Functional basis of the fecundity-longevity reversal in the ant *Temnothorax rugatulus*

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“Evolution has no long-term goal. There is no long-distance target, no final perfection to serve as a criterion for selection, although human vanity cherishes the absurd notion that our species is the final goal of evolution.”

— Richard Dawkins

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ZUSAMMENFASSUNG

Der Alterungsprozess ist eine evolutionäre Ungereimtheit. In den meisten Lebewesen findet ein „Trade-off“ zwischen „Life-history“ Merkmalen wie Lebensdauer und Fortpflanzung statt. In sozialen Insekten hingegen können Langlebigkeit und Fekundität miteinander einhergehen. Obwohl soziale Insekten dadurch ideale Modellorganismen zur Erforschung des Alterns und der Regulierung des „Life-history Trade-offs“ darstellen, sind viele Aspekte ihrer Lebendauer und Fekundität bislang unerforscht.

In meiner Arbeit habe ich Verhaltensbeobachtungen, experimentelle Manipulationen, molekulare Labortechniken und Transkriptomik angewandt, um die Grundlage und die Abhängigkeit von Langlebigkeit und Fekundität in der Ameise *Temnothorax rugatulus* zu untersuchen. Die Königinnenmorphologien dieser Spezies weisen unterschiedliche Fortpflanzungsstrategien auf. In **Kapitel 1** gebe ich neue Einblicke in den Zusammenhang zwischen den Merkmalen von Königinnen und Arbeiterinnen, der Zusammensetzung der Kolonie und Umweltfaktoren. Nach dem Verlust der Königin können Arbeiterinnen von *T. rugatulus* Eier legen, die sich zu Männchen entwickeln. **Kapitel 2 und 3** behandeln daher die Fertilität der Arbeiterinnen in Hinblick auf das Verhalten, die Entwicklung der Ovarien und die Genexpression. Außerdem weise ich auf die Rolle der Histonacetylierung bei der Regulierung von fertilitätsrelevanten Genen hin. **Kapitel 4** vertieft die epigenetischen Grundlagen der Fekundität der Königinnen, um festzustellen, ob die durch Eiwegnahme induzierte Erhöhung der Fekundität von Königinnen auf eine Genregulierung durch Histonacetylierung zurückzuführen ist. Mein Versuch der Herunterregulierung neu entdeckter fertilitäts- und langlebigkeitsrelevanter Gene war nicht erfolgreich. Die Ergebnisse aus **Kapitel 5** gehen daher näher auf die Herausforderungen, die mit RNAi-Interferenz einhergehen, ein. Nährstoff-sensitive Signalwege spielen eine wichtige Rolle bei der Regulierung von „Life-history“ Merkmalen. Daher befasst sich **Kapitel 6** mit dem Einfluss des Proteingehaltes der Nahrung auf die Langlebigkeit und die Fertilität von Arbeiterinnen in Abwesenheit von Königinnen. Hier zeige ich, dass Proteine die Lebensdauer der Arbeiterinnen verkürzen, während die Fertilität unbeeinflusst bleibt, und ziehe Parallelen zu dem, was bei solitären Insekten zu beobachten ist. Schließlich zeige ich in **Kapitel 7** mit Hilfe von Genexpressions-, Anreicherungs- und Wortsuchanalysen die Unterschiede in der Genexpression im physiologisch aktiven Fettkörper zwischen jungen Brutpflegerinnen und alten Erkunderinnen.

Insgesamt vertiefen meine Ergebnisse unser Verständnis für die Mechanismen, die der Verbindung von Langlebigkeit und Fekundität bei sozialen Insekten zugrunde liegen. Ich habe Gene identifiziert, die mit der plastischen Fertilität und dem Polyethismus bei Arbeiterinnen in Verbindung stehen. Zusätzlich mache ich auf die Relevanz der Ernährung für die Regulierung von Langlebigkeit und Fekundität aufmerksam. Darüber hinaus trägt meine Arbeit zu anderen Studien bei, die epigenetischen Modifikationen eine große Bedeutung für die Regulierung der phänotypischen Plastizität sozialer Insekten zusprechen. Ich zeige eine neue Rolle der Histonacetylierung, da diese bei der Erhöhung der Fekundität in *T. rugatulus* zu Tragen kommt. Letztlich setzen meine auf jüngsten Fortschritten bei molekularen Methoden und assoziierten bioinformatischen Analysen basierenden Ergebnisse die Grundlage für weitere Forschungen über die außergewöhnliche „Life-history“ sozialer Insekten.

SUMMARY

The process of aging is an evolutionary incongruity. Life-history traits such as lifespan and reproduction are traded-off in most living organisms. Social insects challenge this view as longevity and fecundity can be positively associated. Hence, social insects offer great opportunities to deepen our understanding of aging and the regulation of life-history trade-offs. Nevertheless, many questions remain to be addressed regarding the mechanisms that underlie the long and fecund lives of social insect reproductives.

For my thesis, I used behavioral observations, experimental manipulations, laboratory techniques, and transcriptomics, to characterize the functional basis of the fecundity-longevity reversal in the ant *Temnothorax rugatulus*. In this ant species, queen morph is associated with alternative reproductive strategies. I provide novel insights on the association between queen and worker traits, colony composition, and environmental factors in **Chapter 1**. Following the loss of their queen, *T. rugatulus* workers can start laying male-destined eggs. **Chapter 2** and **Chapter 3** characterize the modalities of worker reproduction in terms of behavior, ovarian development, and gene expression. Moreover, I suggest a role of histone acetylation in the regulation of genes associated with worker reproduction. The role of epigenetics in reproduction is investigated in the queen caste in **Chapter 4**, as I determine whether histone acetylation affects gene regulation to facilitate the experimental increase in queen fecundity induced by egg removal. My attempt to knock down newly-found genes associated with longevity and fecundity in workers was unsuccessful. However, results from **Chapter 5** bring attention to the challenges associated with RNAi-mediated gene silencing. Nutrient-sensing signaling pathways are major contributors to the regulation of life-history traits. Hence, **Chapter 6** focuses on the influence of dietary proteins on the survival and fecundity of workers under queenless conditions. I demonstrate a detrimental effect of proteins on worker survival, while worker fecundity remains unaffected, and draw parallels with what is found in solitary insects. Finally, I used gene expression, enrichment, and word search analyses to examine the molecular underpinnings of worker polyethism in **Chapter 7**. I reveal different transcriptomic signatures between young brood carers and old foragers in the fat body, a physiologically active tissue.

Altogether, my findings deepen our understanding of the mechanisms that underly the lack of longevity-fecundity trade-off in social insects. I identify genes that are associated with plastic reproduction and polyethism in ant workers. I also bring attention to the importance of nutrition for the regulation of longevity and fecundity. Moreover, my thesis contributes to the growing evidence that epigenetic mechanisms are of major importance for the regulation of social insect phenotypic plasticity. I propose a novel role of histone acetylation, as this epigenetic mark might alter gene expression in a way that allows both *T. rugatulus* queens and workers to increase their fecundity. In addition, my results open promising avenues for further research on the extraordinary life history of social insects by exploiting recent advances in molecular methods and associated bioinformatic analyses.

GENERAL INTRODUCTION

Marina Choppin

Aging and associated life-history traits

Aging can be defined as the intrinsic deterioration of an organism leading to decreased survival and fertility over time (Rose 1991). The fact that such a detrimental process has been maintained throughout evolution and is widespread across the tree of life makes aging an incongruity that still puzzles the scientific community. The "programmed death" theory argues that aging is adaptive because it regulates population size and speeds up generation turnover (Weismann 1889), allowing organisms to keep up with ever-changing environments. However, as senescence does not appear to be the main cause of death in the wild, this theory has been refuted (Kowald and Kirkwood 2016). Indeed, free-living organisms mainly die from extrinsic causes such as starvation, diseases, or predation (Hill et al. 2019). Hence, how long an organism lives negatively correlates with the risk of extrinsic mortality it faces, and animals that can fly or those of larger size generally live longer than terrestrial or smaller ones (Speakman 2005; Healy et al. 2014). The lack of old organisms in the wild suggests that the strength of natural selection decreases over time, a phenomenon described as "selection shadow" (Charlesworth 1994). From the concept of selection shadow emerged the first theory of aging: the "mutation accumulation" theory (Medawar 1952). Based on this theory, the declining force of selection with age allows the accumulation of deleterious allele mutations in the genome, leading to increased damage that ultimately causes senescence. Another aging theory, the so-called "antagonistic pleiotropy", states that some mutations with beneficial outcomes in early life stages might be detrimental in late life stages, but will not be selected against as they enhance the fitness of young individuals with reproductive potential (Williams 1957). Finally, the "disposable soma" theory proposes that, because resources are scarce, they must be allocated between body maintenance (soma) or

reproduction (germline) (Kirkwood 1977). The two latest theories introduce the concept of trade-offs, which is very important to understand the evolution of aging.

Survival and fertility represent two basic fitness components that are traded off in most organisms (Reznick 1985; Kirkwood and Rose 1991), conforming with the disposable soma theory. Not only do long-lived individuals produce fewer offspring, but an experimental arrest of reproduction extends lifespan in different species (Drewry et al. 2008; Flatt et al. 2008). When looking at organismal lifespan and reproduction, it appears relevant to consider body size, as larger animals usually live longer (Gillooly et al. 2005; Speakman 2005). This positive correlation between body size and lifespan has been attributed to the lower metabolic rate per gram of tissue of larger individuals (Gillooly et al. 2005; Speakman 2005) and this finding is central to the "rate of living" theory of aging (Pearl 1928; Sohal 1986). Yet, this theory faces several limitations (Stark et al. 2020), and other confounding factors like temperature or food intake make it challenging to investigate the relationship between lifespan and metabolism (Hulbert et al. 2004; Vaanholt et al. 2009). In addition, Baudisch (2011) suggests paying closer attention to the "pace and shape of aging" to rigorously compare how different species age. Another trait commonly assumed to be traded off with lifespan is immunity. Indeed, having a vigorous immune system can help fight diseases and infections, but inflammatory responses are energetically costly and can cause damage that shortens lifespan (Lochmiller and Deerenberg 2000; DeVeale et al. 2004). This has been experimentally demonstrated in various organisms including bumblebees (Moret and Schmid-Hempel 2000), fruit flies (Fabian et al. 2018; Yamashita et al. 2021), crustaceans (Little and Killick 2007), and house sparrows (Martin et al. 2003). Conversely, long-lived nematodes have more robust immune responses than short-lived ones (Amrit et al. 2010). Hence, there seem to be compromises between beneficial and detrimental effects of

immune responses that affect the aging process and might vary across species and/or life stages (DeVeale et al. 2004). Finally, in the last decade, researchers have brought attention to the concept of healthspan emphasizing that long lives do not necessarily mean healthy lives (Bansal et al. 2015) and that maximizing healthspan might be more relevant than maximizing lifespan (Hansen and Kennedy 2016; Quigley and Amdam 2021). Altogether, the aging process seems entangled with many life-history traits, and how organisms invest in these traits might depend on their phylogeny and ecology.

The study by Jones et al. (2014) sheds light on the immense diversity of mortality and reproduction patterns across the tree of life. Mortality rates are intuitively thought to increase over time, but they actually remain constant in some species of *Hydra* (Jones et al. 2014; Schaible et al. 2015). Surprisingly, some organisms even show negative senescence, which is characterized by a decline in mortality with age after reproductive maturity (Vaupel et al. 2004; Jones et al. 2014). In terms of fertility, most trajectories are bell-shaped and centered at young ages, but several species like the agave *Agave marmorata* show increased fertility over time (Jones et al. 2014). Besides, post-reproductive lifespans are not only found in humans but also in killer whales and some nematodes (Jones et al. 2014), making this trait more common than originally thought (Cohen 2004). Jones et al. (2014) also reveal the clustering of fertility and mortality patterns on broad taxonomic levels. These findings not only emphasize how different aging might look from one species to the other, but the diversity in demographic trajectories raises questions on the regulation of aging and associated life-history trade-offs, and whether aging regulatory mechanisms are conserved between different organisms.

Proximate mechanisms of aging

Because aging has broad social and economic implications, many studies have investigated its proximate mechanisms (Kirkwood and Austad 2000; Blagosklonny 2012), giving birth to the mechanistic theories of aging. The popular "molecular damage" theory claims that aging is caused by the unavoidable accumulation of molecular damage over time due to incomplete somatic repair (Kirkwood and Holliday 1979). Molecular damage can stem from mistakes in DNA-related processes like replication or transcription, but also biochemical errors and the production of reactive oxygen species (ROS) (Sun et al. 2016; Yousefzadeh et al. 2021). ROS are core elements of the "free radical" theory that considers them as the main source of molecular damage, also called oxidative damage (Harman 1955). Supporting this theory, the upregulation of superoxide dismutase and catalase enzymes positively affects lifespan in *Drosophila* fruit flies (Orr and Sohal 1994) and the nematode *Caenorhabditis elegans* (Melov et al. 2000; Sampayo et al. 2003). Although the relevance of this theory has been empirically demonstrated, an increasing number of studies minimize the central role of oxidative damage in aging and favor the molecular damage theory (Wickens 2001; Gladyshev 2014). Finally, the "hyperfunction" theory states that senescence is caused by an excess of protein synthesis resulting from growth (Blagosklonny 2006), but the significance of this theory has been highly disputed in the past years (Gems and De La Guardia 2013; De Verges and Nehring 2016; Gems 2022). Overall, it seems challenging to characterize the proximate mechanisms of aging because many interconnected biological processes contribute to senescence while leading to similar outcomes, e.g. cellular damage. Besides, the diversity of demographic trajectories seems to be reflected on the molecular level, as aging regulating mechanisms might differ across organisms. Hence, looking at conserved signaling pathways might provide a better framework to understand aging and associated life-history trade-offs.

The two highly conserved pathways target of rapamycin (TOR) and insulin/insulin-like growth factor 1 signaling (IIS) can detect nutrient intake (especially the amino acid methionine) and are associated with the regulation of many biological traits including longevity and fecundity (Kenyon 2005, 2010; Narasimhan et al. 2009; Efeyan et al. 2015). In insects, changes upstream IIS subsequently affect the production of downstream components such as juvenile hormone (JH) and ecdysteroids that are major regulators of development and physiology (Jindra et al. 2013; Li et al. 2019). However, surgical removal of the corpus allatum that produces JH extends lifespan in several insect species (Herman and Tatar 2001; Hodkova 2008), and reducing JH titers is sufficient for *Drosophila* flies to live longer (Yamamoto et al. 2013), pointing to the versatility of insect JH. These endocrine hormones also play a major role in life-history correlations and trade-offs (Zera and Harshman 2001). Indeed, JH contributes to vitellogenesis (Wyatta and Daveyb 1996; Sheng et al. 2011; Zhu et al. 2021), a process leading to the production of female-specific glycoproteins (vitellogenins) which are involved in egg maturation (Byrne et al. 1989). Hence, the upregulation of IIS benefits reproduction but negatively affects lifespan due to the pleiotropic effects of JH (Flatt and Kawecki 2007; Toivonen and Partridge 2009). The downregulation of TOR also extends lifespan in yeast (Powers III et al. 2006), worms (Hansen et al. 2007), and flies (Kapahi et al. 2004). And dietary restriction results in lifespan extension from yeast to humans (Fontana et al. 2010; Kapahi et al. 2017), which has been attributed to an inactivation of TOR leading to increased autophagy (Hansen et al. 2008; Minina et al. 2013; Madeo et al. 2015). Autophagy is a self-cleaning process conducted by the cells that plays an important role in aging (Rubinsztein et al. 2011). On the other hand, an excess of protein overstimulates TOR, leading to a shortening of lifespan (Simpson and Raubenheimer 2009). For these reasons, TOR has been described as a “key modulator of aging” (Johnson et al. 2013). Most above-

mentioned studies have performed experimental alterations of nutrient-sensing signaling pathways using short-lived model organisms such as mice, *Drosophila*, and *C. elegans*. However, despite the challenges that come with studying non-model species, the longer-lived eusocial insects have great potential to enrich our understanding of aging and the regulation of life-history trade-offs, as they appear to circumvent them at least partially.

The fascinating case of social insects

Eusociality is the most advanced form of biological complexity that has evolved so far (Nowak et al. 2010) and can be defined by three characteristics: cooperative brood care, overlapping generations of adults coexisting in the same nest, and reproductive division of labor among females (Michener 1969; Crespi and Yanega 1995; Nowak et al. 2010). Eusocial species include mostly insects, and eusociality evolved seven times in Hymenoptera (ants, bees, and wasps) (Wilson and Hölldobler 2005) versus only once each in Isoptera (termites) (Lo et al. 2000) and Thysanoptera (thrips) (Crespi 1992). Colonies of eusocial insects have been described as "superorganisms" wherein reproductives (queens, and kings in termites) represent the germline while non-reproductives (workers) constitute the soma (Wheeler 1911; Hölldobler and Wilson 2009). Eusociality thus adds another level of selection (the colony) that changes aging patterns compared to solitary organisms (Korb and Heinze 2004, 2021; Negroni et al. 2016). Long lifespans indeed strongly correlate with the evolution of eusociality in insects (Keller and Genoud 1997). As mentioned above, insect societies operate following a reproductive division of labor: queens monopolize reproduction while workers take over various tasks in the colony such as brood and nestmate care, guarding, and foraging (Hölldobler and Wilson 1990). Tasks are associated with distinct phenotypes (polyphenism), and queens highly differ from workers in many traits including size and

fecundity (Hölldobler and Wilson 1990). Polyphenism can even occur among workers of the same species (Wilson 1943). Queens and workers also show extreme lifespan differences as queens can live up to 30 years versus a few years maximum for workers (Keller and Genoud 1997; Promislow et al. 2022). Interestingly, female social insects share the same genome but evolve different phenotypes through developmental plasticity (Corona et al. 2016). Besides, epigenetics has repeatedly been suggested to explain the remarkable phenotypic plasticity displayed by social insects (Bonasio 2012, 2014; Herb 2014; Yan et al. 2014; Vaiserman 2015; Maleszka 2016; Opachaloemphan et al. 2018; Vaiserman et al. 2018) and an increasing number of empirical studies demonstrate the role of epigenetic mechanisms such as histone modification in the regulation of social insect traits (Bonasio et al. 2010; Spannhoff et al. 2011; Foret et al. 2012; Simola et al. 2013, 2016; Glastad et al. 2019; Libbrecht et al. 2020). However, the functional importance of DNA methylation in social insect genomes remains highly debated (Libbrecht et al. 2016; Glastad et al. 2017; Cardoso-Júnior et al. 2021).

