoidance behavior, depending on its substrates (GR81)	**0.02106**	**Pvalue: 0.0228 t value: 2.794**	**Pvalue: 0.8327 t value: 0.578**	**Pvalue: 0.0833 t value: -2.216**
Facilitated trehalose transporter Tret1a	**0.05407**	**Pvalue: NA t value: NA**	**Pvalue: NA t value: NA**	**Pvalue: NA t value: NA**
Facilitated trehalose transporter Tret1b	**0.00252**	**Pvalue: 0.0018 t value: -3.770**	**Pvalue: 0.0722 t value: -2.284**	**Pvalue: 0.3106 t value: 1.486**
Facilitated trehalose transporter Tret1-2 homolog	**0.0005**	**Pvalue: 0.0154 t value: 2.956**	**Pvalue: 0.3848 t value: -1.338**	**Pvalue: 0.0004 t value: -4.294**

Supplementary Table 4: Statistical results of the genes of interest's expression comparison between queens with workers, with no workers, and queens separated from workers by a wire mesh. The text in bold indicates genes showing a main effect between the three categories, with a difference in expression between queens with and with removed workers, and with a t-value indicating that the effect is in the same direction as that observed in queens without workers. Genes meeting these conditions were considered to follow the same expression pattern as the queen's behaviors in Majidifar et al (2024).

Genes of interest	Main effect (Pvalue)	Two-by-two comparison: No workers / Presence of workers (Pvalue & t test)	Two-by-two comparison: No workers / Separated workers (Pvalue & t test)	Two-by-two comparison: Presence of workers / Separated workers (Pvalue & t test)
Krueppel homolog 1 (Kr-h1)	**4.028e-05**	**Pvalue: 0.0007 t value: -4.122**	**Pvalue: 0.5678 t value: 1.024**	**Pvalue: 0.0001 t value: 4.954**
3-phosphoinositide-dependent protein kinase (dPDk1)	**7.762e-06**	**Pvalue: 0.0005 t value: -4.248**	**Pvalue: 0.2529 t value: 1.619**	**Pvalue: <.0001 t value: 5.669**
Insulin	**0.00219**	**Pvalue: 0.0090 t value: -3.182**	**Pvalue: 0.9048 t value: 0.427**	**Pvalue: 0.0044 t value: 3.461**
Juvenile hormone esterase	**0.00033**	**Pvalue: 0.0010 t value: -4.005**	**Pvalue: 0.9991 t value: 0.041**	**Pvalue: 0.0015 t value: 3.859**
Esterase E4	0.1727	Pvalue: NA t value: NA	Pvalue: NA t value: NA	Pvalue: NA t value: NA
Protein doublesex	**4.157e-06**	**Pvalue: <.0001 t value: -5.488**	**Pvalue: 0.9867 t value: -0.156**	**Pvalue: 0.0001 t value: 5.077**
Protein henna	**1.728e-05**	**Pvalue: 0.0001 t value: 4.769**	**Pvalue: 0.9215 t value: -0.386**	**Pvalue: 0.0001 t value: -4.933**
Prohormone-2	0.04476	Pvalue: 0.0428 t value: 2.531	Pvalue: 0.8199 t value: 0.602	Pvalue: 0.1827 t value: -1.811
Pheromone-binding protein Gp-9	0.134	Pvalue: NA t value: NA	Pvalue: NA t value: NA	Pvalue: NA t value: NA
Gustatory receptor which mediates acceptance or avoidance behavior, depending on its substrates (GR81)	0.01583	Pvalue: 0.0165 t value: 2.939	Pvalue: 0.8310 t value: 0.581	Pvalue: 0.0834 t value: -2.221
Facilitated trehalose transporter Tret1a	**0.00032**	**Pvalue: 0.0009 t value: 4.040**	**Pvalue: 1.0000 t value: 0.004**	**Pvalue: 0.0016 t value: -3.848**
Facilitated trehalose transporter Tret1b	**0.00033**	**Pvalue:0.0016 t value: -3.831**	**Pvalue: 0.9254 t value: 0.376**	**Pvalue: 0.0010 t value: 4.028**
Facilitated trehalose transporter Tret1-2 homolog	**8.495e-05**	**Pvalue: 0.0028 t value: 3.637**	**Pvalue: 0.3649 t value: -1.377**	**Pvalue: 0.0001 t value: -4.845**

Supplementary Table 5. Statistical results of the genes of interest's expression comparison between queens with larvae and without larvae, and between queens without workers and with workers. The text in bold indicates genes showing either no differences of expression between queens with and without larvae, or with an estimate with a different sign than the Log2FoldChange in the comparison of expression of queens without and with workers. Genes meeting these conditions were considered unaffected by contact with larvae, as we hypothesized that if a gene is influenced by contact with larvae, its expression between the queen with larvae should be similar to that of the queen without workers who take care of the brood, and inversely with queens without larvae and queens with workers.

Genes of interest	Gene expression comparison between queens with and without larvae (Estimate & Pvalue)	Gene expression comparison between queens without and with workers (Log2FoldChange & Padj)
Krueppel homolog 1 (Kr-h1)	**Estimate: -28.229 Pvalue: 0.0046**	**Log2FoldChange: 0.9188 Pvalue: 0.0031**
3-phosphoinositide-dependent protein kinase (dPDk1)	**Estimate: -212.36 Pvalue: 0.0166**	**Log2FoldChange: 0.4396 Pvalue: 0.0010**
Insulin	**Estimate: 6.970 Pvalue: 0.6234**	**Log2FoldChange: 1.0101 Pvalue: 0.0004**
Juvenile hormone esterase	**Estimate: -7.873 Pvalue: 0.866**	**Log2FoldChange: 1.0888 Pvalue: <0.0001**
Esterase E4	**Estimate: 6.299 Pvalue: -0.0725**	**Log2FoldChange: -1.2817 Pvalue: 0.046**
Protein doublesex	**Estimate: -23.32 Pvalue: 0.1172**	**Log2FoldChange: 0.3128 Pvalue: 0.0026**
Protein henna	**Estimate: 71.44 Pvalue: 0.0616**	**Log2FoldChange: -1.9646 Pvalue: 0.0014**
Prohormone-2	**Estimate: -850.6 Pvalue: 0.138**	**Log2FoldChange: -0.2271 Pvalue: 0.0055**
Pheromone-binding protein Gp-9	**Estimate: 662.8 Pvalue: 0.0055**	**Log2FoldChange: -1.0982 Pvalue: 0.0250**
Gustatory receptor which mediates acceptance or avoidance behavior, depending on its substrates (GR81)	**Estimate: 41.32 Pvalue: 0.235**	**Log2FoldChange: -0.4692 Pvalue: 0.0054**
Facilitated trehalose transporter Tret1a	**Estimate: 998.8 Pvalue: 0.112**	**Log2FoldChange: -1.2914 Pvalue: 0.0015**
Facilitated trehalose transporter Tret1b	**Estimate: -1118.5 Pvalue: 0.1079**	**Log2FoldChange: 0.8909 Pvalue: 0.0001**
Facilitated trehalose transporter Tret1-2 homolog	**Estimate: 69.70 Pvalue: 0.0007**	**Log2FoldChange: -0.6123 Pvalue: 0.0002**