Not only does the common longevity-fecundity trade-off appear to be missing in insect societies, but these two traits can be positively correlated (Parker 2010; Monroy Kuhn and Korb 2016; Negroni et al. 2016). Highly fecund queens live longer than less fecund ones in the ant *Cardiocondyla obscurior* for example (Schrempf et al. 2017). Moreover, workers can start reproducing following the loss of their queen in some ants (Heinze et al. 2002; Helanterä and Sundström 2005; Heinze 2008; Giehr et al. 2020b), wasps (Wenseleers et al. 2005), and bees (Oldroyd et al. 2001; Beekman and Oldroyd 2008; Princen et al. 2020), and fertile workers live longer than infertile ones (Hartmann and Heinze 2003; Kohlmeier et al. 2017; Negroni et al. 2020, 2021b; **Chapter 2**). How can queens achieve extraordinary long lifespans while retaining high reproductive potential throughout their long lives is still under investigation (Korb 2016; Law 2021). As

discussed previously, trade-offs commonly stem from resource limitation. However, queens are pampered from the emergence of their first workers so they might not have to compromise between reproduction and lifespan (Kramer and Schaible 2013). Although this would suggest that workers do face trade-offs, whereas they live relatively long lives compared to solitary insects and show positive effects of reproduction on lifespan. In addition to being protected by the nest, queens benefit from social immunity (Cremer et al. 2007) which might allow them to invest less in their immune system in favor of their reproduction and lifespan (Negroni et al. 2019). With the rapid advances of laboratory techniques and associated bioinformatic analyses, researchers have increasingly focused on the molecular underpinnings of the peculiar aging trajectories and life-history correlations observed in social insects. Interestingly, the free radical theory of aging does not seem to explain the lack of longevity-fecundity trade-off as long-lived castes do not consistently show increased antioxidant production (Parker 2010; Kramer et al. 2021), and no differences in telomere length were found between queens and workers of the ant *Lasius niger* (Jemielity et al. 2007). Supporting these findings, old termite queens do not show signs of senescence until they eventually die (Monroy Kuhn et al. 2021). And the seeming absence of selection shadow at old ages might come from late fitness gains via the production of sexuals in ant queens (Jaimes Nino et al. 2021). It has also been suggested that nutrient-sensing signaling pathways like IIS might operate differently in social insects (Pamminger et al. 2016; Rodrigues and Flatt 2016; Rüppell et al. 2016). Indeed, ovary activation correlates with high titers of ecdysteroids in honey bee queens (Robinson et al. 1991) and JH titers measured in ant queens of one *Diacamma* species were consistently low (Sommer et al. 1993). These findings challenge what is found in most solitary insects wherein JH primarily regulates vitellogenesis and thus fecundity. Besides, vitellogenins have an ancestral fecundity function as egg yolk precursors in most

insects (Amdam et al. 2003), but during social insect evolution, vitellogenin genes experienced multiple events of duplication, diversification, and subfunctionalization, and vitellogenin orthologues are now associated with caste differentiation (Feldmeyer et al. 2014; Morandin et al. 2014), behavior (Kohlmeier et al. 2018), oxidative stress resistance (Seehuus et al. 2006), and other anti-aging mechanisms (Parker 2010). Until recently it was unclear whether the changes in life history that social insects exhibit were associated with similar alterations in genes and pathways across different species of ants, bees, and termites. Gene expression results from a recent study revealed more species-specific mechanisms underpinning social insect aging and fecundity than originally thought (Korb et al. 2021). The authors also proposed the TI-J-LiFe (TOR/IIS – JH – Lifespan and Fecundity) network as a "conceptual framework" to better understand the regulation of aging and fecundity in social insects. Besides, the TI-J-LiFe network encompasses around 120 genes originally described in the solitary insect *Drosophila* (Flatt et al. 2005; Yamamoto et al. 2013), allowing comparisons with results from transcriptomic studies conducted in ants, bees, and termites. Despite extensive efforts to unravel the secrets of the long and fecund lifespans of social insect reproductives, experimental work remains challenging with non-model organisms and many functional aspects of longevity and fecundity are yet to be elucidated. Nevertheless, the great phenotypic plasticity in lifespan and reproduction displayed by both queens and workers offers the opportunity to better understand the unusual fecundity-longevity reversal that characterizes insect societies.

Biology of the study species

Temnothorax rugatulus is a small Myrmicine ant distributed in the Western South of the United States and the Northern part of Mexico. Ant colonies of this species can be found in cool forests of coniferous trees, inside rock crevices or under stones. Colonies contain

from around 50 to more than a thousand workers (Figure 1A) and are relatively easy to find, collect, and maintain under laboratory conditions, facilitating experimental manipulations. In this species, two queen morphs of different sizes occur (Figure 1B) and are associated with alternative reproductive strategies (Rüppell et al. 1998, 2001a; Negroni et al. 2021c; **Chapter 1**). The large macrogynes found their colony independently and reside as sole queens in their colony, while small microgynes are usually readopted by their mother colony and co-exist with other queens. In some colonies, both queen morphs co-exist (Rüppell et al. 2001a). Multiple-queen colonies containing microgynes are more often found at higher elevations (Heinze and Rüppell 2014), suggesting that microgynes are an alternative reproductive morph better adapted to difficult environmental conditions (**Chapter 1**). *Temnothorax* queens can live up to 20 years (Plateaux 1986; Keller and Genoud 1997), and because of this time constraint, I mostly used shorter-lived workers to answer my research questions. Indeed, worker lifespan varies between a couple of months to a couple of years in *T. rugatulus*. Also, despite not having spermathecas, workers of this species can start laying haploid, male-destined eggs in absence of the queen, and fertile workers live longer and show transcriptomic changes in the fat body (Majoe et al. 2021; Negroni et al. 2021b; **Chapter 2**). These two traits (shorter lifespans and inducible fecundity) make *T. rugatulus* workers very suitable to investigate the molecular underpinnings of fecundity and longevity in ants.

Scientific questions

The research I conducted for my thesis focused on the proximate mechanisms that regulate fecundity and longevity in the ant *Temnothorax rugatulus* (Figure 2). **Chapter 1** provides novel information on queen, worker, and colony traits using morphometrical

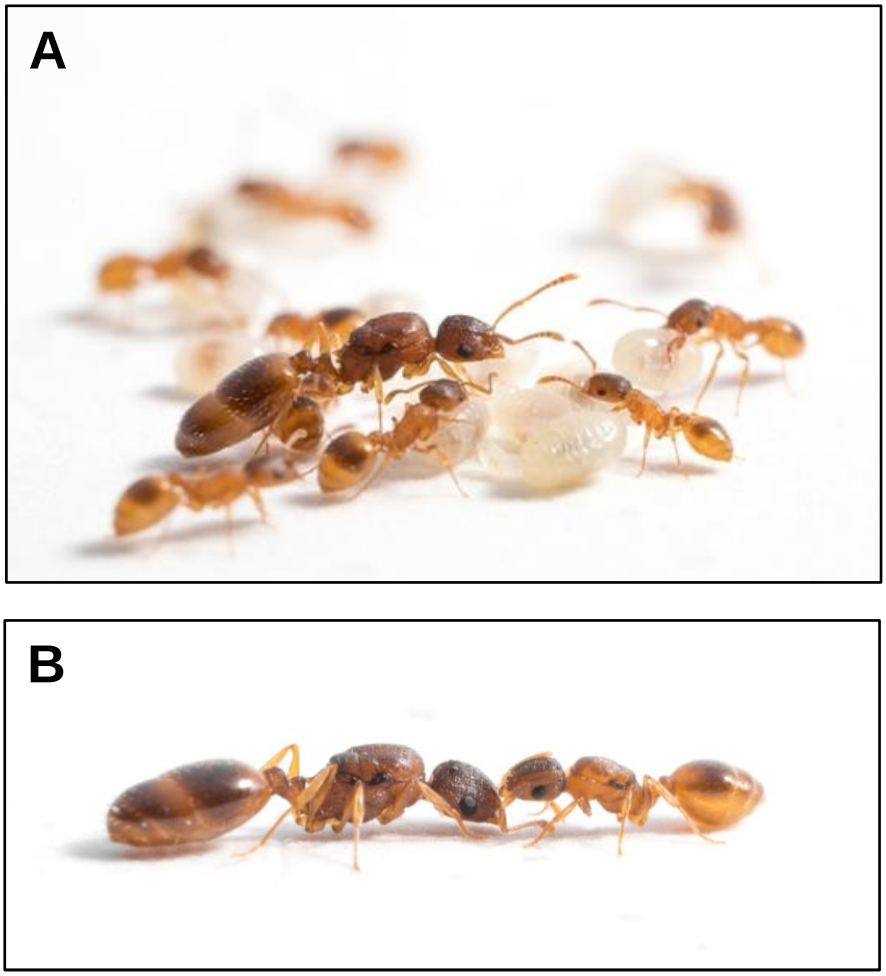

Figure 1. (A) Macrogyne queen with workers and larvae. **(B)** Two queens of *Temnothorax rugatulus* facing each other; one large macrogyne (left) and one small microgyne (right). Both pictures were taken by Dr. Romain Libbrecht.

measurements, behavioral observations, chemical assays, and demographic analyses. Results indicate that microgynes of *T. rugatulus* do not show parasitic tendencies as opposed to other ant species (Hora et al. 2005; Lenoir et al. 2010; Schär and Nash 2014), and suggest that microgynes of this ant species might be an alternative reproductive morph better adapted to difficult environmental conditions since they are found at higher elevations (Heinze and Rüppell 2014).

Because an increasing number of studies demonstrate that epigenetics contributes to the regulation of social insect phenotypic plasticity (Bonasio et al. 2010; Spannhoff et al. 2011; Foret et al. 2012; Simola et al. 2013, 2016; Glastad et al. 2019; Libbrecht et al. 2020), I investigated the role of histone acetylation in worker plastic reproduction in **Chapter 2**. As expected, workers in queenless colonies survived better and developed their ovaries, but their ovarian development was impeded when treated with chemical inhibitors of histone acetylation C646 and deacetylation Trichostatin A (TSA). Both queen removal and chemical inhibitor treatment altered the expression of many genes with longevity and fecundity functions, and workers in queenright colonies shared a large number of differentially expressed genes with C646-treated workers in queenless colonies. Hence, I suggest that histone acetylation might play a role in the regulation of genes associated with worker reproduction in ants.

In **Chapter 3** I characterized worker reproduction in greater lengths, using a similar setup to the first worker experiment described above. I monitored the establishment of worker reproductive hierarchy following queen removal using behavioral observations, assessed worker fecundity at the end of the experiment, and plan on associating gene expression patterns with histone acetylation profiles using cleavage under targets and tagmentation (CUT&TAG; Kaya-Okur et al. 2020). Worker aggressive interactions in queenless colonies peaked during the first three days following

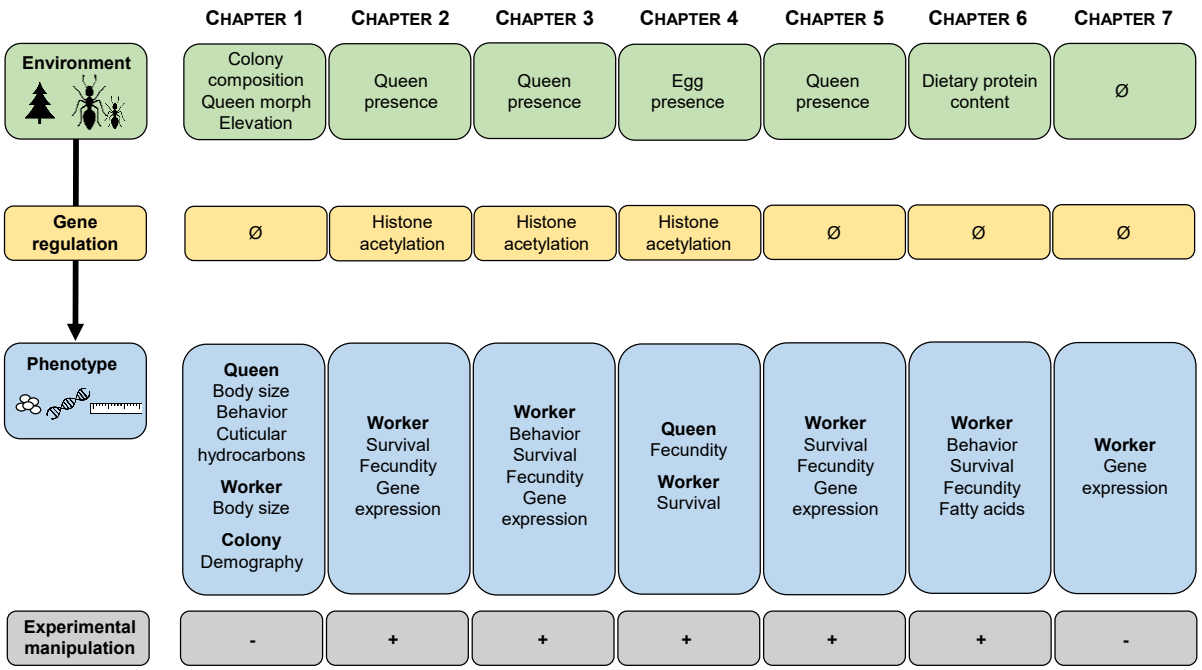

Figure 2. Changes in environmental factors can lead organisms to plastically adjust their phenotype in order to be better adapted to their novel environment. Such phenotypic plasticity requires changes in gene expression that are regulated by several molecular mechanisms, including epigenetic modifications. Social insects are great models of phenotypic plasticity. Thus, I took advantage of this phenotypic plasticity framework to investigate how life-history traits are regulated in the ant *Temnothorax rugatulus*, with an emphasis on fecundity and longevity. I summarized the content of each chapter in terms of environmental factors that were manipulated or not, associated traits that were measured, and potential regulatory mechanisms that were investigated.

queen removal. However, levels of aggression were higher and lasted longer in colonies treated with C646 and TSA, and worker ovarian development was lower in these colonies, as found previously. Thus, the two components of worker reproduction (aggressive behavior and ovarian development) might be regulated differently, like in the closely related species *Temnothorax longispinosus* (Konrad et al. 2012). Sequencing both RNA and histone-associated DNA from worker fat bodies will permit linking the expression of specific fecundity and longevity genes with different histone acetylation levels, confirming the role of this epigenetic mark in the regulation of worker reproduction.

I tested whether our results on the epigenetic regulation of worker reproduction could be extended to the queen caste in **Chapter 4**. Social insect queens live extremely long lives while retaining high reproductive potential (Keller and Genoud 1997; Korb 2016; Schrempf et al. 2017). I used egg removal to increase queen egg-laying (Schrempf et al. 2017; Negroni et al. 2021a) while feeding colonies the inhibitors of histone acetylation C646 and deacetylation TSA. Egg removal increased egg production as expected, but this plastic increase in fecundity was impaired by the chemical inhibitors of histone (de)acetylation, similarly to what was found in our worker experiments. Therefore, I propose that histone acetylation might facilitate the experimental increase of queen fecundity induced by egg removal via the regulation of specific fecundity genes.

In **Chapter 5** I aimed to validate the longevity and fecundity functions of candidate genes that were upregulated by fertile *T. rugatulus* workers in previous transcriptomic studies (Negroni et al. 2020, 2021b; **Chapter 2**). I fed whole colonies with dicer-substrate small interfering RNA (dsiRNA) to prevent workers from expressing the genes “alpha-tocopherol transfer-protein”, “transketolase protein 2”, and “vitellogenin 1” following queen removal. Workers in queenless colonies fed with dsiRNA still developed their ovaries. However, the fecundity-related gene “vitellogenin 1” was downregulated in

workers from colonies where dsiRNA was fed, including the positive control, pointing to a detrimental effect of siRNAs themselves.

Nutrient-sensing pathways like IIS or TOR contribute to the regulation of many life-history traits, including fecundity and longevity, in most organisms (Kenyon 2005, 2010; Narasimhan et al. 2009; Efeyan et al. 2015), pointing to an important role of nutrition in regulating reproduction and lifespan. I investigated how the dietary protein content affects ant worker traits in **Chapter 6**. Interestingly, the diet with a high protein to carbohydrate ratio (2:1) reduced worker survival but did not alter worker egg production. As evidenced by these results, ingesting too much protein might have life-shortening effects in social insect workers, similarly to what is found in solitary insects like *Drosophila*.

Finally, in **Chapter 7** I analyzed the fat body gene expression of nurses (brood-carers) and foragers. Worker task is associated with many factors including age in social insects (Wilson 1971). Thus, I expected to find differences in transcriptomic signatures related to age and/or task between the two worker groups. Foragers upregulated many genes associated with immunity and stress-response, which might be connected to both foragers' dangerous lifestyle outside the colony and their older age. Besides, several histone genes were upregulated in foragers, supporting recent findings on the role of histone acetylation in the regulation of worker foraging behavior in ants (Simola et al. 2016; Libbrecht et al. 2020). Altogether, these results add to the existing knowledge about the molecular mechanisms underlying worker polyethism.

CHAPTER 1

Queen and worker phenotypic traits are associated with colony composition and environment in *Temnothorax rugatulus*, an ant with alternative reproductive strategies

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Abstract

Alternative reproductive strategies are often associated with distinct morphological phenotypes. Some ant species display two queen morphs: larger queens (macrogynes) conduct mating flights followed by independent colony foundation, whereas smaller queens (microgynes) are readopted by their mother colony. In some cases, microgynes can evolve into social parasites that seek adoption into non-natal colonies. Here, we used morphometric measurements, behavioral experiments, chemistry, and demographic analyses to characterize queen alternative reproductive strategies in the ant *Temnothorax rugatulus* and question whether there is evidence for the evolution of social parasitism in microgynes. We show that body size is differently affected by colony composition in the two queen morphs. Interestingly, worker body size is also influenced by queen morph and colony composition, and the smallest workers are found in colonies with a single microgyne. Colony composition changes across collection sites, and colonies with microgynes are more frequent at higher elevations, suggesting that alternative reproductive strategies might be primarily associated with environmental conditions in this species. Behavioral experiments revealed a similar, low likelihood of both morphs to be accepted by non-natal colonies, which is consistent with microgynes being a non-parasitic, reproductive morph. This finding is corroborated by similar chemical profiles between queen morphs, which are again rather influenced by colony composition. Our study highlights the association between colony composition, environmental factors, and queen dimorphism, giving more insights into the evolution of alternative reproductive strategies in ants.

Introduction

Social insects are great models for the study of intraspecific polymorphism. Societies of ants, bees, and termites exhibit a reproductive division of labor, where the queens represent the main reproductive caste, while workers perform all other necessary tasks for the colony such as foraging, guarding, and brood and nestmate care (Hölldobler and Wilson 1990). Reproductive and non-reproductive individuals display strong phenotypic differences, the queens being typically winged and larger than the workers. In most social insects, caste is not genetically determined but arises from phenotypic plasticity (Corona et al. 2016). Indeed, environmental influences during development such as different temperatures, food quality or quantity affect caste determination (Libbrecht et al. 2013a; Berens et al. 2015). Interestingly, beyond the typical differences between queens and workers, polymorphisms can also occur within the queen or worker caste, shedding light on the evolution and maintenance of alternative phenotypes (Ribeiro et al. 2006).

In ants, polymorphism within the queen caste is often associated with alternative reproductive strategies (Rüppell and Heinze 1999; Keller and Heinze 2000; Wolf and Seppä 2016). Queens differ in traits associated with reproduction and dispersal like fat content, body size, or wing presence (Hakala et al. 2019). In some ant species, queen size is bimodally distributed. The larger queens (macrogynes) are provided with sufficient body reserves to found their colony independently, whereas the smaller queens (microgynes) seek readoption into their mother colony, and lay eggs alongside the other queens (McInnes and Tschinkel 1995; Rüppell et al. 1998). In the latter case, workers assist queens during the dependent colony foundation, and the colony reproduces by budding or fission (Peeters and Molet 2009). In other ant species, primarily wingless queens evolved to not disperse, but mate within or near their mother colony (Foitzik et al. 2010; Peeters 2012). These strategies of dependent colony foundation have evolved

repeatedly in ants (Cronin et al. 2013). They maximize queen survival by avoiding the risky solitary founding phase (Molet et al. 2009), which can be advantageous in habitats where resources or nest sites are limited (Heinze 1993; Bourke and Heinze 1994). Besides, microgynes can evolve a parasitic strategy, by seeking adoption into non-natal colonies headed by macrogynes and exploiting their workforce (Foitzik and Heinze 1998; Keller and Heinze 2000). In some cases, speciation facilitates further adaptations including the loss of the worker caste and the evolution of true inquilinism, a form of obligate social parasitism (Heinze and Buschinger 1989; Savolainen and Vepsäläinen 2003; Leppänen et al. 2015; De La Mora et al. 2020).

In our model species *Temnothorax rugatulus*, two queen morphs are associated with alternative reproductive strategies (Rüppell et al. 2001a, 2002). The large macrogynes predominantly found their colony independently, benefiting from higher fat content, and mostly residing as the sole queens in monogynous colonies. The small, worker-sized microgynes are instead seeking readoption into their mother colonies, and are thus commonly found in polygynous colonies with up to several dozens of queens. Most colonies contain queens of a single morph, but mixed colonies with both macrogynes and microgynes occur and make up about 8% of the colonies in the wild (see methods). Microgynes exhibit a similar egg-laying rate as macrogynes in small colonies, which is most likely mediated by their higher metabolic rate, which they maintain by being fed more often by the workers (Matteo Negroni, pers. comm.). In mixed colonies, microgynes produce proportionally fewer workers and more sexual offspring than macrogynes (Rüppell et al. 2002), a trait which has been associated with social parasitism before (Hora et al. 2005; Schär and Nash 2014).

In this study, we enrich previous work on queen alternative reproductive strategies in *Temnothorax rugatulus* (Rüppell et al. 1998, 2001a, b, 2002; Heinze and

Rüppell 2014) by adding novel information on queen, worker, and colony traits. Using morphometric measurements, we analyzed how queen and worker body size varies with colony composition. We then investigated an association between queen alternative reproductive strategies and environmental conditions. We predicted that microgynous colonies would be prevalent at higher elevations since microgynes use dependent colony foundation. Besides, we used readoption experiments to investigate whether microgynes are more likely to successfully intrude non-natal colonies. Such a tendency would be indicative of early steps towards the evolution of a parasitic strategy, despite the lack of genetic differentiation between the two queen morphs (Rüppell et al. 2001a). Finally, we extracted and analyzed the cuticular hydrocarbons from queens of the two morphs, which have been shown to differ between social morphs in *Solenopsis invicta* (Keller and Ross 1998), workers from different social origins in *Formica selysi* (Meunier et al. 2011) or social parasites (Nehring et al. 2015; Kleeberg et al. 2017).

Methods

Ant collection and maintenance

Temnothorax rugatulus is a small ant species distributed throughout the western part of North America. These ants inhabit high elevation coniferous forests, residing mostly in rock crevices or under stones. In August 2018, 857 colonies were collected from nine different locations in the Chiricahua Mountains (Arizona, USA; Table S1a). The ant species was identified by Susanne Foitzik in the field and later confirmed in the laboratory using a determination key (MacKay 2000). In the laboratory, each colony was kept in a box (9.7 × 9.7 × 2.9 cm) with three chambers connected by holes. Each colony was provided with a microscopic slide nest covered with a red foil to block the light and a lid. Ant colonies were maintained at 21 °C and 70% humidity with a 12:12 light:dark cycle and fed weekly

with half a cricket and honey. To increase ants' activity for the behavioral experiment (see below), the ants were moved to 25 °C and 70% humidity with a 12:12 light:dark cycle. These colonies were fed weekly during the experimental period with honey and an artificial diet composed of honey, eggs, agar, and crickets.

Morphometric measurements

After transfer to the laboratory, the number of workers in each colony was counted, and the head width, thorax width, and thorax length of all queens from all colonies (N = 2227 individuals) were measured. Queens were assigned to the macrogyne or microgyne morph based on their thorax width and additional criteria (see online supplementary material). Additionally, the head and thorax width of two nurses (i.e., workers close to the brood) and two foragers (i.e., workers collecting food) from twelve colonies of each colony morph (macrogynous, microgynous) and social structure (monogynous, pure polygynous) were measured, using a full factorial design (N = 192 individuals). Head width is the most used proxy for body size in workers. Measuring the thorax width was also interesting since macrogynes and microgynes strongly differ in this trait. Photos were taken of live ants, immobilized in modeling clay (Play-Doh) on a wooden ball under a Leica stereomicroscope (magnification × 20), and measures were done using the Leica software LAS v4.5. The following size index by Rüppell et al. (1998) was used as a proxy for queen body size:

$$\text{size index (mm)} = \frac{\sqrt{\text{thorax length} * \text{thorax width}} + \text{head width}}{2}$$

In their natural environment, queens of the two morphs occur by themselves in monogynous colonies or in polygynous colonies with other queens of their own morph. Occasionally, they also occur in large, mixed colonies with queens of both morphs. Thus,

the influence of queen morph (macrogyne, microgyne) in interaction with colony composition (monogynous, pure polygynous, mixed polygynous) on queen body size was investigated using a linear mixed-effects model with the R package "lme4" (Bates et al. 2015). Post-hoc Tukey comparisons were then carried out using the package "multcomp" (Hothorn et al. 2008). Linear mixed-effects models were also used to investigate the influence of colony morph (macrogynous, microgynous), social structure (monogynous, pure polygynous), and their interaction on worker head and thorax widths. Colony identification (ID) was used as a random factor in all models to account for inter-colony variability. The models' fit was assessed using visual inspections of the residual distributions. Alpha was set at 0.05 for all statistical tests. All analyses were conducted in R v3.5.1 (R Core Team 2020).

Demographic analyses

To address whether the ecological background influences *Temnothorax rugatulus* colonies, the data were combined with additional data from a previous collection trip in August 2015, when 557 ant colonies were collected at 15 sites throughout the Chiricahua Mountains (Arizona, USA; Table S1b). The number of colonies of each composition is summarized in Table S2. First, the collection site influence on colony composition (monogynous macrogynous, polygynous macrogynous, monogynous microgynous, polygynous microgynous, and polygynous mixed) was investigated using a Chi-square test. Then, the effect of the collection sites' elevation on the proportion of colonies containing microgynes, as well as the proportion of polygynous colonies, was tested using linear models. Collection sites with fewer than eight colonies were removed from the models to avoid unreliable proportions. Differences in the number of workers per queen depending on the colony morph (macrogynous, microgynous) were also investigated

using a linear mixed-effects model with the collection site as a random factor. Here, the terms macrogynous and microgynous colonies encompass both monogynous and polygynous colonies. The models' fit was assessed using visual inspections of the residual distributions.

Behavioral experiments

Readoption experiments were carried out to test the microgynes' ability to successfully intrude non-natal colonies, in comparison with macrogynes (Figure S1). Initially, 32 source colonies containing queens of both morphs were used. From each source colony, a macrogyne and a microgyne were tested. The tested queens were marked with wire loops (0.02 mm Elektrisola, Eckenhagen, Germany) between the petiole and post-petiole. Each queen was tested for each of four host colony compositions, one after the other in a pseudo-randomized order. The queen was confronted with (i) her natal colony, (ii) a non-natal monogynous colony with a single macrogyne, (iii) a non-natal polygynous colony with multiple macrogynes, and (iv) a non-natal polygynous colony with multiple microgynes. In total, 20 host colonies of each colony composition were used. Host colonies with a single microgyne could not be used because of their limited number.

The host colony was placed in the left chamber of a three-chambered box. The left chamber (2.9 × 9.7 cm) served as an arena for the experiment and was isolated from the rest of the box using tape to cover the connecting hole. The tested queen was placed about 1 cm in front of the nest entrance of the host colony using clean forceps. Scans were performed every five minutes during the first hour following the start of the trial (N = 12 scans per trial). For each scan during the first hour, the location of the queen (inside the nest, outside the nest) and the type of interaction between queen and workers (aggression, grooming) were reported. After 24 hours, the location of the queen and

whether she was alive or not was reported, before returning the queen to her colony. Each queen had a 5-day break in between each trial. It was not possible to collect the data blind since queen morph can be identified visually. Three source colonies were removed from the experiment because the queen died before being tested or lost her wire (final N = 29). Because the queens were often mutilated by the workers, the death rate was higher than expected, and the final number of trials was N = 64 for the macrogynes and N = 42 for the microgynes (Table S3).

First, the likelihood of queens of both morphs to be accepted by their natal colony compared with a non-natal one was investigated. Generalized linear mixed-effects models (GLMMs, binomial family) were used to test for the effect of colony origin (natal, non-natal) on (i) the queen location after one hour and the proportion of scans where at least one (ii) aggression or (iii) grooming event occurred during the first hour. Queen ID was always used as a random factor because each source colony provided one queen of each morph. The GLMMs were tested for overdispersion using the package "DHARMa" (Hartig 2020). The survival and location of the queen 24 hours after being introduced to the host colony was tested using a Fisher test, because the survival and success rates of queens entering their natal colony were 100% for this analysis and thus, binomial models did not converge. Dead queens were removed from the data set when analyzing the effect of colony origin on the location after 24 hours.

Similar binomial models with queen ID as a random factor and model inspection methods were used to investigate whether the two queen morphs had different likelihoods of successfully intruding non-natal colonies, and whether the composition of these non-natal colonies played a role. The effects of queen morph (macrogyne, microgyne), colony composition (monogynous macrogynous, polygynous macrogynous, and polygynous microgynous), and their interaction were tested on (i) the queen location

after one hour and the proportion of scans where at least one (ii) aggression or (iii) grooming event occurred during the first hour and queen (iv) survival and (v) location 24 hours after the introduction to the host colony. Only non-natal colonies were kept as host colonies here. Using colony composition as a single response variable was the only way to analyze the data rigorously since the experimental design was not fully factorial, due to the lack of monogynous microgynous host colonies. Additionally, none of the queens could be used for all the four trials as originally planned due to high mutilation and death rates. Consequently, host colonies were used variably, between one and four times. Thus, independent data sets were created to test for the effects of colony origin and queen morph in interaction with colony composition, where only the host colonies used once were kept and one trial for the host colonies used multiple times was randomly selected. The order of trial was included as a covariate in all the models to test for a potential effect on the queens' performance, and then removed since no significant effect was found.

Chemical analyses

Cuticular hydrocarbon (CHC) profiles of macrogynes and microgynes were analyzed using two separate data sets: (A) CHC profiles of ten macrogynes and ten microgynes from the same mixed colonies, and macrogynes from ten pure macrogynous colonies (data collected in September and October 2019) and (B) CHC profiles of ten microgynes and ten macrogynes from pure colonies (data collected in February 2020) (Table S4). Due to potential experimenter and biological biases, the analysis of these data sets was done separately.

For both data sets, gas chromatography-mass spectrometry (GC-MS) was used to determine whether the cuticular hydrocarbon profiles of macrogynes and microgynes differed. After spending two weeks under the same conditions (25 °C and 70% humidity

with a 12:12 light:dark cycle and fed weekly with honey and half a cricket), the queens were individually frozen in glass vials and stored at -20 °C. To extract the CHCs, each ant was covered in n-hexane for ten minutes and a standard (100 ng n-octadecane solved in 10 µl n-heptane) was added for absolute quantification of the CHCs. The extracts were transferred to micro inserts, evaporated under a nitrogen stream, and injected into a gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) coupled to a mass selective detector (5975C, Agilent). A Zebron Inferno DB5-MS capillary column (length 30 m, diameter 0.25 mm, 0.25 µm coating, Phenomenex Ltd, Aschaffenburg, Germany) was used as stationary phase and helium with a flow rate of 1.2 ml / min as a carrier gas. The temperature was initially at 60 °C, then increased to 200 °C at 60 °C / min and finally to 320 °C at 4 °C / min, where it was kept constant for ten minutes. The mass spectrometer had an ionization voltage of 70 eV and fragments were scanned 40 to 550 m / z. Manual integration of the derived peaks was conducted using the software MSD ChemStation E02.02 (Agilent 2008). The integrated peaks were then manually aligned, and hydrocarbons were identified using their retention index as well as diagnostic ions in Microsoft Excel.

One outlier was removed in each data set because their CHC composition was strongly deviating from all the other samples in multivariate analysis plots, and was thus considered an artifact. Differences between CHC profiles of the queens were tested with a permutational MANOVA (999 iterations) using the programs PRIMER 6 (v6.1.14; Clarke and Gorley 2006) and PERMANOVA+ (v1.0.4; Anderson et al. 2008) (both Primer-E Ltd, Plymouth, England). Queen morph was used as a fixed factor in both the analyses of the data set A (macrogyne, microgyne) and the data set B (macrogyne pure colony, macrogyne mixed colony, and microgyne pure colony). For the analysis of the data set A, colony ID was used as a random factor and pairwise tests were carried out. Using the data

set A, differences in the proportion of alkanes between the two queen morphs were additionally investigated using a linear mixed-effects model with colony ID as a random factor. The model fit was assessed using visual inspections of the residual distributions.

Results

Morphometry

We calculated a size index according to Rüppell et al. (1998) as a proxy for queen body size and obtained a clear bimodal distribution (N = 1784 macrogynes, 0.819 mm ± 0.034 mm; N = 387 microgynes, 0.697 mm ± 0.031 mm; Figure 1). Body size changed depending on queen morph (X^2 = 2563.029, df = 1, $p < 0.001$) and colony composition (X^2 = 130.602, df = 2, $p < 0.001$), and we found a significant effect of the interaction between the factors (X^2 = 58.656, df = 2, $p < 0.001$; Figure 2 and Table S5). Macrogynes from multiple-queen colonies showed a reduction in body size compared with macrogynes from single-queen colonies (z = -9.387, $p < 0.001$) and were even smaller when sharing their colony with microgynes (z = -9.401, $p < 0.001$). Interestingly, a different pattern was observed in microgynes, which were significantly smaller in monogynous (z = 2.770, $p < 0.05$) and pure polygynous colonies that contained only microgynes (z = -3.340, $p < 0.01$), compared with mixed colonies with both morphs.

Worker head and thorax widths were highly correlated (F = 289.7, df = 1, r^2 = 0.602, $p < 0.001$; Figure S2). We found a significant effect of social structure on worker head width (X^2 = 8.020, df = 1, $p < 0.01$), and workers from polygynous colonies had larger heads compared with workers from monogynous colonies. Workers from polygynous colonies also tended to have larger thoraces (X^2 = 3.699, df = 1, p = 0.054). We then compared thorax width of workers from macrogynous and microgynous colonies and found a strong effect of colony morph on thorax width (X^2 = 20.318, df = 1, $p < 0.001$), and

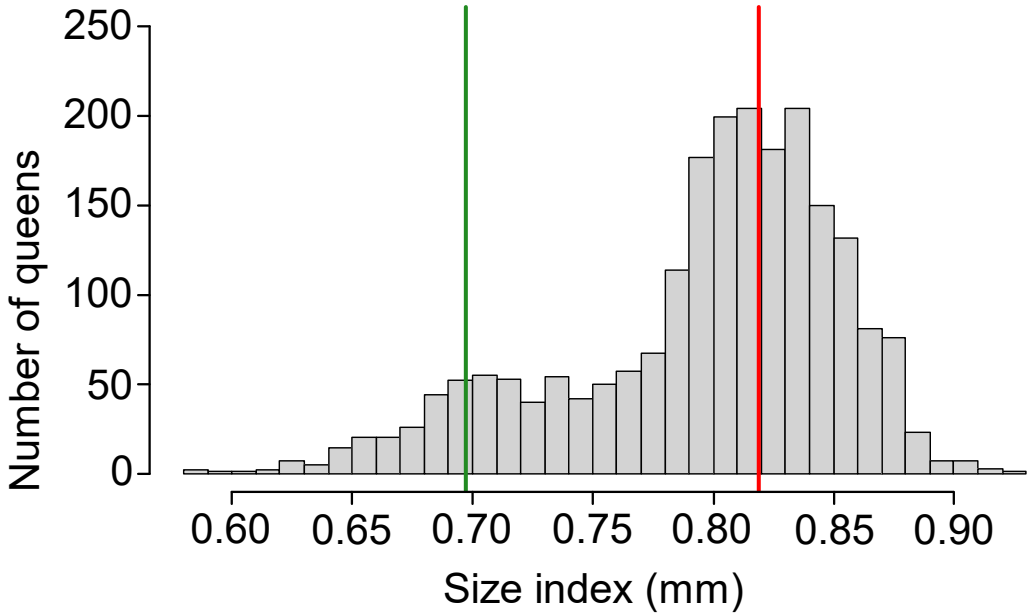

Figure 1. Queen body size distribution in *Temnothorax rugatulus* showing the mean body size of macrogynes (red) and microgynes (green).

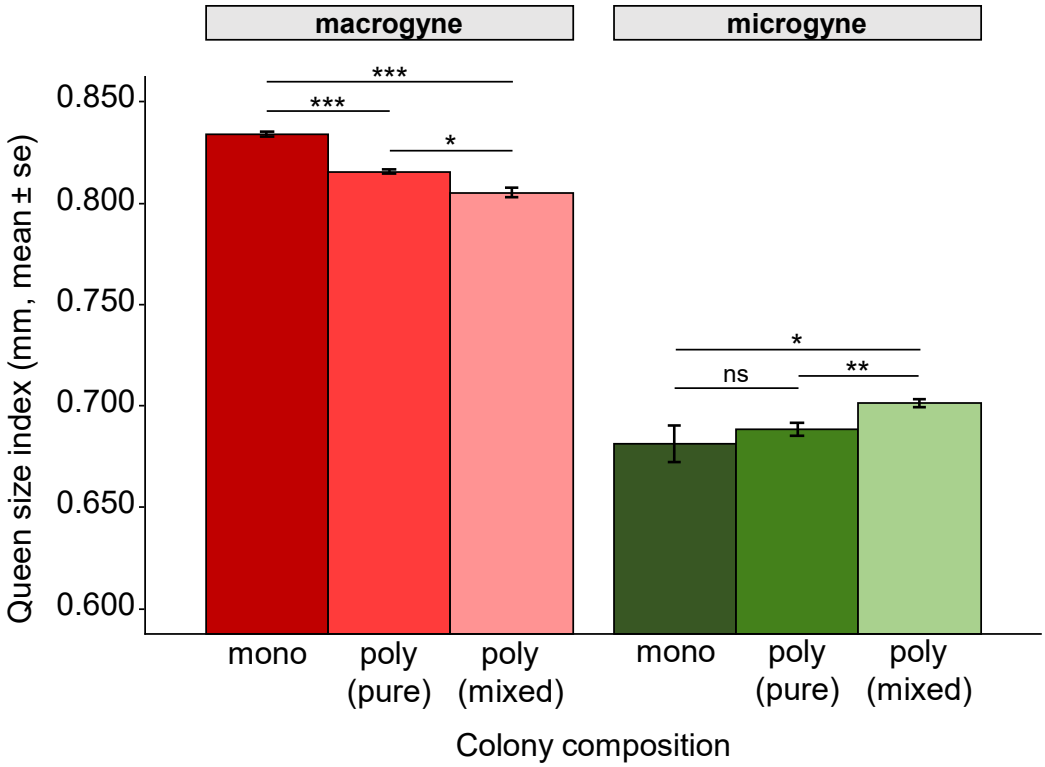

Figure 2. Interactive effects of queen morph and colony composition (mono = monogynous and poly = polygynous) on queen body size. The levels of significance are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. To improve readability, only the significance of intra-morph comparisons is provided.

workers from microgynous colonies, that is, produced by microgynes, had smaller thoraces than those from macrogynous colonies. We also detected a significant interaction between social structure and colony morph on thorax width (X^2 = 5.671, df = 1, p < 0.05; Figure 3 and Table S6). Workers produced by microgynes had larger thoraces in polygynous compared with monogynous colonies (z = 3.044, p < 0.05), while social structure did not influence worker thorax width in macrogynous colonies.

Demography

The Chiricahua Mountains in Southeastern Arizona rise to 2976 meters and *Temnothorax rugatulus* colonies reside in rock crevices on the harsh mountaintops as well as in the valleys. First, we tested whether colony composition changes with the site of collection and did find differences between sites (Pearson's Chi-squared test, X^2 = 366.09, df = 56, p < 0.001; Figure S3). Then, we tested whether the elevation of the collection sites had an influence on queen morph and colony composition in *T. rugatulus* colonies. Indeed, the proportion of colonies containing microgynes increased with elevation (F = 6.116, df = 1, p < 0.05; Figure 4). However, we did not find an effect of elevation on the proportion of monogynous versus polygynous colonies (F = 3.249, df = 1, p = 0.102). Finally, queens from microgynous colonies had fewer workers compared with queens from macrogynous colonies (X^2 = 33.82, df = 1, p < 0.001).

Behavior

First, we tested whether queens, regardless of their morph, were more likely to be accepted by their natal colony than by a non-natal colony. After one hour, queens were more often found inside the nest of their natal colony compared with non-natal colonies (X^2 = 4.712, df = 1, p < 0.05; Figure 5). They were also less often attacked (X^2 = 6.938, d =

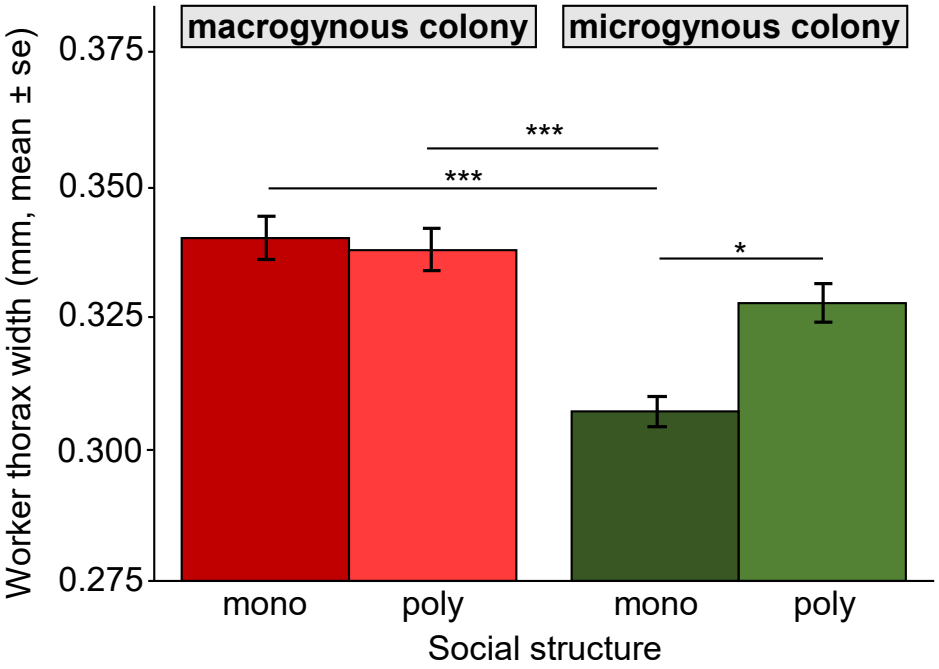

Figure 3. Interactive effects of colony morph and social structure (mono = monogynous and poly = polygynous) on worker body size. The levels of significance are indicated as follows: * $p < 0.05$, *** $p < 0.001$.