Supplementary Figure 1: Queen's ability to antennate with workers through the wire mesh. Half of the queens (n = 5) were observed passing their antennae through the wire mesh to touch a worker's antennae, showing that the wire mesh enabled the queen to antennate with workers. Each label represents a queen separated from three callow workers by a wire mesh. The queens' behaviors were monitored using focus animal sampling during 50 mn per 1 hour video, as we did not analyze the first and last 5 min to avoid potential disturbances due to the experimental manipulations.

General Discussion

Division of labor is one of the key aspects of the ecological success of social insect societies. These societies consist of non-reproductive workers, which maintain the colony and reproductive queens. By definition, the ant queen is the pivotal individual within a colony, yet it is frequently reduced to its reproductive role, neglecting other aspects. My dissertation demonstrates that this reduction obscures a far richer reality: queens retain a suite of behavioral capability with an underlying molecular machinery that can be reshaped by the social environment, mating status, and species-specific life-history strategies.

Chapter 1: “Evidence for social control of queen specialization across the ant phylogeny” establishes that queen behavioral plasticity, *i.e.,* the ability of the queen to adjust her behavior accordingly to social environment, which had previously been reported for only a few ant species, is, in fact, a widespread phenomenon across the ant phylogeny. With an investigation into 38 species, across 6 subfamilies, we revealed queen behavioral plasticity in 19 species. The effect is detectable in taxa with radically different life histories, ranging from monogynous species that found colonies independently to the opposite extreme of dependent founding, polygynous species. Furthermore, we detected in five species that queens express brood care regardless of worker presence. In monogynous species the behavioral response to the presence of brood was markedly stronger than in the polygynous species. These results indicate that queen behavioral flexibility is neither a rare occurrence nor a trait specific to certain lineages.

Chapter 2: “Unmated queens show worker-like behavior and gene expression in polygynous colonies of the ant *Stigmatomma pallipes*” focuses on a single species, *Stigmatomma pallipes*, in which queens of polygynous colonies, although morphologically identical, can be differentiated based on activity and behavior, revealing high-mobility and low-mobility queens. While developmental processes determine caste during larval development, we reveal that the underlying molecular framework remains flexible enough to permit worker-like gene expression in certain queens. For example, high-mobility queens and workers differed little in their gene expression in the brain and the fat body, while, in comparison, there were extensive differences with low-mobility queens in the range of two to four thousand genes. Importantly, factors external to morphology, most notably the queen’s mating status, contribute to the differences between queens. Both types of queens interact with broods in the presence of workers, underscoring that queen-worker identity can be fluid. This finding emphasizes that queen morphology alone does not dictate behavioral repertoire.

Chapter 3: “Transcriptomic changes associated with the socially regulated behavioral specialization of ant queens” demonstrates that socially induced changes in queen behavior are accompanied by systematic shifts in gene expression. We detected 123 differential expressed genes comparing queens in the presence and absence of workers. Among these, we found 13 genes of interest, already known to regulate insect behavior, such as *Krüppel-homolog 1* (*Kr-h1*), and other components of the juvenile hormone and insulin-like signaling pathways. Furthermore, we identified a subset of four candidate genes, whose expression was reversible and could not simply be explained by the presence of larvae, therefore directly tracking the queens behavioral flexibility. In conclusion, queen behavioral plasticity draws on an often-utilized genetic toolkit rather than on novel, queen-specific pathways. The conserved nature of the toolkit suggests that similar regulatory modules may be coopted in many ant lineages. Furthermore, in Appendix 1, we attempted to knock down one of the genes of interest, which was upregulated in the presence of workers, *dPDK1*. While the attempt was unsuccessful, it highlights the development of the first methodological approaches to investigate the function of these genes.

Taken together, the three chapters elucidate the range of queen behavioral plasticity at multiple levels of organization. In the following sections of the discussion, I will highlight the interactions of the behavioral and molecular insights we gained in these chapters, before putting them into a broader perspective.

Queen Behavior in Mature Colonies is more Variable than Expected

Most ant queens have to go through a risky founding stage, in which they must establish the first nesting site, raise the first brood, and, in some species, go out and forage (Brown and Bonhoeffer 2003; Keller 1991). Only once workers emerge can queens shift their focus entirely to reproduction. Yet, until recently, little was known about how flexible queen behavior remains in established colonies (Majidifar et al. 2024; Ortiz-Alvarado and Rivera-Marchand 2020).