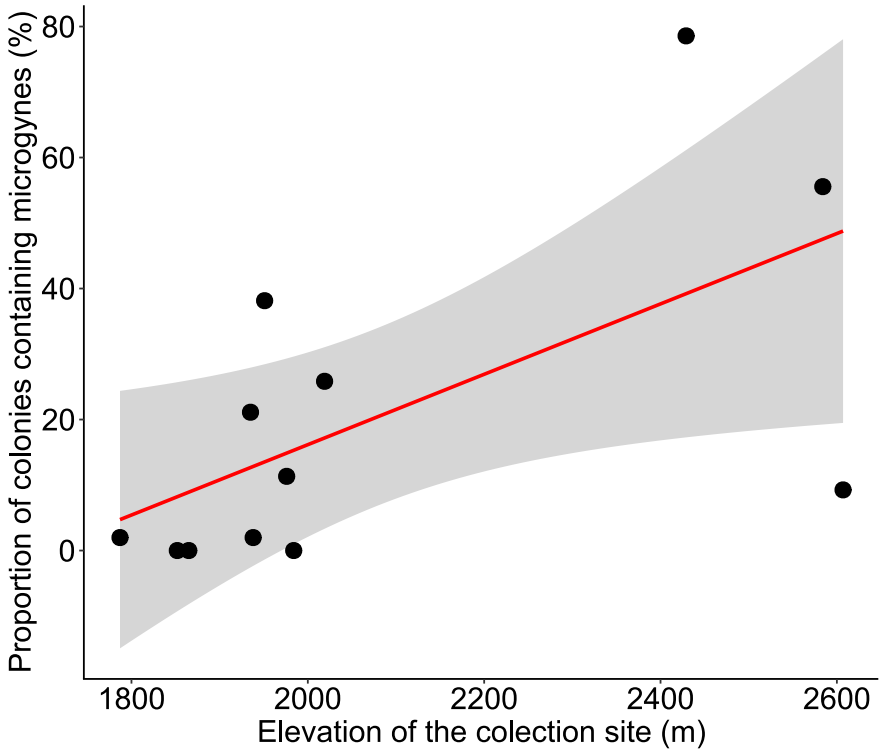

Figure 4. Proportion of colonies containing microgynes depending on the elevation of the collection site. Each dot represents one collection site.

1, $p < 0.01$) and more often groomed ($X^2 = 7.231$, df = 1, $p < 0.01$) by their own workers. After 24 hours, all queens that entered their natal colony were found alive inside their colony, whereas 37% of the queens which tried to enter a non-natal colony were found dead (Fisher's Exact Test, $p < 0.05$). Among the living queens, only 55% were found inside the nest of the non-natal colony, and the remaining 45% were found outside the nest (Fisher's Exact Test, $p < 0.01$).

We then tested whether queen morph influenced the success of intruding non-natal colonies and whether the colony composition (monogynous macrogynous, polygynous macrogynous or microgynous) of the host colonies played a role. Queen morph did not affect the likelihood of being found inside a non-natal colony after one hour ($X^2 = 0.002$, df = 1, $p = 0.969$), nor did the composition of the non-natal colony ($X^2 = 1.641$, df = 2, $p = 0.440$). We did not find an interaction between queen morph and colony composition on the success rate of the queens ($X^2 = 2.052$, df = 2, $p = 0.359$). Similar patterns were found regarding the aggression and grooming behaviors of the workers towards the intruding queens since queen morph, colony composition, and their interaction did not have significant effects (Table S7). The results were similar after 24 hours (Tables S7 and S8). In summary, microgynes and macrogynes were both more likely to be accepted by their natal colony and had a similar, low likelihood of successfully intruding a non-natal colony.

Chemistry

We investigated potential differences in the cuticular hydrocarbon (CHC) profiles of macrogynes and microgynes (Table S9 and Figure S4) from different colony compositions. Cuticular hydrocarbon profiles of microgynes and macrogynes from the same mixed colonies did not differ (PERMANOVA: $t = 0.762$, df = 18, $p = 0.770$; Figure 6A), nor was

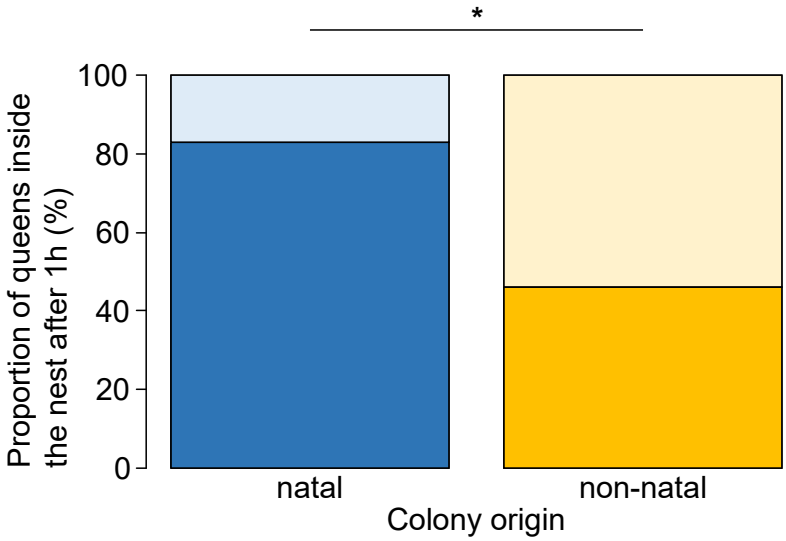

Figure 5. Proportion of queens found inside the nest (darker shade) of natal and non-natal colonies one hour after their introduction to the colony. The level of significance is indicated as follows: * $p < 0.05$.

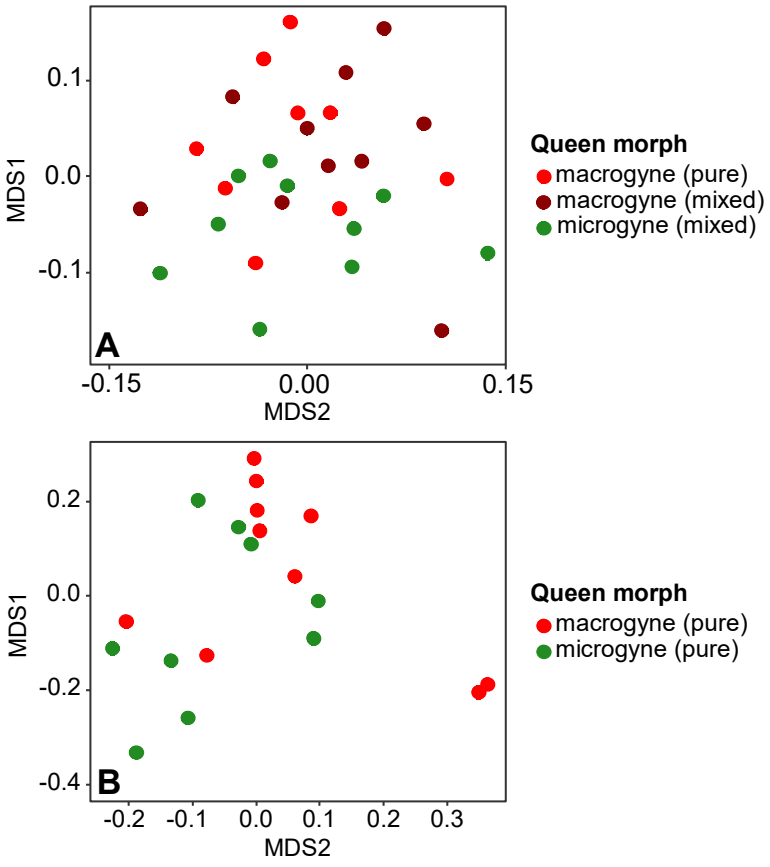

Figure 6. Non-Metric Multidimensional Scaling (NMDS) plots showing the cuticular hydrocarbon (CHC) profiles of **(A)** macrogynes from pure and mixed colonies and microgynes from mixed colonies and **(B)** macrogynes and microgynes from pure colonies. Each dot represents one individual.

there a difference in CHC profiles of microgynes and macrogynes from different pure colonies (PERMANOVA: t = 1.898, df = 1, p = 0.135; Figure 6B). However, macrogynes from pure colonies were different from microgynes of mixed colonies (t = 1.897, df =17, p < 0.01; Figure 6A) and also tended to differ from macrogynes of mixed colonies (t = 1.522, df =17, p = 0.051; Figure 6A). Macrogynes and microgynes did not differ in their proportion of alkanes (X^2 = 0.001, df =1, p = 0.970).

Discussion

Alternative reproductive strategies evolve when environmental or social conditions fluctuate, and are often associated with variation in phenotypic traits matching the respective strategy (Gross 1996; Taborsky and Brockmann 2008). Here, we used multiple approaches to characterize queen alternative reproductive strategies in *Temnothorax rugatulus*. Our results shed light on different traits associated with queen alternative reproductive strategies, which are extending to the worker caste and could potentially be associated with environmental conditions. However, we found no evidence of social parasitic tendencies in microgynes.

In ants, dependent colony foundation usually leads to functional polygyny where several queens reproduce in a single colony, and is often associated with a reduction in queen body size since readoption and budding do not require large body reserves (Keller 1995; Libbrecht and Kronauer 2014; Wolf and Seppä 2016). Our data confirm this size reduction in polygynous macrogynes. However, we observe a different pattern in microgynes, which are larger when occurring in mixed colonies with macrogynes, compared with monogynous and polygynous colonies with microgynes only. Since body size is correlated with fecundity in insects (Honek 1993), we can hypothesize that both queen morphs display fitness optima when living in the colony composition associated

with their reproductive strategy – monogynous colonies for macrogynes versus mixed polygynous colonies for microgynes – and where they are most often found in nature. Supporting this hypothesis, microgynes produce fewer and smaller workers in pure microgynous colonies, while they can keep up their egg-laying rate with macrogynes in mixed colonies (Matteo Negroni, pers. comm.). Also, monogynous microgynous colonies are relatively rare in nature (2.7% of our collected colonies).

Our worker data reflects the relationship between body size, colony composition, and fitness observed in queens since the smallest workers (i.e., workers with the smallest thoraces) are found in colonies with a single microgyne. Interestingly, monogynous microgynes are also the smallest queens. It has been previously suggested that maternal effects are responsible for body size transmission in *Temnothorax rugatulus* (Rüppell et al. 2001b), which is supported by our data showing that the smallest queens produce the smallest workers. Similar results are found in *Myrmica ruginodis*, where microgynes produce smaller workers (Elmes 1991). In our species, however, we cannot rule out that both queens and workers are smaller in monogynous microgynous colonies because of resource limitation, which could be linked to the poor colony fitness discussed above. In general, it seems that worker size is rather plastic in *T. rugatulus*, which contrasts with other species with queen polymorphism such as *Solenopsis invicta,* where alleles on the gene Gp-9 have been shown to affect the body mass of field-collected workers (Goodisman et al. 1999). Finally, our data revealed that workers from polygynous colonies have larger heads, which could suggest stronger territoriality in habitats where polygynous colonies are denser (Adams 2016), and agonistic interactions with unrelated individuals consequently more frequent.

Dependent colony foundation can be associated with ecological factors (Heinze 1993; Bourke and Heinze 1994) and colonies of *Temnothorax rugatulus* inhabit diverse

habitats from valleys to mountaintops. Thus, we decided to investigate whether the ecological background influences different colony traits in our model species. Interestingly, colony composition strongly varies with the site of collection. Since our collection sites appeared to be ecologically different, we investigated whether the site effect we found could be driven by differences in elevation, and found that colonies containing microgynes occur more often at higher elevations. This finding goes in a similar direction to what was previously found in this species (Rüppell et al. 2001a; Heinze and Rüppell 2014), although we were unable to demonstrate a significant increase in the proportion of polygynous colonies with elevation, most likely because our range of elevations was much smaller. Habitats at higher elevations are most likely harsher and resources might be limited, leading to food restriction for the growing larvae, which in turn might result in smaller queens producing smaller workers (Rüppell et al. 2001b). A similar strategy is found in *Myrmica ruginodis,* where the polygynous microgyne form is more common in rapidly changing habitats (Seppä et al. 1995), highlighting once more the similarity with our system in terms of reproductive strategies. Thus, producing smaller queens and workers might be an advantageous strategy for colonies inhabiting difficult environments. However, we should keep in mind that those results might be specific to the population we studied (i.e., Chiricahua mountains) since microgynes are less common in other populations of *T. rugatulus* (Susanne Foitzik, pers. comm.), and other environmental factors correlating with elevation might be involved in shaping colony traits (Foitzik et al. 2004).

Along with previous studies, we provide insights into queen size dimorphism in association with reproductive strategies in *Temnothorax rugatulus* (Rüppell et al. 1998, 2001b, a; Heinze and Rüppell 2014), and our data suggest that the production of microgynes could be an adaptation to difficult environmental conditions. However, the

existence of a microgyne morph has been previously discussed as a route to social parasitism in social insects (Wolf and Seppä 2016) and parasitic microgynes are found in several ant species like *Ectatomma tuberculatum* (Hora et al. 2005) or *Myrmica rubra* (Schär and Nash 2014). Moreover, in some ant species, the role of microgynes is not clear yet (Lenoir et al. 2010). For these reasons, we investigated queens' behavior in readoption experiments and analyzed their chemistry, to better characterize the two queen morphs and rule out the potential parasitic nature of microgynes.

Our behavioral experiments revealed that both queen morphs were more likely to be accepted by their natal colony compared with a non-natal colony. Not surprisingly, *Temnothorax rugatulus* ants seem to possess an effective nestmate recognition system and do not accept unrelated individuals in their colony, like many other ant species (Sturgis and Gordon 2012). Microgynes did not more likely successfully intrude non-natal colonies compared with macrogynes, probably because they are usually readopted by their mother colony (Rüppell et al. 2001a, 2002). Whereas in the ant *Myrmica rubra*, which displays intraspecific social parasitism, the survival rate of microgynes intruding non-natal colonies is high (Schär and Nash 2014). Our cuticular hydrocarbon (CHC) analyses also give evidence for microgynes being a reproductive morph rather than a social parasitic one since they do not exhibit a higher proportion of alkanes, like some parasitic ants do to avoid recognition by their hosts (Keller and Ross 1998; Nehring et al. 2015).

Additionally, we did not find evidence that CHC profiles of microgynes differ from macrogynes, as opposed to *Solenopsis invicta* and *Formica selysi* where social morphs (*S. invicta*) and workers from different social origins (*F. selysi*) have a different chemistry (Keller and Ross 1998; Meunier et al. 2011). In those species, queen morph and colony composition are determined by a so-called social chromosome (Wang et al. 2013; Purcell

et al. 2014; Brelsford et al. 2020; Yan et al. 2020). The difference in our results could be explained by the genetic proximity of the two queen morphs in *Temnothorax rugatulus* (Rüppell et al. 2001a). Interestingly, CHC profiles seemed rather to be influenced by colony composition (pure colonies with only one queen morph versus mixed colonies where the two queen morphs co-occur). Whether mixed colonies have different chemical signatures compared with pure colonies due to the mix of queens from different morphs and workers from different queen morphs, or whether these differences are due to environmental effects (i.e., mixed colonies are more frequent at higher elevations) still need to be elucidated. Currently, little is known about the respective contribution of genetics and environmental factors to CHC profiles (Menzel et al. 2017a), but environmental conditions, especially humidity and temperature, affect CHC profiles and their plasticity (Menzel et al. 2017b; Sprenger and Menzel 2020).

Using multiple approaches, we shed light on a combination of traits characterizing queen alternative reproductive strategies in the ant *Temnothorax rugatulus* and confirm earlier work on the role of microgynes in that species. These traits seem tightly linked to colony composition, and we propose that both queen morphs show fitness optima in their respective most frequent colony composition: monogynous colonies for macrogynes and mixed polygynous colonies for microgynes. Interestingly, colony composition not only affects queen traits but also extends to the worker caste. As we found no evidence for parasitic strategies of microgynes and revealed a link between queen alternative reproductive strategies and environmental conditions, we suggest that colonies with multiple queens including microgynes have evolved as an adaption to harsher environments (i.e., higher elevations). Ultimately, macrogynes and microgynes in *T. rugatulus* could potentially evolve towards becoming different ecotypes if the gene flow between the two morphs starts to diminish (Nosil 2012; Wolf and Seppä 2016). To

summarize our findings, we highlighted the important role of colony composition and environmental factors in association with phenotypical traits linked to queen alternative reproductive strategies in *T. rugatulus*, pointing to the importance of ecology for the evolution of reproductive strategies in social insects.

Author contributions

Ant collection was conducted by MC, BF, and SF. The study was conceived by MC, SG, BF, and SF. Morphometric measurements were done by MC. Behavioral experiments were conducted by SG. Extraction and analyses of cuticular hydrocarbons were conducted by MC and SG, with the help of FM. Statistical analyses were conducted by MC, with significant input from RL. MC wrote the first draft of the manuscript and all co-authors contributed to data interpretation and improvement of the manuscript.

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CHAPTER 2

Histone acetylation regulates the expression of genes involved in worker reproduction in the ant *Temnothorax rugatulus*

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Note: Figures and Tables are not numerically ordered because their numbers were kept from the published version where the method section was after the discussion.

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Abstract

In insect societies, queens monopolize reproduction while workers perform tasks such as brood care or foraging. Queen loss leads to ovary development and lifespan extension in workers of many ant species. However, the underlying molecular mechanisms of this phenotypic plasticity remain unclear. Recent studies highlight the importance of epigenetics in regulating plastic traits in social insects. Thus, we investigated the role of histone acetylation in regulating worker reproduction in the ant *Temnothorax rugatulus*. We removed queens from their colonies to induce worker fecundity, and either fed workers with chemical inhibitors of histone acetylation (C646), deacetylation (TSA), or the solvent (DMSO) as control. We monitored worker number for six weeks after which we assessed ovary development and sequenced fat body mRNA. Workers survived better in queenless colonies. They also developed their ovaries after queen removal in control colonies as expected, but not in colonies treated with the chemical inhibitors. Both inhibitors affected gene expression, although the inhibition of histone acetylation using C646 altered the expression of more genes with immunity, fecundity, and longevity functionalities. Interestingly, these C646-treated workers shared many upregulated genes with infertile workers from queenright colonies. We also identified one gene with antioxidant properties commonly downregulated in infertile workers from queenright colonies and both C646 and TSA-treated workers from queenless colonies. Our results suggest that histone acetylation is involved in the molecular regulation of worker reproduction, and thus point to an important role of histone modifications in modulating phenotypic plasticity of life history traits in social insects.

Introduction

Eusocial insect societies exhibit a reproductive division of labor where one or a few females (often called queens) reproduce, whereas workers perform all other tasks including brood care, nest defense, and foraging (Hölldobler and Wilson 1990). Workers thus sacrifice their own reproduction and this evolutionary incongruity is commonly explained by Hamilton's inclusive fitness theory, which states that genes of sterile altruists can be transmitted indirectly to the next generation by helping closely related reproductives (Hamilton 1964). The proximate mechanisms underlying the maintenance of worker sterility in social insect colonies have been extensively investigated. Worker reproduction is regulated via chemical signals emitted by the queen or her brood (Endler et al. 2004; Matsuura et al. 2010; Van Oystaeyen et al. 2014), or through social control meditated by the queen or by the workers themselves (Foster and Ratnieks 2000; Oldroyd et al. 2001; Ruhland et al. 2020). However, following the loss of their queen or even sometimes in queenright colonies, social insect workers can circumvent those restraints and successfully gain direct fitness benefits by laying haploid, male-destined eggs (Monnin and Peeters 1999; Beekman and Oldroyd 2008; Heinze 2008; Giehr et al. 2020a, b). Reproduction has strong effects on the physiology and immunity of workers, who can become more resistant to oxidative stress and often live longer (Kohlmeier et al. 2017; Lopes et al. 2020; Negroni et al. 2020; Majoe et al. 2021). These positive effects of reproduction have been linked to the activation of signaling pathways such as the insulin/insulin-like growth factor 1 (IIS), the target of rapamycin (mTOR), and the alpha-ketoglutarate (alpha-KG) (Negroni et al. 2021b). In fact, gene expression changes profoundly in workers after queen loss in social wasps (Taylor et al. 2021), honey bees (Cardoen et al. 2011), and ants (Wurm et al. 2010; Negroni et al. 2021b) and in various tissues from the brain to the fat body. Similarly, gene expression differs between

reproductive and sterile bumblebee workers (Marshall et al. 2019). Worker fecundity thus appears to be a highly plastic trait positively linked to lifespan (Heinze and Schrempf 2008). This opens up exciting new avenues to study the molecular regulation of plasticity in fecundity and longevity in social insect workers (Monroy Kuhn and Korb 2016). Indeed, the transcriptomic changes linked to worker reproduction have been well characterized, while the underlying gene regulatory mechanisms remain largely unexplored.

Epigenetic mechanisms including DNA methylation and histone modifications have been proposed to play a major role in the extraordinary phenotypic plasticity exhibited by social insects (Bonasio 2012, 2014; Herb 2014; Vaiserman 2015; Yan et al. 2015; Maleszka 2016; Vaiserman et al. 2018). In Carpenter ants, histone modifications have been associated with behavioral differences between major and minor ant workers (Simola et al. 2013, 2016; Glastad et al. 2019) and worker polymorphism (Alvarado et al. 2015). Histone acetylation has also been associated with the ability of workers to adjust to new daily rhythms (Libbrecht et al. 2020). Besides, there is growing evidence for the role of histone modifications in caste differentiation. In honey bees, queen development is largely controlled by royal jelly, a secretion that has histone deacetylase inhibitor (HDACi) activity (Spannhoff et al. 2011). Moreover, caste-determined female larvae exhibit genome-wide differences in histone acetylation and methylation patterns, which are linked to caste-specific gene expression (Wojciechowski et al. 2018). Besides, the transition of non-reproductive to reproductive workers (also called gamergates) has been associated with transcriptomic changes linked to epigenetic pathways in the ant Harpegnathos saltator (Bonasio et al. 2010).

In this study, we used the ant *Temnothorax rugatulus* to investigate the role of histone acetylation in the regulation of worker reproduction following the loss of their queen. This common Myrmicine ant builds small nests of 50 to 2000 workers with one to

several queens and evolved two queen morphs, the large macrogynes, and the small microgynes, associated with alternative reproductive strategies (Rüppell et al. 1998, 2001a; Choppin et al. 2021b). Queens can live over ten years and their gene expression in the brain and fat body changes with age (Negroni et al. 2019). Following queen loss, *T. rugatulus* workers are known to develop their ovaries, start laying haploid eggs, live longer, and show transcriptomic changes in the fat body, a physiologically active tissue (Negroni et al. 2020, 2021b). Here, we asked whether histone acetylation is required for workers to plastically respond to queen loss by altering their ovary development and associated gene expression. We used queen removal to induce fecundity in workers while feeding them with chemical inhibitors of histone acetylation (C646) or deacetylation (Trichostatin A; TSA). Based on previous studies, we predicted that workers would develop ovaries, survive better, and show transcriptomic changes in the fat body following queen removal. If histone acetylation does play a role in the regulation of worker reproduction, we expected the chemical inhibitors C646 and TSA to prevent workers from developing their ovaries following queen removal, and to alter the expression of fecundity and longevity genes, preventing workers to reproduce and live longer following the loss of their queen.

Methods

Ant collection and maintenance

Temnothorax rugatulus ants are distributed throughout the western part of North America and reside in high elevation coniferous forests, under stones or in rock crevices. In August 2018, we collected colonies from nine different locations in the Chiricahua Mountains (Arizona, USA; Table S1). In the laboratory, each colony was kept in a three-chambered box (9.7 x 9.7 x 2.9 cm) covered with a lid and containing an artificial nest

made of a plastic insert between two glass slides covered by a red foil to block the light. The colonies were maintained at 21°C and 70% humidity with a 12:12 light:dark cycle. They were fed weekly with half a cricket and a drop of honey and were provided with water *ad libitum*.

Colony monitoring

We selected 90 monogynous colonies with 54 to 100 workers and reduced worker number to 50 per colony. To increase behavioral activity, colonies were then moved to a climate chamber at 25°C and 70% humidity with a 12:12 light:dark cycle for two weeks. Before starting the experiment, colonies were randomly assigned to one of five experimental groups with a total of 18 colonies per group (Table 1).

On the first day of the experiment, we removed all eggs, pupae, and males and adjusted the number of larvae to five per colony. Queens from the queen removal groups were removed and returned to their natal colonies. Then, colonies were fed with either the solvent Dimethyl Sulfoxide (DMSO, Carl Roth) only, the inhibitor of histone acetylation C646, which targets the p300/CPB histone acetyltransferases (50 µM in DMSO; Sigma-Aldrich) (Bowers et al. 2010), the inhibitor of histone deacetylation TSA that inhibits class I and II histone deacetylases (50 µM in DMSO; Sigma-Aldrich) (Yoshidas 1990), or a combination of C646 and TSA (both 50 µM in DMSO). All preparations were diluted in 0.102 g/mL sucrose solution. The ants were fed for six weeks every other day with 15 µL of fresh solution per colony (Figure S9). Additionally, each colony received half a cricket every other day and water *ad libitum*. Once a week, we anesthetized all colonies with CO_2. We removed and counted the eggs in queenless colonies to get precise numbers of worker-laid eggs. Once every two weeks, we counted all colony members (queens if applicable, workers, eggs, larvae, and pupae) in colonies from all groups.

Table 1. Group name, manipulation, treatment, and sample sizes (N = colonies at start / colonies for RNA-sequencing of fat body samples) for each experimental group. The queenless control was always used as a reference because it allows comparisons to all the other experimental groups differing in a single factor only i.e., queen presence or inhibitor treatment.

Group name	**Manipulation**	**Treatment**	**Colonies**
Queenright	No queen removal	DMSO	N = 18 / 4
Queenless	Queen removal	DMSO	N = 18 / 5
Queenless+C646	Queen removal	DMSO+C646	N = 18 / 7
Queenless+TSA	Queen removal	DMSO+TSA	N = 18 / 4
Queenless+C646/TSA	Queen removal	DMSO+C646/TSA	N = 18 / 0

We tested the effect of queen removal on worker survival by comparing worker number over time between the groups "queenright" and "queenless" using a linear mixed-effects model (LMM) with the package "lme4" (Bates et al. 2015). In queenless colonies, we investigated the effect of treatment (DMSO, DMSO+C646, DMSO+TSA, and DMSO+C646/TSA) on worker number in interaction with time using a similar model. Colony identification (ID) was used as a random factor in both models to account for inter-colony variability. We assessed the fit of our LMMs using visual inspections of the residual distributions. The effect of treatment on egg production at week six was analyzed using a generalized linear mixed-effects model (GLMM, binomial family) with egg production as a binary variable. Our GLMM was tested for overdispersion using the package "DHARMa" (Hartig 2020). All statistical analyses were conducted in R v3.5.1 (R Core Team 2020).

Experimental usage of chemical inhibitors

Depending on the doses, the chemical inhibitors C646 and TSA can have deleterious or beneficial effects on animals (Wang et al. 2017). They have been repeatedly used in studies investigating plastic changes in ants, including *Temnothorax* ants, showing no negative effects in low concentrations (Simola et al. 2016; Libbrecht et al. 2020). In a pilot experiment, we confirmed that the concentrations used in this study were indeed non-toxic for our ants. Accordingly, we did not observe an increased or decreased mortality following treatment with any of the chemical inhibitors (see results), or an upregulation of detoxification genes. Instead, we found a large overlap of upregulated genes between the "queenright" and "queenless+C646" groups (see results), indicating that the chemical inhibition of histone acetylation prevented workers to respond to queen removal, so that their gene expression resembles the one of workers from queenright colonies. In addition, our Principal Component Analysis (PCA) using all groups (Figure S13) does not indicate

that samples from the chemical inhibitor treatments cluster together, as one might expect if toxins harmed the workers. Finally, other studies on *Temnothorax* ants using the same inhibitors reported increases in behaviors such as foraging or brood care following treatment (Libbrecht et al. 2020; Marina Choppin and Philip Kohlmeier, pers. comm.) attesting to a general "well-being" of treated individuals. We therefore argue that the effects we report below are not mere side-products of the toxicity of the chemical inhibitors, but consequences of the inhibition of histone (de)acetylation.

Dissections, RNA extractions, and fecundity measures

After six weeks, we selected a subset of colonies for dissections. For each colony, we isolated all workers on or next to the brood pile in a Petri dish since brood-carers are typically close to the brood and usually the youngest individuals in the colony, thus more likely to develop ovaries following the loss of their queen (Pamminger et al. 2014; Kohlmeier et al. 2017). We dissected those workers on ice in a drop of a sterile saline solution until two workers with developed ovaries were found (2 to 14 workers dissected per colony). From these two workers, we cleaned the ovaries and took pictures for fecundity measurements using a stereomicroscope as detailed below. The fat bodies, including the first cuticle plate of the gaster, were collected from the two workers and pooled in the same Eppendorf tube containing 50 µl of TRIzol (Thermofisher) for further RNA-sequencing. Tissue collection took less than 10 minutes. The samples were flash-frozen in liquid nitrogen and preserved at -80°C. Before the RNA extraction of each sample, we crushed the fat bodies with a pestle, added 50 µl of phenol:chloroform:isoamyl alcohol (25:24:1) (Carl Roth), mixed manually, and centrifuged at 1200 xg for 15 minutes. Afterward, the upper phase was transferred to a new tube and mixed with 25 µl of ethanol 100% (Carl Roth). RNA was then extracted using a NucleoSpin RNA XS kit (Macherey-

Nagel). After quantity and quality control, 20 samples were sent for sequencing (Table 1). Library preparation was conducted following the standard protocol of BGI (Hongkong), which sequenced 150 bp paired-end reads on an Illumina Hiseq X Ten.

We measured ovariole length and counted the number of white eggs (i.e., eggs in development) and yellow bodies in the ovaries using the Leica software LAS v4.5. Yellow bodies are an indication of recent egg laying in ants (Heinze et al. 1995; Cini 2014; Peeters and Tinaut 2014). We analyzed the effects of queen removal and treatment (DMSO, DMSO+C646, DMSO+TSA, DMSO+C646/TSA) on worker ovariole length using LMMs. We used GLMMs (binomial family) to test for effects of queen removal and treatment on the presence of white eggs and yellow bodies in the ovaries. Colony ID was used as a random factor to account for inter-colony variability. The models' fit was assessed as described above.

Gene expression analysis

Raw reads were trimmed with Trimmomatic v0.39 (Bolger et al. 2014) (Table S2) and quality checked using FastQC v0.11.7 (Andrew 2010). The paired reads were then mapped against the *Temnothorax rugatulus* draft genome (Jongepier et al. 2021) using HISAT2 v2.1.0 (Kim et al. 2015) (Table S2). We converted and sorted the output files using SAMtools v1.7 (Li et al. 2009) and obtained a quality report from Qualimap v2.2.1 (Okonechnikov et al. 2016). A genome-guided transcriptome assembly was created using StringTie v2.1.3 (Pertea et al. 2015) and transcript sequences were extracted using GffRead v0.11.8 on the merged GTF file. Transcriptome quality was assessed using TransRate v1.0.3 (Smith-Unna et al. 2016). Transcripts with an Open Reading Frame (ORF) < 100 bp were removed and the Python script "prepDE.py" from the online StringTie Manual was used to generate the gene count matrix.

We assessed the effect of queen removal on worker gene expression by comparing the groups “queenless” and “queenright”. Then, we tested the effects of the chemical inhibition of histone acetylation and deacetylation by first comparing the groups “queenless” to “queenless+C646”, and then “queenless” to “queenless+TSA”. To avoid factitious DESeq2 results and for each comparison, we first filtered the gene count matrix so at least 70% of samples had a read count of ten or more reads per gene in at least one experimental group. We additionally plotted the maximum cook distance against the average gene expression per sample to identify and remove putative outliers. We used the filtered count matrix (Table S3) to perform the differential gene expression analysis using DESeq2 (Love et al. 2014). An FDR-corrected p-value < 0.05 was set as a significance threshold. We plotted principal component analyses (PCAs) with all genes using the package “ggplot2” (Wickham 2010) to assess the group-based clustering of our samples (Figures S10 to S13). We created heatmaps with the package “pheatmap” (Kolder 2012) to visualize expression differences and clustering between samples. To annotate transcripts we conducted a BlastX homology search with BLAST v2.10.1+ (Altschul et al. 1990) using the non-redundant invertebrate protein database from NCBI (May 2020) and only considered hits with an E-value < 10^{-5}. We combined the blast annotations with gene information from UniProt (https://www.uniprot.org/). We chose to only discuss the top 15 most upregulated or downregulated genes based on adjusted p-values. Additionally, we used TransDecoder v5.5.0 (Haas et al. 2013) to translate nucleotide sequences into amino-acid sequences and then ran InterProScan v5.45-80.0 (Quevillon et al. 2005) to obtain Gene Ontology (GO) term annotations. Then, we performed a GO term enrichment analysis using the R package “topGO” (Alexa and Rahnenführer 2020) with the algorithm “weight01”. We conducted the GO enrichment analysis separately for upregulated and downregulated genes in the groups compared to the queenless control. Statistical

significance was given using Fischer exact tests. We extracted the overlap of upregulated and downregulated genes between the groups "queenright", "queenless+C646" and "queenless+TSA" and assessed whether the overlap size between two groups was larger than expected by chance by resampling random gene lists (500 iterations). Finally, we plotted expression levels (i.e., normalized read counts) of genes of interest using "plotCounts" from DESeq2.

Results

Effects of queen removal

Worker number and fecundity

Worker number decreased less strongly in queenless compared to queenright colonies (interaction time x queen removal: $X^2 = 9.723$, df = 2, p = 0.008; Figure S1). Workers from queenless colonies had longer ovarioles ($X^2 = 30.578$, df = 1, $p < 0.001$; Figure 1A), were more likely to have yellow bodies ($X^2 = 9.588$, df = 1, p = 0.002; Figure 1B), and also tentatively more likely to have white eggs in their ovaries ($X^2 = 2.828$, df = 1, p = 0.093; Figure S2). Yellow bodies are small endocrine structures that remain in the ovaries after an egg has been laid, and thus provide evidence for reproductive activity (Heinze et al. 1995; Cini 2014; Peeters and Tinaut 2014).

Gene expression and functional enrichment

We found 346 differentially expressed genes (DEGs) between workers from the "queenless" and "queenright" groups, among which 206 were upregulated and 140 downregulated in the queenright group. The samples clearly clustered according to treatment in the heatmap based on all DEGs (Figure 1C). The enrichment analysis

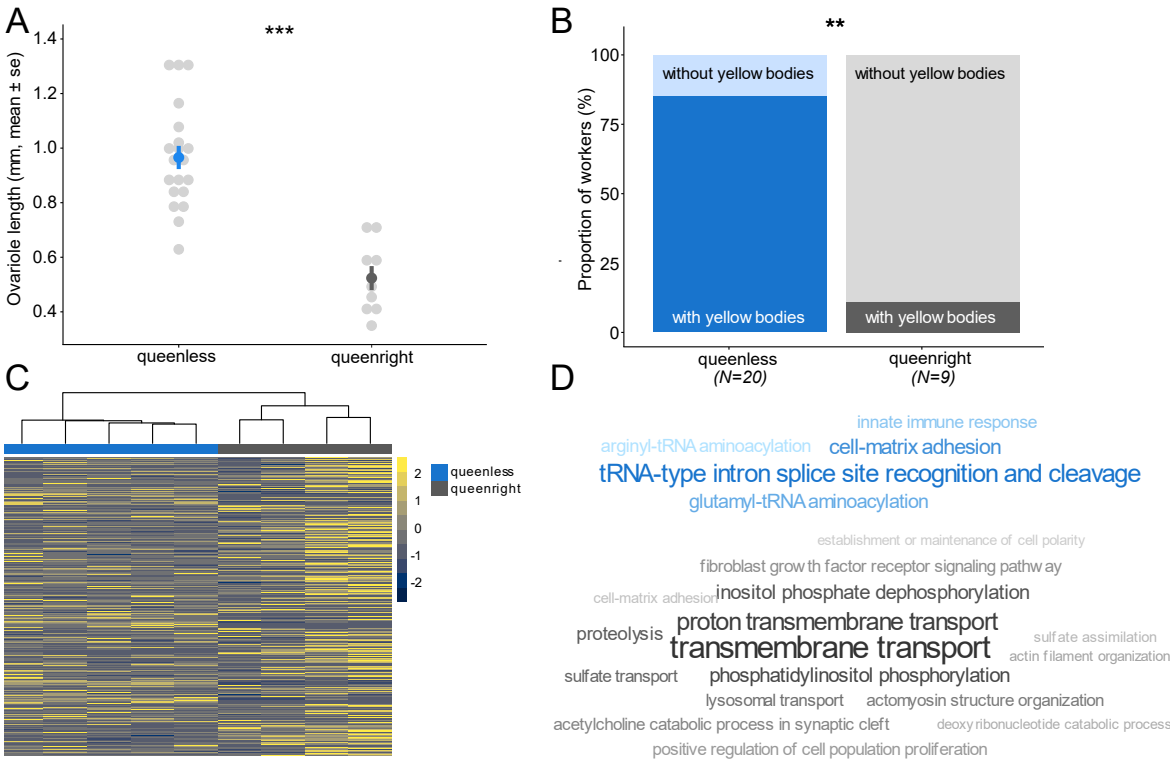

Figure 1. (A) Effect of queen removal on ovariole length and **(B)** the proportion of workers with yellow bodies in their ovaries at the end of the experiment at week 6. Levels of significance are indicated as follows: ** $p < 0.01$ and *** $p < 0.001$. **(C)** Heatmap showing the expression levels of the differentially expressed genes between the "queenless" (control, blue) and "queenright" (grey) groups and the clustering of samples per group. **(D)** Word clouds showing the overrepresented functions associated with downregulated (top, blue) and upregulated (bottom, grey) genes in the group "queenright". In the word clouds, p-value significance is positively correlated with the size and shade darkness of the word.

revealed that workers in presence of their queen downregulated genes related to five functions including "innate immune response" (Figure 1D). This is reflected by multiple immune genes in our list of top 15 downregulated genes in the queenright group including "FK506-binding protein 2 isoform X1" or "chymotrypsin-2-like". Also, a gene coding for "vitellogenin-1-like" (Figure 2) was found to be downregulated in workers from queenright colonies. As vitellogenin copies are so far annotated separately for each genome without orthology inference, the comparison of vitellogenins across species is difficult. Therefore, we investigated where our "vitellogenin-1-like" copy falls within the vitellogenin phylogeny by constructing a maximum likelihood phylogeny with RAxML (Stamatakis 2014) using sequences previously used in (Kohlmeier et al. 2018). We found that it clusters close to the conventional vitellogenins (Figure S3), and thus refer to this vitellogenin copy as "conventional vitellogenin" in the rest of the manuscript. Queen presence also affected the expression of many regulatory genes, such as transcription factors including "zinc finger protein 454-like".