Chapter 1 shows that queens across the ant phylogeny retain the capacity to express brood care behavior upon the removal of workers. Furthermore, in some species, we could show that some queens express brood care regardless of worker presence. Chapter 2 deepens this insight by revealing that in one polygynous species *S. pallipes*, queens can express worker-like behavior, regardless of mating status. This may be linked to the species having low queen-worker dimorphism: with less morphological specialization for reproduction, queens may remain behaviorally flexible enough to care for the brood even alongside

workers. Furthermore, in this species, queens do interact with the brood to gain nutrients (Traniello 1982; Wheeler 1900). This dual evidence confirms that queen caste behavior is more versatile than estimated. While this versatility can be partly explained in monogynous colonies, where queens pass through a stage of independent founding, in many polygynous species, queens are usually always in the presence of workers (Tsuji and Tsuji 1996; Vargo 1993). In those species, we recorded a lower change of brood care behavior. Furthermore, it has been reported that in polygynous colonies, queens can have different reproductive outputs and in some species dominance hierarchies can exist, hinting towards different behaviors expressed between dominant and non-dominant queens (Ito et al. 1996; Bourke 1994; Clutton-Brock 1998).

This variation in polygynous colonies hints towards a further divergence of the social control of queen behavior. On the one side, we have queens that perform little to no brood care at all, such as *Linepithema humile* and *Paratrechina longicornis.* In these species, queens do not perform nuptial flights and remain in the colony their entire life. Therefore, if the lack of flexibility is genuine, in these species, the queen caste has been completely reduced to one apparent purpose, reproduction (Krieger and Keller 2000; Pearcy et al. 2011). On the other side, we have species like *Anoplolepis gracilipes* and *Monomorium pharaonis*, which showed queen behavioral flexibility while sharing many life-history traits with *L. humile* and *P. longicornis* (Buczkowski and Bennett 2009; Hoffmann 2014). Interestingly, for at least *A. gracilipes*, rare independent founding has been reported, hinting towards that queens are not as canalized into reproduction and keep some of the flexibility (Ito et al. 2016). Lastly, we have another highly polygynous invasive species, *Wasmannia auropunctata* (Breton et al. 2004), which always expressed some brood care in our experiment, while in bigger colonies the removal of some of the workers also prompted queens to change behavior (Ortiz-Alvarado and Rivera-Marchand 2020). These findings further exemplify that either the lack of social control or its modulation can channelize the queen caste towards highly specialized queens or more worker-like queens.

Conserved and Novel Gene Regulatory Processes in the Ant Queen

It stands to reason that the molecular machinery described in Chapter 3 underlies the behavioral plasticity observed in Chapter 1 and Majidifar et al. (2024). Genes, such as the transcription factor *Kr-h1,* which has been shown to accompany behavioral changes in the bees (Shpigler et al. 2010; Fussnecker and Grozinger 2008), juvenile hormone and insulin-like peptides, which accompany behavioral transitions in *Lasius niger*, are likely to

serve similar regulatory functions in many other ants (Chandra et al. 2018; Gospocic et al. 2021).

Gene regulation, through epigenetic mechanisms like methylation or the action of transcription factors, for example, drives phenotypic plasticity and has played a central role in the evolution of eusociality (Jones and Robinson 2018; Cristino et al. 2006). For example, in bees it was shown that gene regulation is associated with eusociality, as well as social complexity (Kapheim, Pan, et al. 2015). Similarly, gene regulation is found in association with worker behavior in ants (Ingram et al. 2016; Glastad et al. 2020; Simola et al. 2016). This raises the question of whether the regulation of these pathways is altered in queens that exhibit little plasticity, such as those that either constantly provide brood care or scarcely do. Although these pathways would be predicted to be present, their regulation could differ among species (Vizueta et al. 2025; Kay et al. 2025; Kocher and Kingwell 2024). Furthermore, regulatory processes could also influence how queens perceive, for example, larval signals, as has been shown in reproductive workers of clonal raider ants (Chandra et al. 2018; Libbrecht et al. 2018). To validate the regulatory function of these genes in queens, similarly to workers, one can use molecular methods, such as RNA interference (RNAi) (Chen et al. 2022; 2023a). In Appendix 1, we investigated the knockdown of one of the genes of interest identified in chapter 3, *dPDK1*. Although the attempt was unsuccessful, it underscores the necessity of methodological research on the application of methods to queens versus workers.

Our investigation into queen behavior of *S. pallipes* (Chapter 2), further highlights that in certain species queens can constantly perform brood care behavior, which may be regulated differently, than the highlighted pathways in *L. niger* (Chapter 3). Due to both unmated and mated queens in *S. pallipes* expressing brood care in the presence of workers, it is either likely that the pathway responsible for brood care is always active, or that the switch between totipotent foundress and reproductive unit does not happen in *S. pallipes* to begin with. Moreover, the always-present worker-like transcriptional and behavioral profile of unmated *S. pallipes* queens makes it difficult to disentangle the contribution of brood care per se from caste-specific differences. As *S. pallipes* queens engage in specialized trophic behaviors (e.g., hemolymph feeding) that rely on interactions with the brood (Traniello 1982), the pathways might be altered. In a closely related *Mystrium* species, queens with worker-like morphology have further been fully utilized as the main brood care givers, while workers have been specialized in foraging, further exemplifying the flexibility of the queen caste (Molet et al. 2007; 2009)

In sum, this three-chapter synthesis demonstrates that ant queens retain substantial behavioral plasticity across evolutionary timescales. Furthermore, first insights show that this flexibility could be at least partly orchestrated by a conserved gene-regulatory toolkit, though this will require a comparative genomic and cross-species transcriptomic analysis. After having followed the evolutionary trajectory from major transitions to the emergence of eusociality and finally to the ant model presented in the introduction, I now reverse that analytical direction, slowly broadening our focus to situate these ant-specific insights within a wider evolutionary and comparative framework.

Ant Queens: Not only a “Reproductive Unit”

This evidence regarding the queen's behavioral flexibility across the ants highlights the need to not regard ant queens as a one-dimensional reproductive unit as they are often portrayed, bound by their morphology. While it may be true in specific cases, such as in polygynous species with dependent-colony foundation only (e.g., *P. longicornis*), where queens are always part of the colony, in most ant species, queens must be adapted to dispersal and independent colony founding, which is the ancestral mode (Hölldobler and Wilson 1990). All three stages (dispersal, colony foundation, and reproduction) are of equal importance for the success of a colony. Therefore, reproductive potential in terms of morphology and behavioral specialization can only be maximized until it impacts the other two. Only when one of these three stages are removed can both morphology and behavior be further channeled into reproduction. Nevertheless, once reaching the reproductive stage, in at least monogynous colonies, it is likely safe to attribute the term reproductive unit to the queen. But in polygynous species, it might not be so simple due to the presence of multiple queens relaxing the need to maximize reproduction while introducing potential conflict over reproduction, which can lead to reproductive skews and dominance hierarchies (Bourke 1994; Bourke et al. 1997; Fournier et al. 2004; Ito et al. 1996).