Effects of chemical inhibitors

Worker number, egg production, and fecundity

Here we focus on queenless colonies that were either fed with DMSO only as control or additionally treated with C646, TSA, or both inhibitors. Again, worker number generally decreased over time (X^2 = 719.732, df = 2, p < 0.001), but irrespective of treatment (X^2 = 7.493, df = 6, p = 0.278; Figure S4). After six weeks, 23% of colonies had eggs and the presence of eggs was unaffected by treatment (X^2 = 2.521, df = 3, p = 0.472; Figure S5). However, workers treated with the epigenetic inhibitors had shorter ovarioles (X^2 = 11.569, df = 3, p = 0.009; Figure 3A) and a smaller proportion of treated workers had yellow bodies in their ovaries (X^2 = 9.721, df = 3, p = 0.021; Figure 3B) compared to control

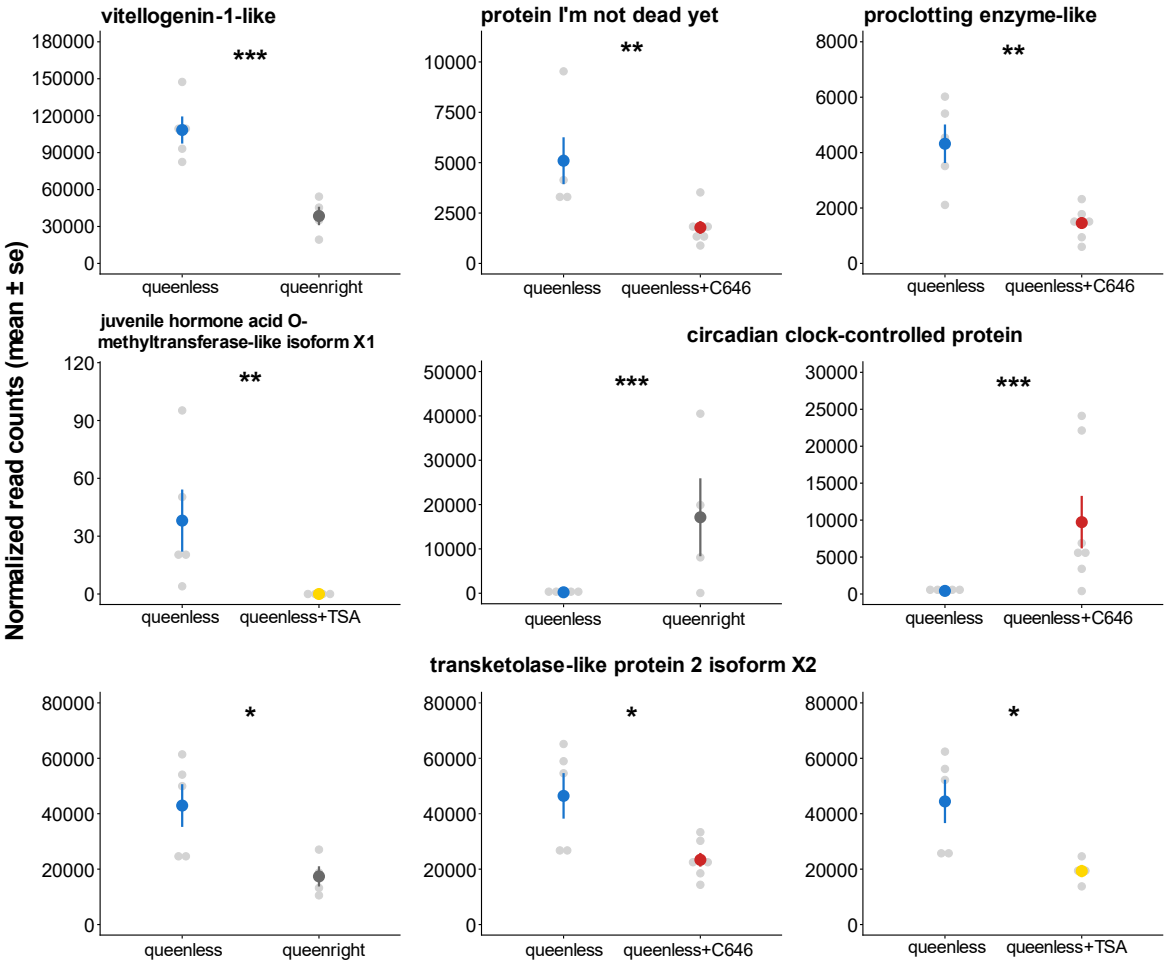

Figure 2. First row: expression levels of the genes "vitellogenin-1-like" ($p < 0.001$) downregulated in the group "queenright" compared to the group "queenless" (control), "protein I'm not dead yet" ($p = 0.004$) and "proclotting enzyme-like" ($p = 0.001$) downregulated in the group "queenless+C646" compared to the group "queenless" (control). Second row: expression levels of the genes "juvenile hormone acid-O-methyltransferase-like isoform X1" ($p = 0.01$) downregulated in the group "queenless+TSA" compared to the group "queenless" (control) and the gene "circadian clock-controlled protein" ($p_{QR} < 0.001$ and $p_{C646} < 0.001$) commonly downregulated in the groups "queenright" and "queenless+C646" compared to "queenless" (control). Third row: expression levels of the gene "transketolase-like protein 2 isoform X2" ($p_{QR} = 0.036$, $p_{C646} = 0.031$ and $p_{TSA} = 0.045$) commonly downregulated in the groups "queenright", "queenless+C646" and "queenless+TSA" compared to "queenless" (control). Levels of significance are indicated as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

workers. More precisely, C646-treated workers exhibited shorter ovarioles (lmer:t_{C646} = -3.290, p_{C646} = 0.002) and were less likely to have yellow bodies in the ovaries (glmer:z_{C646} = -2.366, p_{C646} = 0.018) compared to workers fed with DMSO only. In the TSA treatment, fewer workers had yellow bodies in their ovaries compared to control workers (glmer:z_{TSA} = -2.949, p_{TSA} = 0.003), although ovariole length was unaffected (lmer:t_{TSA} = -0.891, p_{TSA} = 0.379). None of the inhibitors affected the proportion of workers with white eggs in the ovaries (X^2 = 5.817, df = 3, p = 0.121; Figure S6).

Gene expression and functional enrichment

We found 306 differentially expressed genes between workers from the control group and workers treated with the inhibitor of histone acetylation C646, among which 247 were upregulated and 59 were downregulated in the C646-treated workers. The heatmap revealed that C646 samples clustered well together (Figure 3C). C646-treated workers downregulated genes with longevity functionalities such as "protein I'm not dead yet" also called "Indy" (Rogina and Helfand 2013) (Figure 2), or genes with an immune function like "proclotting enzyme-like" (Villanueva-Segura et al. 2020) (Figure 2). Workers fed with C646 also downregulated seven genes related to the synthesis of fatty acids, versus only one in the control. As expected and corroborating the efficacy of our treatment, we found four histone-related genes upregulated in C646-treated workers ("histone H2A-like", "histone H3", "histone PARylation factor 1 isoform X2" and "late histone H1-like"). Our enrichment analysis revealed the overrepresentation of five functions associated with downregulated genes in C646-treated workers including "oxidation-reduction process" (Figure 3D).

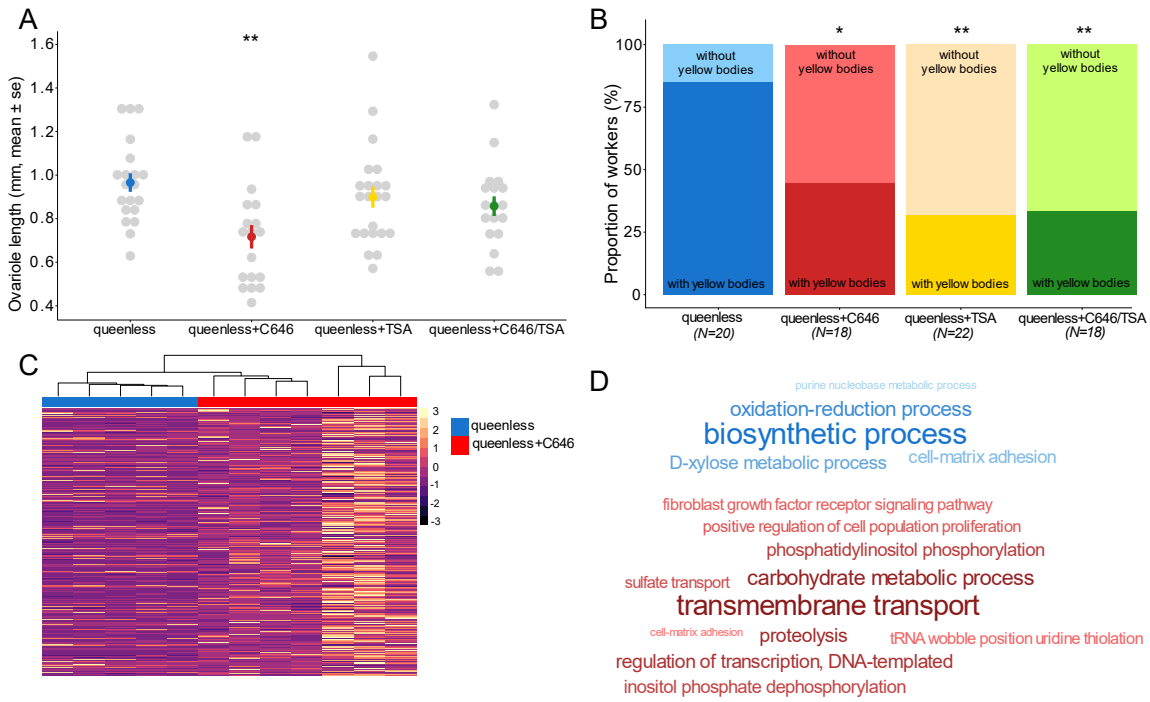

Figure 3. (A) Effect of treatment on ovariole length and **(B)** the proportion of workers with yellow bodies in their ovaries at the end of the experiment at week 6. Levels of significance are indicated as follows: * $p < 0.05$ and ** $p < 0.01$. **(C)** Heatmap showing the expression levels of the differentially expressed genes between the "queenless" (control, blue) and "queenless+C646" (red) groups and the clustering of samples per group. **(D)** Word clouds showing the overrepresented functions associated with downregulated (top, blue) and upregulated (bottom, red) genes in the group "queenless+C646". In the word clouds, p-value significance is positively correlated with the size and shade darkness of the word.

Between workers from the control group and workers treated with the inhibitor of histone deacetylation TSA, we found 33 differentially expressed genes. Three genes were upregulated and 30 were downregulated in the TSA-treated workers. The heatmaps created using the 33 DEGs revealed a good clustering of our samples by group (Figure S7). Based on the low number of DEGs between the two groups we only found the functions "transposition, DNA-mediated" and "autophagy" significantly overrepresented in the DEGs of the TSA-treated workers. Although the TSA treatment had weaker effects on worker fecundity, we did find the aging and fecundity-associated gene "juvenile hormone acid O- methyltransferase-like isoform X1" (Hartfelder 2000; Yamamoto et al. 2013) downregulated in TSA-treated workers (Figure 2).

Overlapping genes between groups

As indicated above, the chemical inhibitors of histone acetylation and deacetylation impaired worker ovary development following queen removal. Thus, we asked whether the transcriptomes of inhibitor-treated workers were similar to the ones of infertile workers from queenright colonies. Indeed, between the groups "queenright" and "queenless+C646" we found 82 genes commonly upregulated (Figure 4A) and five genes commonly downregulated (Figure 4B) in workers. We additionally found five genes commonly downregulated between the groups "queenright" and "queenless+TSA" (Figure 4B). These three numbers of overlapping genes were higher than expected by chance as evidenced by resampling random gene lists (Figure S8). Among the commonly upregulated genes between workers with a queen and C646-treated workers, we found genes associated with circadian rhythm like "circadian clock-controlled protein" (Figure 2). We additionally found many genes associated with digestion like "mucin-5AC-like",

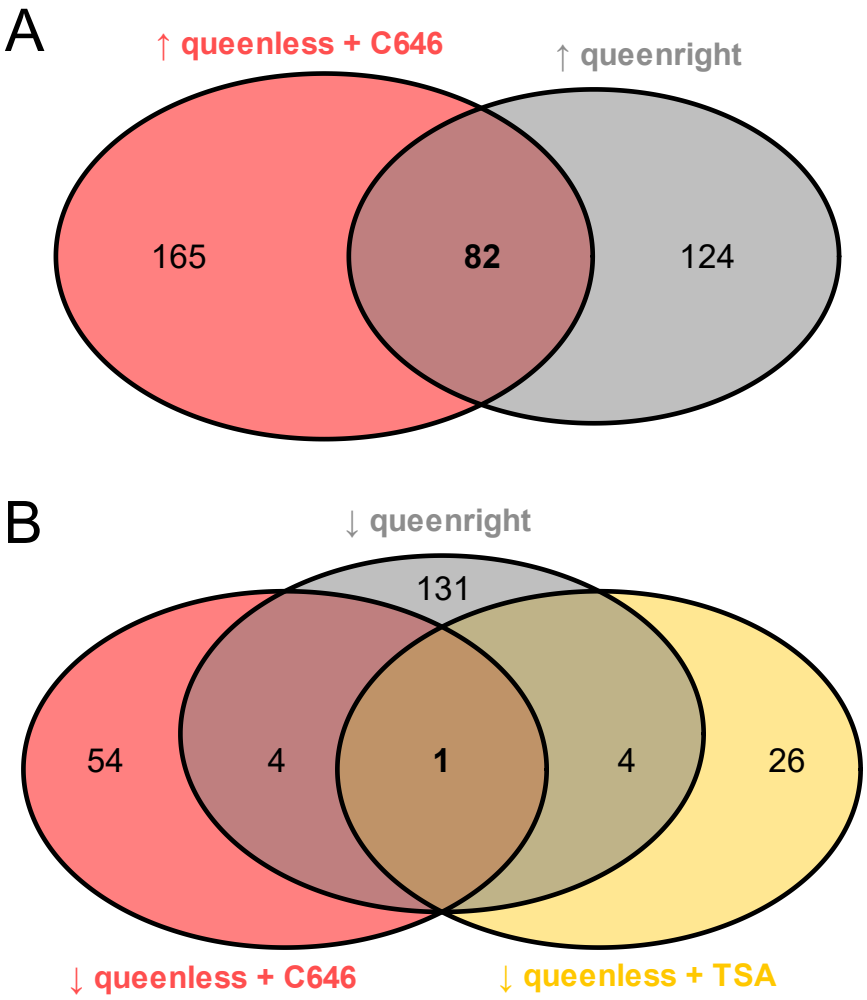

Figure 4. (A) Venn diagram showing the number of genes singly upregulated in the groups "queenless+C646" (165) and "queenright" (124) and the number of genes commonly upregulated between these two groups (82). **(B)** Venn diagram showing the number of genes singly downregulated in the groups "queenless+C646" (54), "queenright" (131), and "queenless+TSA" (26), the number of genes commonly downregulated between "queenright" and "queenless+C646" (4) or "queenless+TSA" (4) and the number of genes commonly downregulated between all three groups (1).

"probable salivary secreted peptide" or "silk gland factor 1". Finally, the gene "transketolase-like protein 2 isoform X2" coding for an enzyme with antioxidant properties (Schenk et al. 1998; Xu et al. 2016; Kovarova et al. 2018) was commonly downregulated in all three groups in comparison to the control (Figure 2 and Figure 4B).

Discussion

In this study, we investigated the role of histone acetylation in the regulation of genes associated with worker reproduction in the ant *Temnothorax rugatulus*. We removed queens from their colonies while feeding the workers with chemical inhibitors of histone acetylation (C646) or deacetylation (TSA). Our phenotypic and transcriptomic results confirmed that worker fecundity and survival increased after queen removal, as shown before in this species (Negroni et al. 2020, 2021b). However, the ovary development of workers from queenless colonies was impaired when treated with the chemical inhibitors, although their survival was unaffected. On a molecular level, the inhibition of histone acetylation using C646 had a stronger effect than the inhibition of histone deacetylation using TSA, as shown by the difference in the number of differentially expressed genes (306 vs 33). Yet, workers from both treatments downregulated many genes related to fecundity, immunity, and longevity, compared to the queenless control. More importantly, a large number of genes were commonly upregulated between infertile workers from queenright colonies and C646-treated workers from queenless colonies, pointing to similarities in gene expression. We additionally found one gene with antioxidant properties commonly downregulated between infertile workers and both C646 and TSA-treated workers, in comparison to workers in the DMSO control, which might be linked to the extended survival of these fecund workers.

Ant workers start to reproduce and live longer after the loss of their queen in many ant species (Heinze 2008; Konrad et al. 2012; Kohlmeier et al. 2017) including our model *Temnothorax rugatulus* (Negroni et al. 2020, 2021b). We took advantage of this plasticity by inducing fecundity in workers using queen removal and confirmed that workers from queenless colonies developed their ovaries, started laying eggs, survived better, and shifted their gene expression in the fat body. Our transcriptomic analysis revealed the upregulation of a conventional vitellogenin (vg) in fecund workers from queenless colonies. During social insect evolution, vg genes underwent several duplications followed by diversification and sub-functionalization. Various vg orthologues now take over different functions in behavior (Kohlmeier et al. 2018) and physiology, including fecundity (Amdam et al. 2003) and aging (Parker 2010), in social insects. Nevertheless, conventional vitellogenins, like the vitellogenin copy we found, have an ancestral fecundity function as an egg yolk precursor (Amdam et al. 2003) rather than a derived role, as in worker caste differentiation (Feldmeyer et al. 2014; Morandin et al. 2014). Additionally, the expression of conventional vitellogenins has been linked to oxidative stress resistance in honey bee workers (Seehuus et al. 2006). Besides, another study on *T. rugatulus* detected an upregulated vg receptor in fecund workers (Negroni et al. 2020), pointing to the importance of vg-associated pathways for worker reproduction.

Longevity is traded off with immunity in many organisms due to the cost of an active immune system and the production of reactive oxygen species during immune reactions (DeVeale et al. 2004; Garschall and Flatt 2018). Nevertheless, our results show that the longer-lived, fecund workers from queenless colonies activate genes with an innate immune response functionality, which is consistent with previous findings in *Temnothorax rugatulus* showing that fecund workers express more immunity genes following an immune challenge, compared to infertile workers (Negroni et al. 2020).

Similarly, the highly fecund young queens of *T. rugatulus* upregulate immune genes in the Toll-pathway (Negroni et al. 2019), which plays a crucial role in insect immunity (Valanne et al. 2011). These findings provide evidence that fecund ants invest in a longer lifespan and immunity concurrently, supporting other studies on social insects indicating that life history trade-offs have shifted during their social evolution (Korb 2016; Schrempf et al. 2017).

Interestingly, ant workers treated with chemical inhibitors following queen removal had a lower ovary development and were less likely to exhibit yellow bodies, which provide evidence for egg-laying (Heinze et al. 1995; Cini 2014; Peeters and Tinaut 2014), compared to control workers from queenless colonies. This indicates that dynamic changes in histone acetylation might be required for workers to shift to fecund phenotypes. In social insects, histone acetylation has previously been linked to various processes including the regulation of foraging behavior and caste determination (Spannhoff et al. 2011; Simola et al. 2013, 2016; Wojciechowski et al. 2018), but evidence for the regulation of life history traits such as fecundity and longevity have been lacking so far. Meanwhile, in solitary insects such as the pea aphid, the inhibition of histone acetylation and deacetylation affects development, fertility, and longevity (Kirfel et al. 2020). In fact, fertility appears to be regulated by this epigenetic mark in various taxonomic groups, which includes other insects such as the planthopper *Nilaparvata lugens* (Zhang et al. 2018), but also mammals including mice (Ma et al. 2012) and men (Wang et al. 2019).

The inhibition of histone acetylation using C646 had more severe consequences on gene expression in worker fat bodies compared to the inhibition of histone deacetylation using TSA, shifting the expression of nearly 10 times as many genes. Among the most strongly downregulated genes in workers treated with the chemical inhibitors, we found

interesting candidates such as the Indy protein, which has been linked to longevity in *Drosophila* (Rogina and Helfand 2013). In these solitary insects, *Indy* knock-outs show extended lifespans, while we found this gene to be upregulated in fecund, longer-lived workers. This reversed link to fecundity and longevity might be due to shifts in gene networks underlying these life history traits in social insects (Negroni et al. 2019; Lin et al. 2021). We additionally found the enzyme proclotting, involved in the innate immune response (Villanueva-Segura et al. 2020), and a gene associated with juvenile hormone, which plays a role in many physiological processes including aging (Yamamoto et al. 2013). We also detected seven downregulated genes associated with the fatty-acid synthesis in the queenless, C646-treated group compared to only one in the queenless control. Fatty acids are involved in the synthesis of cuticular hydrocarbons (Blomquist and Bagnères 2010), and reproductive and non-reproductive individuals exhibit different odors in social insects (Heinze et al. 2002; Monnin 2006) including *Temnothorax* ants (Kleeberg et al. 2017). Besides, many histone-related genes were upregulated in the group where histone acetylation was inhibited (4/247) compared to our control (0/59), attesting to the efficacy of our treatment on a molecular level. Chromatin Immunoprecipitation sequencing (ChIP-sequencing) will be our next logical step to both confirm changes in acetylation on the histone level, and to associate histone acetylation patterns with the expression of genes of interest.

In queenright colonies, workers are mostly infertile due to queen-produced chemical signals such as pheromones (Endler et al. 2004; Matsuura et al. 2010; Van Oystaeyen et al. 2014). Our data show that C646-treated workers remained in a "queenright-like state" and did not develop their ovaries following queen removal. Beyond their phenotypic similarities, C646-treated workers also shared the expression of many genes with workers from queenright colonies, as 33% and 40% of all differentially

expressed genes in the queenright and the queenless group treated with C646 were commonly upregulated, respectively. We propose that this large overlap could be in part linked to the ant circadian rhythm since many of the commonly upregulated genes code for circadian clock-related proteins. On one hand, this is in line with a previous study in another *Temnothorax* species where the use of C646 led to the loss of the ability to adjust to new daily rhythms (Libbrecht et al. 2020). On the other hand, queen presence has been found to affect worker and colony activity in the honey bee (Moritz and Sakofsky 1991; Grodzicki et al. 2020), which could explain the upregulation of circadian rhythm-related genes in workers from queenright colonies. Because both groups contain workers with less developed ovaries, we could alternatively hypothesize that worker sterility is maintained by the upregulation of genes with regulatory functions, which are then downregulated when workers become fecund following queen removal, explaining the large overlap of genes between the two groups.

The candidate gene "transketolase-like protein 2 isoform X2" was commonly downregulated in infertile workers and workers treated with the two chemical inhibitors, despite the relatively small number of genes (i.e., five) in the two lists of commonly downregulated genes. Transketolases are enzymes involved in the non-oxidative part of the pentose phosphate pathway (PPP) in all living organisms (Schenk et al. 1998). They are known to maintain low levels of reactive oxygen species (ROS) and are thus used in cancer treatment (Xu et al. 2016) and parasitic disease control (Kovarova et al. 2018). More broadly, antioxidant production has been positively linked to lifespan in the fruit fly (Orr and Sohal 1994) and the nematode *Caenorhabditis elegans* (Sampayo et al. 2003), and has also been associated with long-lived ant queens (Negroni et al. 2019).

By experimentally manipulating histone (de)acetylation we show that this epigenetic mark might be required for workers to dynamically shift their physiology

following queen removal. Our manipulation did not only affect life history traits such as fecundity, but also shifted the expression of genes with fecundity, immunity, and longevity functionalities. Our results thus provide insights into the molecular regulation of reproduction in social insects, which are prime examples of phenotypic plasticity.

Author contributions

MC, BF, and SF collected the ant colonies and designed the study. MC conducted the experiment and analyzed the transcriptomic data. All three authors contributed to data interpretation, writing, and approval of the final manuscript.

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CHAPTER 3

Inhibiting histone (de)acetylation results in higher aggressiveness, but lower ovarian development in ant workers following queen loss

Marina Choppin, Barbara Feldmeyer, and Susanne Foitzik

Unpublished data

Abstract

Phenotypic plasticity plays an important role in social insects. Not only does it lead to the development of large, long-lived, and fertile queens and small, short-lived, and sterile workers from the same genomic background, but it also allows a rapid response to environmental changes in the adult stage. For example, workers of many ant species can become fertile following the loss of their queen. They establish a new reproductive hierarchy through antagonistic interactions and the resulting dominant workers start to lay eggs, often live longer, and exhibit expression changes in longevity and fertility genes. Nevertheless, the regulatory mechanisms mediating these plastic molecular changes are poorly understood. Here we used chemical inhibitors of histone acetylation (C646) and deacetylation (TSA) to investigate whether gene expression changes induced by this histone modification affect the behavior and fertility of ant workers following queen removal. Queenless workers used aggressions to establish dominance hierarchies, but these aggressive interactions were more frequent and lasted longer in queenless colonies treated with the inhibitors of histone (de)acetylation. This indicates that workers' behavioral plasticity remains unaffected by changes in histone acetylation, but that these changes might influence the ability of workers to remember the outcome of dominance interactions, thus prolonging the fights. Moreover, fewer workers became fertile in queenless colonies where histone (de)acetylation was inhibited, and this reduction of worker fecundity was more pronounced under the inhibition of histone acetylation. Our findings suggest that the two components of workers' plastic response to the loss of their queen (aggressive dominance interactions and ovarian development) might be regulated independently on a molecular level. Future analyses of gene expression and histone acetylation via CUT&TAG will shed more light on the molecular underpinnings of worker reproduction.

Introduction

Phenotypic plasticity can be defined as the ability of genotypes to produce distinct phenotypes under different environmental conditions (West-Eberhard 1989; Whitman and Agrawal 2009). Behavioral, physiological, or even life-history traits can be plastic and organisms highly benefit from modulating these traits in response to changing environments (Ghalambor et al. 2007). In the case of transgenerational plasticity, parents can even modulate the traits of their offspring (Salinas et al. 2013). Well-known examples of phenotypic plasticity include clones of the water flea *Daphnia* that display different morphologies depending on whether they developed in presence of predators or not (Tollrian 1995), or the winter moth *Operophtera brumata* whose hatching time is synchronized with the bud break of its host tree (Dongen et al. 1997).

Phenotypic plasticity is of great importance in social insects, as individuals of the two female castes (queens and workers) have similar genotypes, but very different phenotypes, which are expressed via developmental plasticity depending on environmental factors (Simpson et al. 2011; Corona et al. 2016). In social Hymenoptera, the large, long-lived queens typically monopolize reproduction, whereas workers constitute the sterile, altruistic caste (Hölldobler and Wilson 1990). Nevertheless, workers of many ants (Heinze et al. 2002; Helanterä and Sundström 2005; Heinze 2008; Giehr et al. 2020a), bees (Oldroyd et al. 2001; Beekman and Oldroyd 2008; Princen et al. 2020), and wasps (Wenseleers et al. 2005) can reproduce, especially under queenless conditions. However, eusocial Hymenopteran workers of most species lack a spermatheca and are therefore only able to lay haploid, male-destined eggs, due to their haplodiploid sex-determination system. Therefore, monogynous colonies that have lost their queen will inevitably cease to exist in the long run. But workers in queenless colonies might still get direct fitness benefits from producing sons that will take part in mating flights, thereby

propagating worker genes (Giehr et al. 2020b). Worker reproduction is often physiologically triggered by the absence of fertility-signaling chemicals typically released by a fertile queen (Heinze et al. 2002; Van Oystaeyen et al. 2014; Oi et al. 2021). Queen loss thus opens up novel avenues for workers to gain direct fitness benefits. Workers of some *Temnothorax* ants have even been reported to reproduce in queenless parts of queenright colonies (Giehr et al. 2020a) and honey bee workers were shown to parasitically lay eggs in unrelated colonies (Beekman and Oldroyd 2008). Worker reproduction is also regulated by worker policing via behavioral aggressions or egg cannibalism (Foster and Ratnieks 2000, 2001; Oldroyd et al. 2001; Wenseleers et al. 2005). The intensity of worker policing depends on worker relatedness as it directly correlates with potential fitness consequences and colony efficiency, which should not be diminished by the lack of help from reproducing workers (Hammond and Keller 2004; Wenseleers et al. 2004). Overall, worker reproduction seems to be more widespread and adaptive than originally thought.

During the period when they become fertile, female workers show profound behavioral changes. In many ant species, queenless workers engage in fights leading to the establishment of a novel reproductive hierarchy where the number of reproductive workers varies between species and the size of the colony (Bourke 1988; Monnin and Peeters 1999; Heinze 2008). Similar reproductive hierarchies are found in bumblebees (Princen et al. 2020), social wasps (Dapporto et al. 2006), and honey bees (Page and Robinson 1994; Miller and Ratnieks 2001; Oldroyd et al. 2001). In addition to behavioral changes, fertile workers exhibit different chemical signatures (Peeters et al. 1999; Liebig et al. 2000; Sledge et al. 2001; Heinze et al. 2002; Oi et al. 2021) and longer lifespans (Hartmann and Heinze 2003; Kohlmeier et al. 2017; Negroni et al. 2020, 2021b; Choppin et al. 2021a). The interesting increase in lifespan observed in fertile workers might be

linked to the upregulation of longevity genes in signaling pathways like target-of-rapamycin (TOR) (Negroni et al. 2021b). In fact, the network of TOR and insulin/insulin-like growth factor 1 signaling (IIS) pathways, together with juvenile hormone, is considered to be a major player in the regulation of aging and fecundity in social insects (Korb et al. 2021).

Gene expression changes associated with phenotypic plasticity can be regulated via several epigenetic mechanisms including histone modification, DNA methylation, or non-coding RNA action (Geng et al. 2013; Duncan et al. 2014; Herrel et al. 2020). In Choppin et al. 2021a we suggest that *Temnothorax* ant workers might be able to transition from a short-lived, sterile to a longer-lived, fertile phenotype via changes in gene expression regulated by histone acetylation. Indeed, the contribution of epigenetics to the extraordinary phenotypic plasticity displayed by social insects has been extensively discussed in the past years (Bonasio 2012, 2014; Herb 2014; Yan et al. 2014, 2015; Vaiserman 2015; Maleszka 2016; Vaiserman et al. 2018; Richard et al. 2021) and demonstrated in many empirical studies. For example, in the harvester ant *Camponotus floridanus*, the behavioral differences between major and minor workers are associated with histone acetylation (Simola et al. 2013, 2016; Glastad et al. 2019). When workers of the ant *Harpegnathos saltator* become gamergates (i.e., reproducing workers), they show changes in gene expression that are linked to histone methylation (Bonasio et al. 2010). In the honey bee, queen development is largely controlled by a secretive substance, the royal jelly, that has histone deacetylase inhibitor activity (Spannhoff et al. 2011), and specific histone genes are differentially methylated in the brain of queens and workers (Foret et al. 2012). While these studies highlight the significant role of histone modification for phenotypic plasticity in social insects, DNA methylation appears to be of lesser importance (Libbrecht et al. 2016; Glastad et al. 2017; Cardoso-Júnior et al. 2021).

Similar to Choppin et al. 2021a, we induced worker fecundity by removing queens from their colony, while feeding ant workers inhibitors of histone acetylation (C646; Bowers et al. 2010) or deacetylation (Trichostatin A: TSA; Yoshidas 1990). However, we examined in more detail the plastic changes displayed by workers following queen loss. Indeed, we monitored worker behavior for ten days after queen removal to better characterize the establishment of their reproductive hierarchy. We then reported the number of workers and egg production over four weeks and assessed ovarian development. We collected fat body samples for future gene expression analysis associated with the investigation of histone acetylation profiles via the cutting-edge technology of cleavage under targets and tagmentation (CUT&TAG; Kaya-Okur et al. 2020). If the establishment of workers' reproductive hierarchy is regulated by histone (de)acetylation, we expected the inhibitors to alter the behavior of workers following queen removal. Based on our previous findings, we hypothesized that queen removal would induce worker fecundity while the chemical inhibitors would impair the ovarian development of workers in queenless colonies. Finally, we predict that fertility and longevity genes that regulate worker reproduction will have distinctive acetylation patterns driving their differential expression.

Methods

Ant collection and maintenance

Our study species *Temnothorax rugatulus* is a small North-American ant. Colonies of these ants can be found in coniferous forests at high elevations where they reside under rocks or in crevices. The two queen morphs in this ant species display alternative reproductive strategies (Rüppell et al. 1998, 2001a; Choppin et al. 2021b). The large macrogynes establish their colony independently and occupy their colony only by themselves, while

the small microgynes seek readoption by their mother colony and thus co-exist with other queens in large, polygynous colonies. Ant colonies were collected in the Chiricahua Mountains (Arizona, USA) in August 2018. In the laboratory, they were kept in three-chambered boxes (9.7 x 9.7 x 2.9 cm) with a lid and microscopic slide nests covered with red foil to block off the light. Ant colonies were maintained at 21°C and 70% humidity with a 12:12 light:dark cycle and fed weekly with half a cricket and honey. To increase behavioral activity, we moved the ant colonies used in our experiments to a climate chamber at 25°C with a similar humidity and light:dark cycle. These experimental colonies were fed with a control or treatment diet as described below.

Experimental setting

We selected 64 colonies with a single macrogyne queen and 35 to 140 workers. We removed workers so that colonies contained between 30 and 70 workers in addition to the brood. Each colony was randomly assigned to one of four groups with a total number of 16 colonies per group (Table S1). One week before we started the experiment, we removed eggs, pupae, males, and small callows from the experimental colonies and we counted the number of workers and larvae in each experimental colony. Five days before removing the queens, we started feeding treated colonies with the inhibitor of histone acetylation C646 (50μM in Dimethylsulfoxide: DMSO) or the inhibitor of histone deacetylation TSA (50 μM in DMSO), while control colonies received the solvent DMSO only. All solvents were diluted in 0.102 g/mL of sucrose solution and the ants were fed every other day with 15 μL of the solution per colony, for four weeks. Additionally, each colony received half a cricket every other day and was provided with *ad libitum* water. The experiment was divided into two cohorts with identical experimental designs because conducting the experiment with all colonies at once would have been logistically

challenging. The first cohort was done in June-July 2019 (32 colonies) and the second one in October-November 2019 (32 colonies).

Worker behavior, survival, and fertility

On Day 0 we anesthetized the ants in the colonies using CO_2 to remove the queens and return them to their original colony. We cleaned the forceps using hexane after each queen transfer to avoid spreading colony odor. Queenright colonies were also anesthetized to experience a similar disturbance. We started the behavioral observations on Day 1, 16 hours after queen removal. Before each session, we removed the red foil covering the slide nest and allowed the ants to calm down for five minutes before observing them. Each colony was observed under a stereomicroscope for five minutes, twice per day, during which we recorded all antagonistic interactions between workers (antennal boxing, mandible opening, and biting). The observations were performed blind, for ten days. Additionally, we monitored worker number and egg production in the colonies once per week. Four weeks after the start of the experiment, we dissected on dry ice up to ten randomly chosen workers located near the brood pile. We collected the fat body, head, and thorax of each ant in empty tubes that were then stored at -80°C until further processing. For each ant dissected, we additionally took a picture of the ovaries using a Leica stereomicroscope. We measured ovariole length and counted the number of eggs in development in the ovaries using the Leica software LAS v4.5. The complete experimental timeline can be found in Figure S1.

We analyzed worker aggressiveness by calculating the sum of all aggressive events reported in one colony (antennal boxing, mandible opening, and biting), divided by worker number, and normalizing the response variable using an arcsine transformation. We analyzed the effects of queen removal and treatment on worker aggressiveness using

two separate linear mixed-effects models (LMMs). We also used two LMMs to analyze the effects of queen removal and treatment on worker survival, with worker number, day, and their interaction as response variables. Because *Temnothorax* ants usually have low egg production rates (Stroeymeyt et al. 2007), we analyzed the effects of queen removal and treatment on egg production with two generalized linear mixed-effects models (GLMMs, binomial family) using a binary response variable (presence/absence of eggs in the colony at Day 24, i.e. the last counting day). We calculated the proportion of fertile workers per colony and tested for effects of queen removal and treatment on this measure using two GLMMs (binomial family). Workers were considered fertile when they had at least one egg in development in the ovaries. We analyzed the effects of queen removal and treatment on worker ovariole length with two LMMs. Finally, we analyzed the effects of queen removal and treatment on the number of developing eggs (white eggs) in worker ovaries using two GLMMS (Poisson family). Colony identification (ID) was used as random factor when relevant and cohort was used as a covariate in all models to account for cohort-associated variation. Model fit was assessed by visual inspection of the residual distribution (LMMs) or using the package 'DHARMa" (GLMMs; Hartig 2020). All analyses were conducted in R v4.1.0 (R Core Team 2020).

Results

Queenless colonies showed a peak of worker aggressiveness from Day 1 to Day 3 compared to queenright colonies (Figure 1A). From Day 3 on, the level of aggression in queenless colonies decreased, although it remained higher than in queenright colonies. Hence, we can say that queen removal increased the overall worker aggressiveness (Figure 1B; $X^2 = 49.682$, df = 1, $p < 0.001$). However, changes in worker number over time

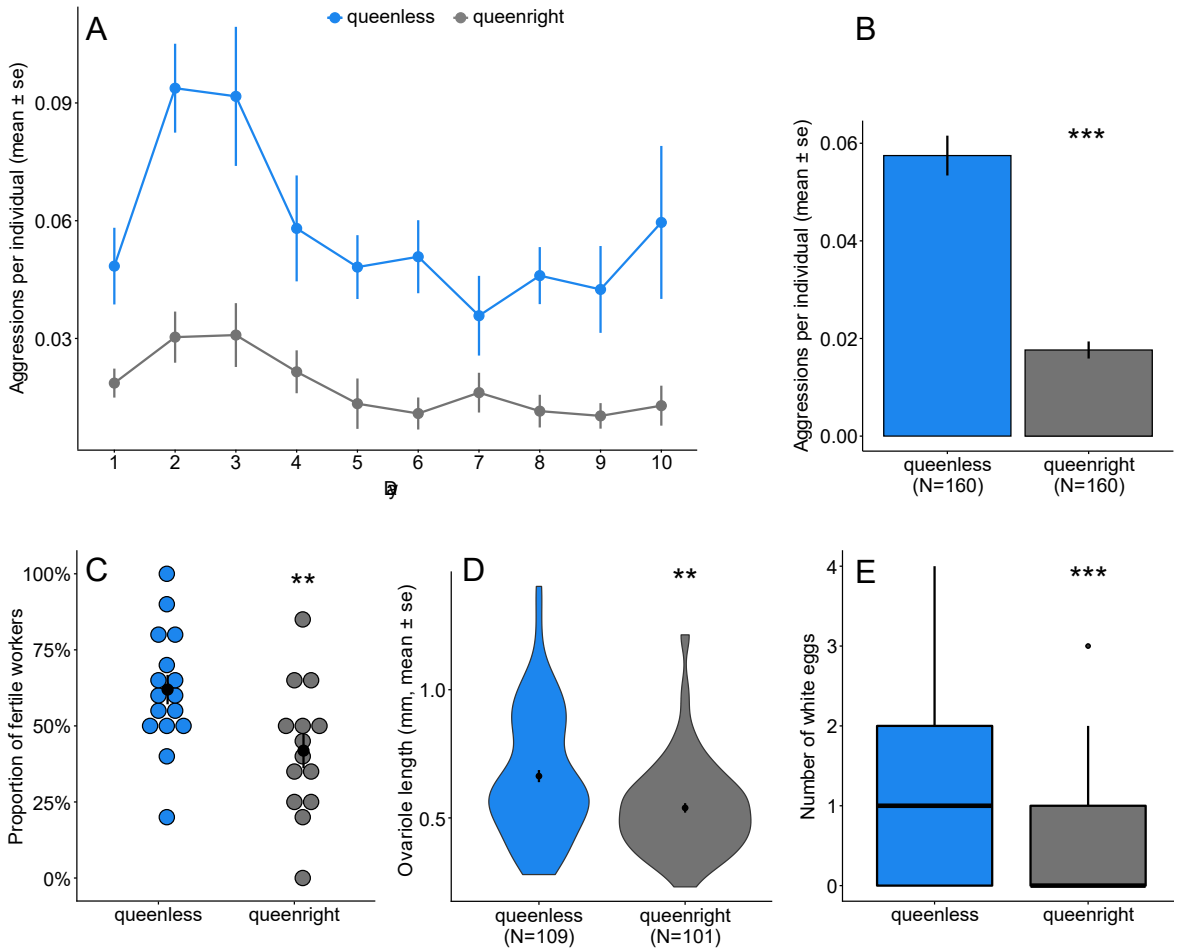

Figure 1. (A) Changes in worker aggression over time in queenless (blue, control) and queenright (grey) colonies. Queens from queenless colonies were removed 16 hours before Day 1. **(B)** Overall effect of queen removal on worker aggressiveness. **(C)** Proportion of fertile workers in queenless and queenright colonies. Each dot represents a colony. Means and associated standard errors are shown by the black dots and vertical bars. Workers were considered fertile when at least one egg in development was counted in their ovaries. Queen removal effects on **(D)** worker ovariole length and **(E)** the number of eggs in development in their ovaries. The levels of significance are indicated as follows: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

were not affected by queen removal (Figure S2; $X^2 = 0.233$, df = 1, p = 0.629). Queenless colonies had significantly more fertile workers (i.e., workers with at least one egg in development in the ovaries) than queenright colonies (Figure 1C; $X^2 = 7.017$, df = 1, p = 0.008), and queenless workers had both longer ovarioles (Figure 1D; $X^2 = 10.166$, df = 1, p = 0.001) and more eggs in development (white eggs) in their ovaries (Figure 1E; $X^2 = 11.837$, df = 1, $p < 0.001$), compared to workers from queenright colonies.