Interestingly, it is not the first time that queens have been reported to be more behavioral flexible than just being reproductive. In two other cases, *Acromyrmex echinatior* and *Ectatomma vizottoi* (Nehring et al. 2012, 2; Vieira et al. 2012), unmated queens have been reported to express worker-like behaviors. These findings together with the results presented in this thesis highlight that morphology alone does not restrict queens in expressing certain behavior, but maybe mating status plays a role, as alluded to in chapter 2. Furthermore, as investigated in chapter 1, the social environment, consisting of worker presence or absence, also modulates queen behavior. For the effects of social environment, we were able to show in chapter 3, that in *L. niger*, key regulatory genes might play a role in the maintenance of specialization. These gene-regulatory tools include heavily studied genes, such as the

transcription factor *Kr-h1*, which has been shown to modulate caste-specific behavior (Gospocic et al. 2021; Chandra et al. 2018). In one example, it shapes the transition of a worker from non-reproductive to reproductive in a poneroid ant, showing that these tools can be evolutionary conserved (Opachaloemphan et al. 2023). Similarly, the insulin-like pathway has been associated with worker behavioral transition and, thus, it is not unsurprising to find this also potentially involved in the queen behavioral transition (Chandra et al. 2018).

Therefore, we demonstrate that in ants the evolution of the queen caste, shaped by diverse morphologies and life-history strategies, has similarly generated a wide spectrum of behavioral flexibility. These behaviors can be modulated by many internal and external factors and are governed by complex gene-regulatory networks. This pattern can be contrasted with, or compared to, other eusocial insects that also possess a morphological distinct queen caste.

Queen Behavioral Plasticity across the Eusocial Insects

Within the eusocial wasps and bees, which are also part of the Hymenoperta, we find a morphologically distinct queen caste. While less is known about the behavioral repertoire of the social wasp queens, they share similar life-history traits to the independent semi-claustral founding queens in the ants, as a single queen has to start the nest, forage for nesting material and food to raise the first larvae (Akre et al. 1976; Leathwick 1997). In comparison, in the bumble bee *Bombus terrestris*, behavioral plasticity, modulating brood care and egg laying behavior depending on the social environment has been shown (Woodard et al. 2013), and it might be similar in the wasps and hornets. Therefore, it seems these mostly annual systems show many similarities to the behavioral plasticity in ants.

The western honey bee *Apis mellifera* remains the best-studied eusocial insect species and a lot of attention has been paid towards the queen caste, focusing heavily on its role in reproduction (Hubhachen et al. 2025; Amiri et al. 2025; Holmes et al. 2025; Kama and Shpigler 2025; Smielevski De Souza et al. 2025). Honey bee queens only fly out to mate and to find another nest via swarming, akin to buddying, where parts of the workers leave with the queen. Similarly to chapter 2, mating status influences queen bee behavior, with unmated queens being more active (Kocher et al. 2010). While honey bee queens express unique behaviors, such as killing competitor queens with an adapted stinger, only once has an unmated individual been observed expressing foraging behavior (Floris et al. 2021). The inability of queen bees to raise broods on their own highlights that these, similar to highly specialized queens in the army ants, have lost their independence from the worker caste.

This aligns them with our results of polygynous queens not expressing any brood care, for example, in *Paratrechina longicornis*. Interestingly, in the Cape honey bee, the queen caste has been lost in favor of clonal workers, similarly to the clonal raider ant in the army ants (Mumoki et al. 2021; Ravary and Jaisson 2002). This hints that upon the loss of independence, the queen caste itself can be lost under specific conditions.

Lastly, we find a dedicated queen caste in the Isoptera, the termites. They go through a similar independent founding stage as the ants (Chouvenc 2023; Higa 1982). Research has shown in the lower termites, queen and king are not able to recover from worker removal (Chouvenc and Su 2017). Meanwhile, the termite queen can go through physiogastry, becoming the literal “egg-laying-machine” with a giant expanded gaster (Bordereau 1982), showing a morphological commitment to reproduction. Hence, until proven otherwise, it seems behavioral plasticity in the queen caste remains a trait of the Hymenoptera.

In summary, queen behavioral plasticity and, therefore, the description of the queen caste swaying away from a pure reproductive unit on multiple occasions, seems to be unique in the Hymenoptera, especially in the ants, due to the sheer number of eusocial species and life history traits they display (Keller 1991; Hölldobler and Wilson 1990).

Queen Behavioral Plasticity and Implications for Superorganisms

The ant colony is often regarded as a superorganism: an integrated biological entity in which the reproductive and non-reproductive castes are distinct, like soma and germline within a multicellular organism (Sanja Maria Hakala et al. 2019; Boomsma and Gawne 2018; Helanterä 2016; Wheeler 1911). This metaphorical analogy has been often used for understanding social insects remains controversial and the striking variability observed within the queen caste invites another reexamination of this analogy.

Both the queen and worker castes are derived phenotypes that arise from the same diploid genome. In holometabolous insects, the adult morphology is fixed at the end of pupation. Consequently, the queen’s morphological and behavioural repertoire must be optimized for every task she will ever perform. In most species this includes an initial phase of dispersal and independent colony founding, followed by a prolonged period of active reproduction (Hölldobler and Wilson 1990; Brown and Bonhoeffer 2003; Peeters and Ito 2001a; Tsuji and Tsuji 1996). The underlying molecular machinery, therefore, must accommodate two very different functional states, and it is at the interface of these states that the observed variability becomes most evident.

When a queen transitions from the foundress to that of the reproductive unit the high degree of optimization precludes a single, irreversible switch once workers have emerged. Strong worker signaling, well documented in *Lasius niger*, can constrain queen behaviour, yet flexibility remains advantageous under adverse conditions (Majidifar et al. 2024). The loss of the first worker would be fatal for a queen lacking behavioural plasticity, so queens that retain the capacity to adjust their actions are more likely to survive. Because workers share the queen's genome, queens can also express worker-like behaviors; for example, unmated queens of *S. pallipes* provide a striking example of such flexibility (Chapter 2).

The need for such flexibility relaxes, for example, via joining another nest, which would typically be a conspecific colony. In more derived polygynous species, mating may even take place within the natal nest, allowing queens to remain in their colony of origin (Pearcy et al. 2011; Keller and Passera 1992; Keller and Fournier 2002). In a few species, temporary social parasitism has evolved: queens invade host colonies, eliminate the resident queen, and take over the nest (Buschinger 1986; Iwai et al. 2021). Joining an already established colony, conspecific or not, may, in theory, require minimal behavioral investment from a queen compared to independent founding.

Yet, even among polygynous species, we observe a wide range of behavioral flexibility. One plausible explanation for this variation could be ecological factors. In stable environments with abundant resources and large, well-established colonies, selection may favor queens who specialize purely in reproduction. In more disturbed or high-mortality settings, however, flexibility becomes advantageous. Similarly, if queens reach their reproductive potential only at a certain age or can differentiate their brood, behavioral flexibility could be an advantage.

Lastly, queen morphology itself is remarkably variable across and sometimes within ant species (Peeters and Ito 2001b). In some lineages, ergatoid queens, worker-like in form, span a spectrum: from those that closely resemble workers to highly derived forms that have maximized fecundity (Heinze 1998; Barth et al. 2014; Brady and Ward 2005). The emergence of macro- and micro-queens, which differ in their colony-founding strategies, further underscores the adaptive diversity within the queen caste (Howard 2006; Negroni et al. 2021). This broad morphological and functional spectrum arises from variation in the underlying molecular machinery, both during development and in adulthood, and ultimately shapes the behavioral and physiological flexibility queens exhibit. In conclusion, ant queens do not always, or entirely, fit the description of a reproductive unit.

While Wheeler's superorganism concept initially faltered due to documented intracolony conflicts, it has reemerged through Boomsma's discussion of the term (Boomsma and Gawne 2018; Wheeler 1911): irreversible reproductive commitment (e.g., morphological

caste differentiation) minimizes conflict by aligning individual and colony-level fitness. Nevertheless, queen behavioral flexibility might allude to a further conflict or control over reproduction versus autonomy in the queen caste. Therefore, ant queens are not invariant reproductive units but context-dependent functional modules, a nuance essential to modern superorganism theory, where colony resilience *depends* on such plasticity. We reveal here that the “egg-laying machine” concept holds for certain species and life history periods, while revealing a far higher variability of behaviors and potential roles in the queen caste of ants.

Future Directions

This thesis demonstrates that queen specialization is not a rigid trait across ants but instead can be highly variable and flexible (Chapter 1). It further explores the underlying molecular framework, showing that queen gene expression can vary substantially depending on external factors, such as mating status (Chapter 2). Finally, queen behavioral flexibility is underpinned by differential expressions in well-studied regulatory pathways (Chapter 3). Here, I propose three complementary approaches to further strengthen these insights: one closely tied to the systems, data and methods established in this thesis; one leveraging well-studied polygynous models; and one requiring the development of new systems to test evolutionary convergence.

To further explore the evolutionary and mechanistic conservation of candidate genes identified in Chapter 3 (*Lasius niger*), it would be highly informative to re-analyze the transcriptomic data of *Stigmatomma pallipes* queens for homologs of these genes. While Chapter 2 focused on mating status (reproductive vs. non-reproductive queens) and Chapter 3 on social environment (worker presence/absence), both studies contrasted queens with divergent behavioral states: highly active/brood-caring queens versus low-activity/ reproductive queens. I hypothesize that if these regulatory pathways are evolutionarily conserved, they should also be detectable in *S. pallipes* queens, which exhibit obligate brood care regardless of social context. Notably, Chapter 3 showed that these candidate genes are not simply responsive to the presence of brood, but to the presence of workers. Moreover, given that both systems are now established, future experiments could probe their nuances, for example, by generating unmated *Lasius niger* queens or manipulating social environments in *S. pallipes* to test behavioral and transcriptional plasticity. Furthermore, it will be important to pursue the functional verification of these genes, as attempted in Appendix 1, to test their role in queen behavioural flexibility. Beyond RNA-seq and RNAi, complementary approaches like DNA methylation and histone modification, which have been shown to be important in worker task allocation (Kohlmeier et al. 2023; Oldroyd and Yagound 2021; Yan et al. 2015), would be promising avenues to explore in the context of queen behavioral flexibility.

To investigate the variability of queen behavioral flexibility in polygynous systems, we can leverage well-established models, such as *Monomorium pharaonis* and *Linepithema humile*, both of which have rich behavioral and genomic resources. Of particular interest would be to revisit the limitations identified in Chapter 1: for instance, the threshold of worker or larval density required to elicit behavioral shifts. By comparing gene expression across species that exhibit little to no brood care versus those with facultative or obligate brood care, we could refine the functional scope of the regulatory networks identified in Chapter 3. I hypothesize

that these networks are not lost in non-plastic species, but either decoupled from brood care entirely or constitutively active, rendering them insensitive to social changes.

Finally, a notable limitation of the above approaches is their focus on the formicoid clade, largely overlooking the poneroid clade, where low queen-worker dimorphism often coincides with polygynous social structures, as seen in *S. pallipes*. Investigating this parallel lineage could reveal whether queen behavioral specialization evolved via conserved molecular pathways or through convergent, lineage-specific mechanisms. A logical first step would be to re-analyze recently published transcriptomic datasets from poneroid species to identify potential homologs of Chapter 3 candidates. From there, one to three target species could be selected for in-depth behavioral and molecular characterization, ideally those exhibiting clear queen behavioral polymorphisms. Such comparative work would not only deepen our understanding of queen plasticity but also illuminate the broader evolutionary principles underlying eusocial caste evolution in the ants.

These efforts will advance our understanding of how queen behavioral flexibility emerges and is maintained across ant societies, a fundamental question in the evolution of eusociality.

Appendix 1: Workflow for RNAi knockdown of *dPDK1* in *Lasius niger* queens

Background

Transcriptomic-based analysis of *Lasius niger* queen brains identified 3-phosphoinositide-dependent protein kinase (dPDK1; gene Lnig_g11740) that was consistently upregulated when queens were in the presence of workers (Chapter 3). Because dPDK1 is involved in the insulin signaling pathway (Wick and Liu 2001; Storz and Toker 2002), which regulates division of labor in many eusocial insects (Wick and Liu 2001; Chandra et al. 2018; Ament et al. 2008; Chen et al. 2023b; Ferreira et al. 2025; Libbrecht et al. 2013; Okada et al. 2010; Weger and Rittschof 2024; Yan et al. 2022), we selected it to functionally validate its role in the regulation of queen behavioral specialization. To do so, we established step-wise procedures to arrive at a reliable RNAi-knockdown. This included several steps which needed to be established and whose methodology and results will be discussed here.

Step 1: Establishing a non-lethal injection protocol

Methods

To reliably inject the RNAi-probe into the queens, both injections and the resulting injury should be non-lethal and not hinder the expression of behavior. Furthermore, as we analyzed the expression of dPDK1 in the brain, the injection should be as close as possible. To evaluate injection protocol, we used 20 queens from established *L. niger* colonies collected from nuptial flights from 2022 in Mainz, Germany. One queen at a time was first chilled on ice for three minutes until motionless. Then it was immobilized on a piece of rubber, which was innervated by a 0.1 mm nylon thread. The nylon thread was lifted by gently squeezing the piece of rubber and the queen placed under the thread, in such a way that the head was fixated straight on the rubber and the loop was tightened behind the head capsule, which was enough to prevent displacement. A glass capillary (3.5" Drummond capillaries, Drummond, USA) was pulled on a capillary puller (Narishige PC-100, Narishige Group, Japan) to produce a tip of 10–15 µm diameter and subsequently mounted onto a micro-injector (Nanoject II, Drummond, USA), which was attached to a three-axis micromanipulator (Nanoject Micromanipulator, Drummond, USA). For perforation, a metal needle (Size 0# Globleland DE, Germany) was stabilized by affixing it between two blue Eppendorf pipette tips with a thin film of super-glue, creating a rigid “needle-holder” that prevents lateral drift during insertion. This needle was placed on top of the glass capillary.

Under a stereomicroscope, the needle was positioned mid-dorsally, roughly in the middle between the ocelli and the clypeus. Using the micromanipulator, a constant force was applied until the cuticle yielded (Figure 1 A). The metal needle was immediately retracted to minimize damage to the underlying tissue and removed from the micro-injector, revealing the glass capillary. Immediately, thereafter, the calibrated micro-injector was used to deliver 180 nl of phosphate-buffered saline (PBS) into the head capsule (Figure 1 B). Afterwards, the queens were returned to a glass tube partly filled with water and a cotton plug.

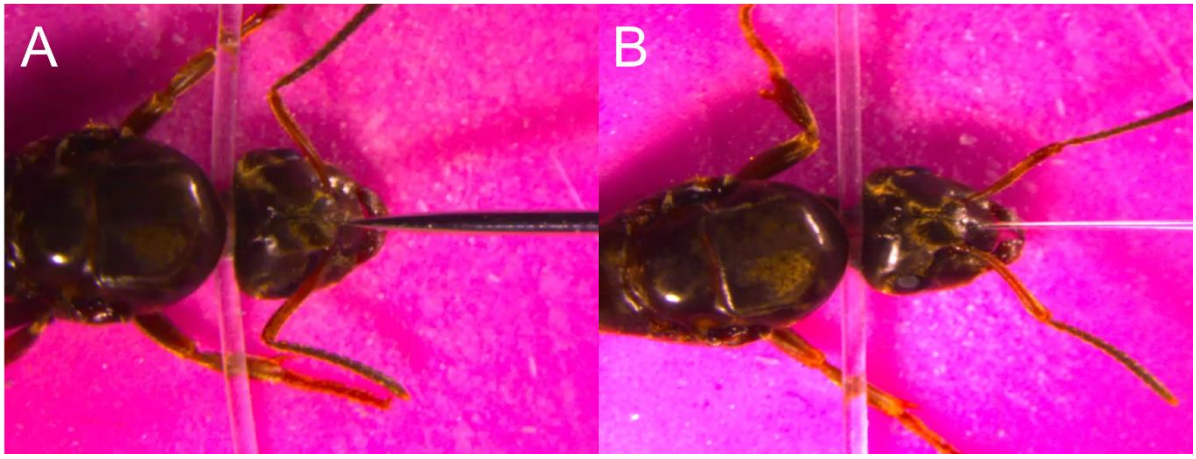


Figure 1: Nonlethal perforation and injection into the head of *Lasius niger* queens. Both images show a *Lasius niger* queen fixated between a piece of rubber and a nylon string. A) shows the perforation of the head capsule via a steel needle while B) shows the injection into perforation via a micro-injector needle.

Result

A pilot survival assay involved 20 queens that received a PBS injection and were maintained in a climate-controlled chamber (22 °C) post-injection. After 14 days, 19 queens were still alive (95 % survival), confirming that the injection procedure does not cause acute mortality and can be used for downstream RNAi experiments.

Step 2: Investigation into dPDK1 expression via injection and qPCR quantification

Methods

To test whether the injection itself perturbs dPDK1 expression, a separate set of 20 queens, from the same cohort received either an injection of 180 nl PBS or no injection (Step 1). Next, the queens were housed either with five workers and five larvae or with larvae only (five replicates per condition). After 24 h, brains were harvested and placed in 100 µl of TRIzoland

stored at –80 °C until RNA extraction (for details on dissection and RNA extraction see Chapter 2).

A total of 4 µl of RNA was used to generate complementary DNA (cDNA) via reverse-transcription with a QuantiTect Reverse Transcription Kit (Qiagen, USA). Quantitative PCR was carried out on a real-time cycler (Bio Molecular Systems Mic qPCR Cycle,Bio Molecular Systems, USA) using SYBR-Green master mix (Biozym Blue S'Green qPCR Kit Separate ROX) in 10 µl reactions. Three primer sets were used: a housekeeping pair for 18S rRNA (N_2_18s_F/R); a second housekeeping pair for elongation-factor-α (q_EA_Ln_F/R); and a gene-specific pair for PDPK1 that amplifies a region outside the RNAi target (q_PDPK1) (Table 1). All amplicons were around 80 base pairs.

Primer efficiencies were verified on a five-point dilution series (90–110 %). Each sample was run in technical triplicate, and no-template (negative) controls were included on every plate.

Relative expression was calculated using the $\Delta\Delta C_t$ method, normalizing each target C_t to the geometric mean of the two housekeeping C_t values. Statistical analyses were performed in R (v4.4) using linear mixed-effects models (lme4) with treatment, time point, and their interaction as fixed effects and batch as a random effect.

Table 1: Primers developed for RNA interference probe and quantitative PCR. For each primer pair, the name and sequence (5'-3') are provided.

Name	Sequence
A_PDPK_Ln_F	GCACCTTCCAGGACACCGATA
A_PDPK1_Ln_R	GTCTGGGTCTCCGTCCGA
T7PDPK1_Ln_F	TAATACGACTCACTATAGGGAGCACCTTCCAGGACACCGATA
T7PDPK1_Ln_R	TAATACGACTCACTATAGGGAGTCTGGGTCTCCGTCCGA
qPDPK1_Ln_F	GGTCGCGGTGAAGCCAAGAAGG
qPDPK1_Ln_R	GCCGCATCCTGATCTGCTCCG
N_2_18s_F	GCGCTACACTGAAGGAATCAGC

N_2_18s_R	TCATGGGGAACAATTGCAAGCC
qEA_Ln_F	GCTCGCCTTTACTCTCGGCGTC
qEA_Ln_R	TCGGAGTACGGAGGCTCGGTGG

Result

Queens injected with PBS displayed dPDK1 transcript levels indistinguishable from non-injected controls ($\Delta\Delta C_t$ difference = 0.12 ± 0.08, $p = 0.31$). The presence or absence of workers during the 24 h post-injection period also had no detectable effect ($p = 0.34$), indicating that the injection itself does not influence downstream expression analyses.

Step 3: Design and synthesis of the dsRNA probe

Methods

To design a suitable doubled stranded RNA (dsRNA) probe to knock down PDPK1 expression, the PDPK1 coding sequence from the newly assembled *L. niger* genome assembly was examined with AlphaFold (Jumper et al. 2021). The predicted active-site region (≈341 bp) was selected as the RNAi target. Previously generated cDNA was used as a template. Gene-specific primers (A_PDPK_Ln_F/R) were designed to amplify this fragment and to incorporate the T7 promoter at the 5′ end of each strand (T7PDPK1_Ln_F/R, Table 1).

An initial PCR using a Qiagen Multiplex PCR Kit (Qiagen, USA) amplified a product of ~500bp. A second, nested PCR with the T7-tailed primers, produced the 341 bp template for the dsRNA synthesis. Double-stranded RNA was synthesized using the MEGAscript™ RNAi Kit (ThermoFisher, USA), according to the manufacturer's instructions. The dsRNA stock was stored at –20 °C. A four aliquot was reversed transcribed into cDNA and sent for Sanger-based sequencing to confirm amplification target was correct (StarSEQ, Germany)

Result

Product integrity and size were confirmed on a 2 % agarose gel (Figure 2) and sequencing and blast againsz the dPDK1 sequence verified the correct target sequence (Table 2).

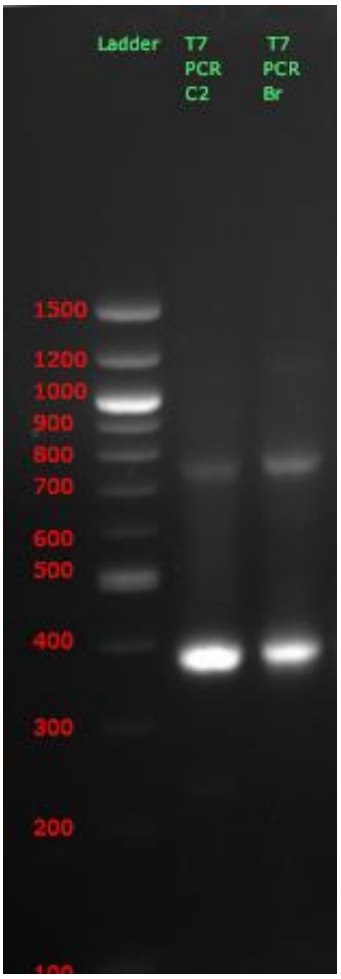


Figure 2: PCR product of nested PCR on the active site of dPDK1. Visualisation of PCR products (amplicons) on a 2 % agarose gel. For size comparison of amplified products, a 100 bp DNA ladder is shown in the first lane while second and third lanes show amplification of products of targeted size.

Step 4: RNAi knockdown

Methods

To investigate the functional role of PDPK1 on queen behavioral flexibility, we designed a knockdown experiment consisting of three batches running on successive days to control for temporal variation. Each batch comprised 32 queens from established colonies, distributed across four post-injection timepoints (6, 24, 48, 72 h). For each timepoint, four queens received dsRNA (treatment) and four received PBS (control), yielding a total of 96 queens (3 batches × 4 timepoints × 2 treatment × 4 replicates). Queens were randomly assigned to treatment groups using a random-number generator, and the order of injections was

alternated (every two queens for the 6 h group, every six queens for the later groups) to minimize systematic needle-swap effects.

Immediately after injection, each queen was placed in a Petri dish (50 mm diameter, nine mm height; Falcon, USA), which was layered with moist plaster, and contained five workers and five larvae. Petri dishes were positioned on a tray that could be moved beneath a camera (Sony FDR-AX33 Handycam, Sony Corporation, Japan) for behavioral recording. The tray layout was randomized daily to avoid spatial biases. At the designated post-injection interval, filming was performed for 1 h, after which, the queen was snap-frozen in liquid nitrogen and stored at –80 °C until dissection.

Brains were dissected and transferred to 1.5 ml tubes prefilled with 100 µl of TRIzol and store at -80 C until extraction. Total RNA was isolated and 4 µl aliquots were used to generate cDNA utilizing a reverse-transcriptase kit and PDPK1 was quantified via quantification PCR as described above.

Result

Across all three batches, the $\Delta\Delta C_t$ values for dsRNA-treated queens did not differ significantly from PBS controls (Figure 3). At 6 h, RNAi treatment had no significant effect on relative expression (β = 0.18 ± 0.15 SE, t = 1.21, p = 0.242). Similarly, at 24 h, the RNAi effect remained non-significant (β = –0.17 ± 0.13 SE, t = –1.32, p = 0.202). At 48 h, the treatment effect was essentially absent (β = –0.001 ± 0.13 SE, t = –0.006, p = 0.996), again indicating no measurable difference between groups. In contrast, at 72 h, RNAi significantly increased expression levels (β = 0.33 ± 0.14 SE, t = 2.37, p = 0.027), representing the only timepoint with a clear treatment response. Overall, RNAi produced no detectable expression changes during the first 48 hours, followed by a delayed up-regulation at 72 hours. Due to this, the behavior was not analyzed.

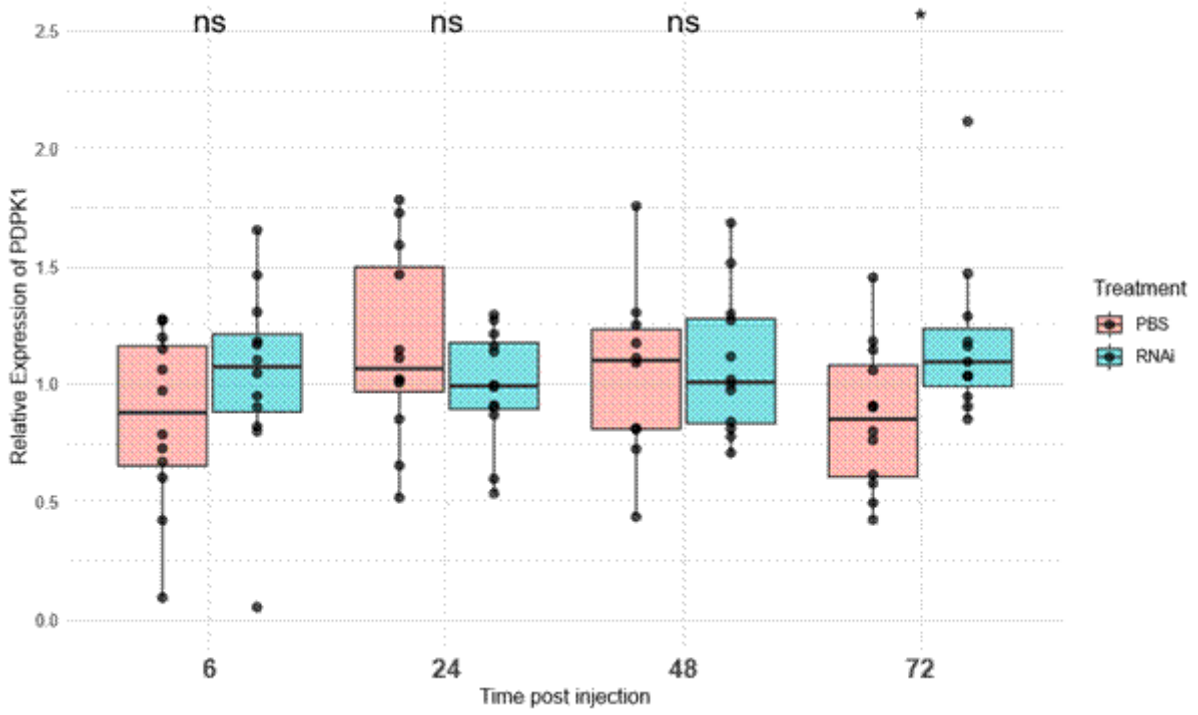


Figure 3: Relative expression of *PDPK1* after injection of PBS or RNAi probe across four timepoints (6 h, 24 h, 48 h, 72 h). Colored bars show mean expression ± SE, and black dots represent individual samples. Expression did not differ between treatments at 6 h, 24 h, or 48 h, but RNAi-treated individuals showed significantly higher expression at 72 h based on the results of linear mixed-effects model.

Discussion

We established a head-injection protocol for RNAi in *Lasius niger* queens and successfully generated a dsRNA probe targeting the active site of dPDK1. Despite confirmed probe integrity and delivery, no transcript knockdown was observed, with only a modest upregulation at 72 h, potentially reflecting compensatory feedback.

At the time of the RNAi experiment, it was unknown whether dPDK1 expression responds to social context, specifically, whether worker removal would trigger changes in its expression (as explored later in Chapter 3). Step 2 of the analysis, however, revealed that dPDK1 transcript levels remain stable even after worker removal, indicating that its expression is not socially regulated under these conditions.

RNAi in ants is typically achieved via feeding assays in workers, which allow higher, sustained but uncontrollable dsRNA exposure (Chen et al. 2023; Meng et al. 2020). Our direct head injection aimed to bypass the mass/dilution problem in queens, but without increasing dose or improving brain uptake, it was insufficient.

To further verify and improve the RNAi method in queens, one could: (i) increase the amount of RNAi probe delivered to account for queen size and dilution; (ii) confirm brain delivery using fluorescently labelled dsRNA or in situ hybridization; and (iii) investigate downstream pathways or compensatory regulators to assess whether the probe triggers biological feedback, helping to distinguish true functional knockdown from indirect effects.

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“You always meet twice in life”

Statement of the usage of generative AI

The author used generative AI (chatgpt/ki-chat.uni.mainz) to improve flow, grammar, spelling and wording in the thesis as well as generating and adjusting code. All ideas, projects, experiments and initial written text were generated by the author first. Text was always further adjusted and modified. The author verified all generated text.