The chemical inhibitor treatment affected the aggressiveness of queenless workers (Figures 2A-B; $X^2 = 7.270$, df = 2, p = 0.026), and both C646 (t = 2.170, $p < 0.035$) and TSA (t = 2.471, $p < 0.017$) treated workers were more aggressive than queenless workers fed with DMSO only. Changes in worker number over time was not affected by the chemical inhibitor treatment (Figure S3; $X^2 = 0.451$, df = 2, p = 0.798). Very few eggs were laid during the four weeks of experiment (1.5 on average in queenless, control colonies) and the number of eggs found in queenless colonies at Day 24 was not affected by the chemical inhibitor treatment (Figure S4; $X^2 = 0.743$, df = 2, p = 0.690). Nevertheless, the inhibitor treatment affected the proportion of fertile workers in queenless colonies (Figure 2C; $X^2 = 10.967$, df = 2, p = 0.004), and both C646 (z = -3.228, p = 0.001) and TSA (z = -2.090, p = 0.037) treated colonies had fewer fertile workers compared to control colonies. Additionally, the inhibitor treatment impaired ovarian development in terms of ovariole length (Figure 2D; $X^2 = 6.508$, df = 2, p = 0.039) and white eggs (Figure 2E; $X^2 = 7.527$, df = 2, p = 0.023). C646-treated workers had both shorter ovarioles (t = -2.534, p = 0.015) and fewer white eggs in the ovaries (z = -2.694, p = 0.007), while TSA-treated workers only tended to have shorter ovarioles (z = -1.651, p = 0.099).

In short, queen removal increased aggressiveness and induced fecundity in ant workers, as expected from earlier studies. However, C646 and TSA treatments resulted in

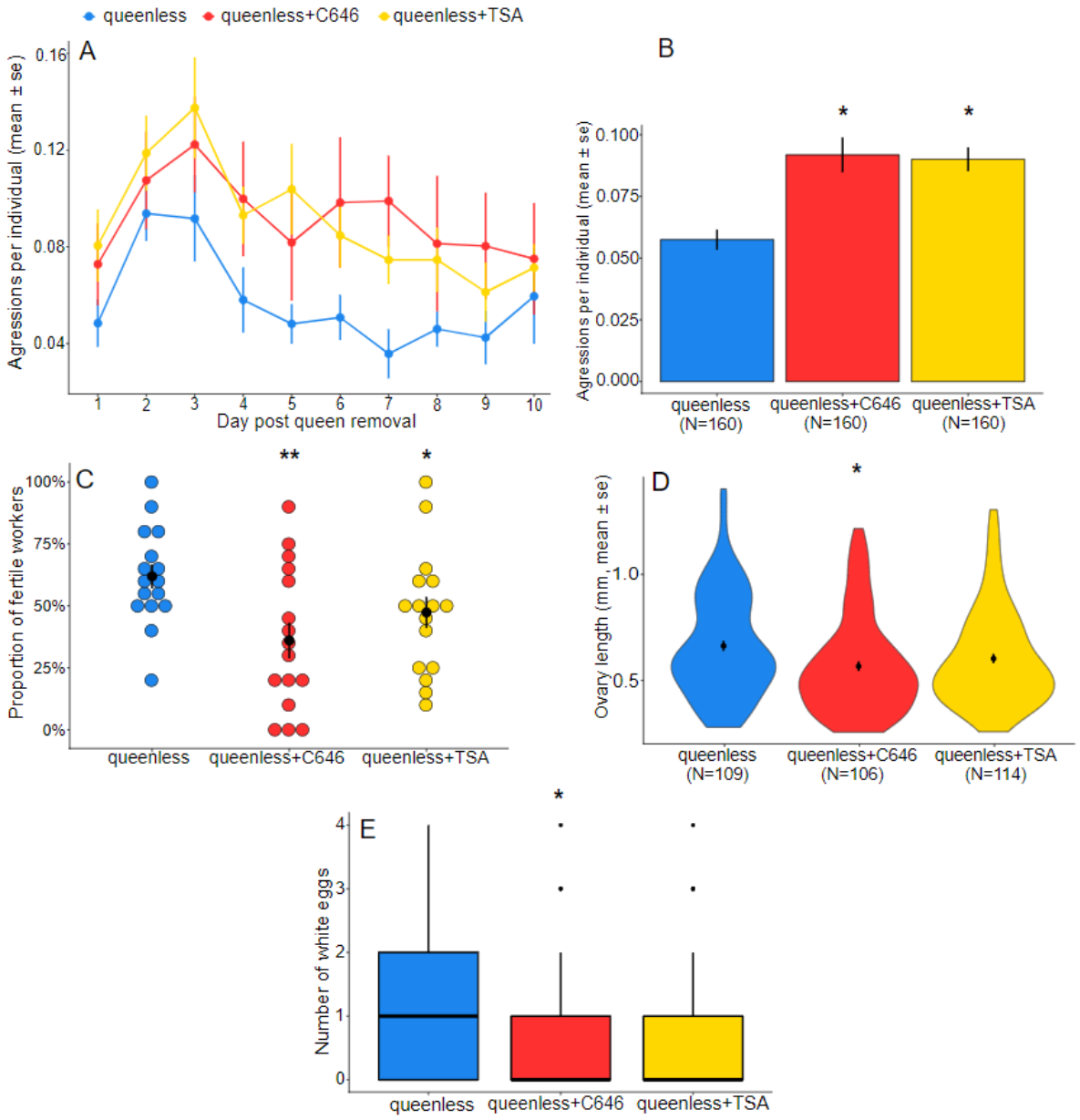

Figure 2. (A) Changes in worker aggressiveness over time in queenless (blue, control), C646-treated (red), and TSA-treated (yellow) colonies. Queens from queenless colonies were removed 16 hours before Day 1. **(B)** Overall treatment effect on worker aggressiveness. **(C)** Proportion of fertile workers in queenless colonies treated or not with the chemical inhibitors. Each dot represents a colony. Means and associated standard errors are shown by the black dots and vertical bars. Workers were considered fertile when at least one egg in development was counted in their ovaries. Treatment effects on **(D)** worker ovariole length and **(E)** the number of eggs in development in their ovaries. The levels of significance are indicated as follows: * $p < 0.05$ and ** $p < 0.01$ and correspond to comparisons with the queenless control.

even higher levels of aggressiveness while impairing ovarian development when the queen was absent.

Discussion

In social insects, histone modification has recently been acknowledged as one of the most important epigenetic regulators of caste differentiation. In this study, we investigated whether histone acetylation is associated with the increase in fecundity and aggressive behavior during the establishment of reproductive hierarchies in ant workers. In line with the results of our previous study, we found that histone acetylation might indeed play a role in the regulation of worker reproduction in ants (Choppin et al. 2021a). We found that the chemical inhibitors of histone acetylation C646 and histone deacetylation TSA reduced the proportion of fertile workers in queenless colonies, and C646 even impaired ovarian elongation and oocyte development after queen removal. Interestingly, workers in queenless colonies that were fed with C646 or TSA were more aggressive than workers in queenless colonies fed with the solvent DMSO only. Indeed, aggression levels in the inhibitor-treated colonies remained higher than in control colonies throughout the ten days of observation.

The induction of worker fertility via queen removal exists in multiple social insect species (Miller and Ratnieks 2001; Oldroyd et al. 2001; Helanterä and Sundström 2005; Heinze 2008; Lopes et al. 2020; Giehr et al. 2020b; Princen et al. 2020; Negroni et al. 2021b). However, the underlying regulatory mechanisms are still poorly understood. Our study shows that fewer workers were fertile in C646 and TSA-treated colonies and that C646-treated workers had shorter ovarioles and fewer developing eggs in the ovaries. Two studies demonstrated an association between histone (de)acetylation and fertility in other insect species, the brown planthopper *Nilaparvata lugens* (Zhang et al. 2018) and

the pea aphid *Acyrthosiphon pisum* (Kirfel et al. 2020), which is consistent with our results. However, we were not able to detect a difference in the number of eggs laid between treated and control colonies. We can speculate that four weeks under queenless conditions might have been too short to detect treatment effects on egg production. Indeed, the overall egg production was very low as only 1.5 eggs were laid in the queenless, control colonies on average after four weeks. This number is not too surprising knowing that *Temnothorax* ants have low egg production rates (Stroeymeyt et al. 2007). In addition, worker policing via egg cannibalism has been observed in many social insect species (Page and Robinson 1994; Foster and Ratnieks 2001; Wenseleers et al. 2005), and this behavior could also explain the low number of eggs laid in the colonies. Nevertheless, the presence of eggs in development in the ovaries indicates that workers were in the process of producing eggs.

In contrast to most organisms, fecundity and longevity are positively correlated in social insects, as queens monopolize reproduction while living for several decades. The same association has been observed in fecund workers after queen removal, including in the focal species of this study (Negroni et al. 2020, 2021b; Choppin et al. 2021a; Majoe et al. 2021). However, here, worker number was neither affected by queen removal nor by the chemical inhibitor treatment. Once again, the duration of our experiment was probably too short to detect an effect of queen removal on survival. Indeed, we monitored worker number for four weeks, against six weeks in our previous study wherein queenless workers survived better (Choppin et al. 2021a). In fact, the lifespan extension observed in workers from queenless colonies is much clearer in long-term studies both in our species *Temnothorax rugatulus* (Negroni et al. 2020, 2021b; Majoe et al. 2021) and other ant species (Kohlmeier et al. 2017; Majoe et al. 2021). Such lifespan extension of fertile workers has also been observed in other insect species like the neotropical

stingless bee *Scaptotrigona* aff. *postica* (Lopes et al. 2020), the honey bee *Apis mellifera* (Kuszewska et al. 2017), and the bumblebee *Bombus terrestris* (Blacher et al. 2017).

In Konrad et al. 2012 the authors used the closely related species *Temnothorax longispinosus* to investigate the effects of behavioral and chemical signals from the queen on workers. They found that workers without a queen but exposed to her volatiles developed their ovaries, but did not change their behavior, while workers in complete separation from the queen and her fertility signals both developed their ovaries and showed aggressive dominance interactions. These results suggest an independent regulation of worker behavior and fecundity, and resemble the pattern observed in our study. Indeed, although workers treated with the chemical inhibitors of histone (de)acetylation remained in a queenright state in terms of fecundity after queen removal, their aggressive behavior was similar to the one of workers in queenless, control colonies. Although worker aggressive behavior in treated colonies was even more pronounced and longer-lasting. Hence, we propose that the physiological and behavioral components of worker plastic response to queen removal might be independently regulated on the molecular level.

The role of epigenetics in memory-related processes is well known and has been extensively discussed (Levenson and Sweatt 2005; Day and Sweatt 2011). In mice, the crucial function of the histone acetyltransferase CREB-binding protein (CBP) in the consolidation of memories has been demonstrated using the inhibitor of histone acetylation C646 (Maddox et al. 2013) and by knocking out CBP in the hippocamp (Barrett et al. 2011). In the honey bee, experimental manipulations have revealed the association of olfactory memory with both DNA methylation (Lockett et al. 2010) and histone acetylation (Lockett et al. 2014). These studies led us to speculate that the higher aggressiveness seen in inhibitor-treated colonies might be linked to the alteration of

worker memory induced by the disruption of histone acetyltransferases and deacetylases' activity. Indeed, studies showed that novel reproductive hierarchies formed by workers are well structured with dominant workers monopolizing egg-laying while subordinate workers continue to perform their usual tasks in the colony (Bourke 1988; Monnin and Peeters 1999; Trunzer et al. 1999; Heinze 2008). The maintenance of these newly-formed reproductive hierarchies likely requires the use of worker memory and can be reinforced by other signals such as chemical signatures that indicate the reproductive status of individuals (Peeters et al. 1999; Liebig et al. 2000). Therefore, feeding the ants with chemical inhibitors of histone (de)acetylation might have altered their ability to store information about the outcome of dominance interactions and consequently disrupted the establishment and maintenance of novel reproductive hierarchies, explaining the higher levels of aggression in treated colonies during the ten days of observation.

Alternatively, the higher aggressiveness of treated colonies could be explained by the direct regulation of worker behavior via histone acetylation. Behavioral epigenetics has received increased attention in the past years, especially in social insects that represent great model organisms because of their caste polyethism (Yan et al. 2014; Opachaloemphan et al. 2018). Using experimental manipulations, it has been demonstrated that histone acetylation regulates foraging behavior in the ant *Camponotus floridanus* (Simola et al. 2016) and plays a role in the adjustment to daily rhythms in *Temnothorax longispinosus* (Libbrecht et al. 2020). Thus, workers' behavioral response to queen loss in *T. rugatulus* might involve gene expression changes in the brain that are regulated by histone acetylation. As potential gene candidates, we can cite tachykinins which are neuropeptides regulating behavior in many organisms including invertebrates (Severini et al. 2002). Aggression is positively correlated with *Tachykinin* expression in the brain of *Acromyrmex echinatior* workers (Howe et al. 2016). On a similar note, the

Africanized and European honey bees show very different levels of aggression that have been associated with changes in the regulation of genes involved in the response to alarm pheromone (Alaux et al. 2009). Finally, genes associated with reproductive dominance in the paper wasp *Polistes metricus* were found to be linked with aggressive behavior in other insects and mammals (Toth et al. 2014). One way to get insights into the potential epigenetic regulation of such candidate genes is to investigate protein-DNA interactions using protocols like CUT&TAG.

The CUT&TAG technology allows to target proteins of interest like transcription factors or histone marks using antibodies and to retrieve associated fragments of DNA using a fusion protein pre-loaded with sequencing adapters (Kaya-Okur et al. 2020). The DNA is then extracted, amplified, and sequenced. Bioinformatic analyses using peak-calling software are used to determine what regions of the genome are differentially acetylated. Using this technology in combination with RNA-sequencing, we want to investigate whether candidate genes with behavior, longevity, and fecundity functions have different acetylation profiles, which would allow us to confirm the role of histone acetylation in the regulation of worker reproduction in our ant species. In the fat body, we will focus on gene candidates found in previous studies such as "vitellogenin 1" which is upregulated in queenless workers (Choppin et al. 2021a) and has a well-known reproductive function in insects (Amdam et al. 2003). We also want to assess acetylation patterns in combination with the expression of other genes coding for proteins that have known longevity and fecundity functions such as transketolases (Xu et al. 2016; Kovarova et al. 2018), juvenile hormone (Hartfelder 2000; Matsuura et al. 2010), or catalases (Negroni et al. 2021b). In the brain, we want to look at genes associated with aggression, like tachykinins (Howe et al. 2016), among others discussed above.

To summarize, we know that ant workers that are mostly sterile in the presence of the queen can plastically respond in absence of fertility signals. By doing so, they display profound changes in behavior, chemical profiles, and ovary development. On a molecular level, some genes have been associated with the plastic transition from sterility to fertility in workers, but the regulatory mechanisms underlying these changes in gene expression are yet to be elucidated. Our study sheds light on how histone modification could be one of these regulatory mechanisms, as histone acetylation seems to play a role in worker reproduction. Our results also indicate that the behavioral and physiological responses of workers to queen removal might be differentially regulated, highlighting the complexity of the molecular mechanisms regulating phenotypic plasticity in social insects.

Author contributions

MC, BF, and SF collected the ant colonies and designed the study. MC conducted the experiment and analyzed the data. MC wrote the first draft of the manuscript and the three authors contributed to its improvement.

Acknowledgments

Many thanks to Dr. Romain Libbrecht for his help with the statistical analysis of the behavioral data.

Supplementary material

Table S1. Group name, manipulation, treatment, and sample size for each experimental group. The queenless control is our reference because it can be compared to all the other experimental groups while differing in one factor only: queen presence or inhibitor treatment.

Group name	Manipulation	Treatment	Colonies
Queenright	No queen removal	DMSO	N = 16
Queenless	Queen removal	DMSO	N = 16
Queenless+C646	Queen removal	DMSO+C646	N = 16
Queenless+TSA	Queen removal	DMSO+TSA	N = 16

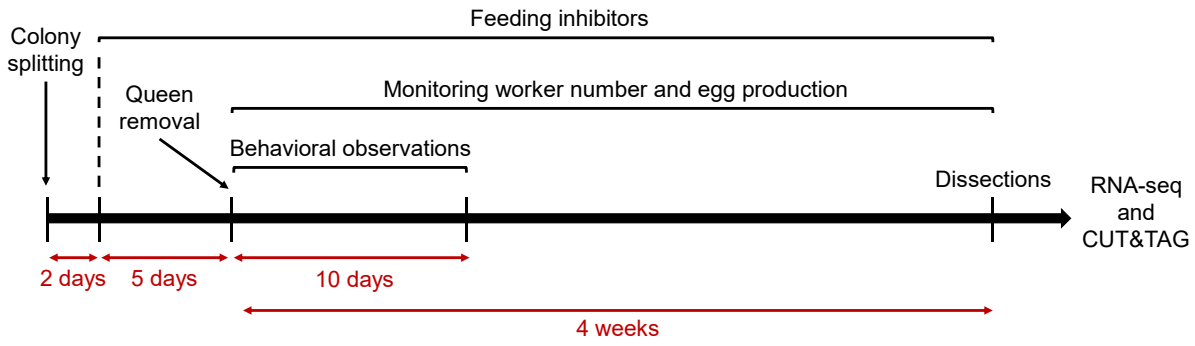

Figure S1. Experimental timeline

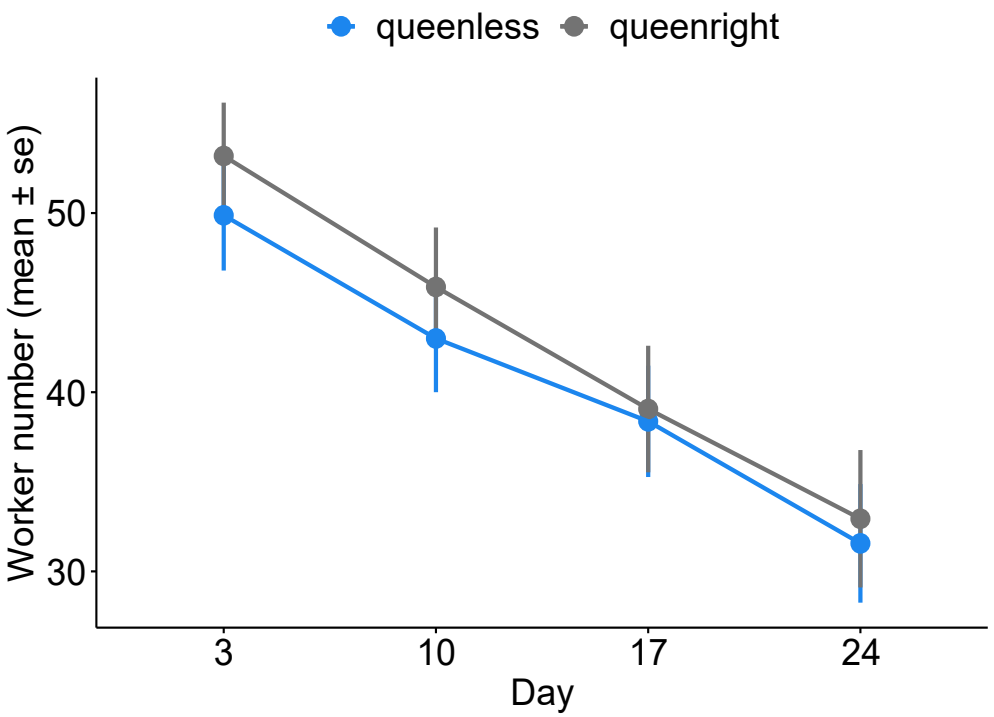

Figure S2. Changes in worker number over time in queenless (blue, control) and queenright (grey) colonies.

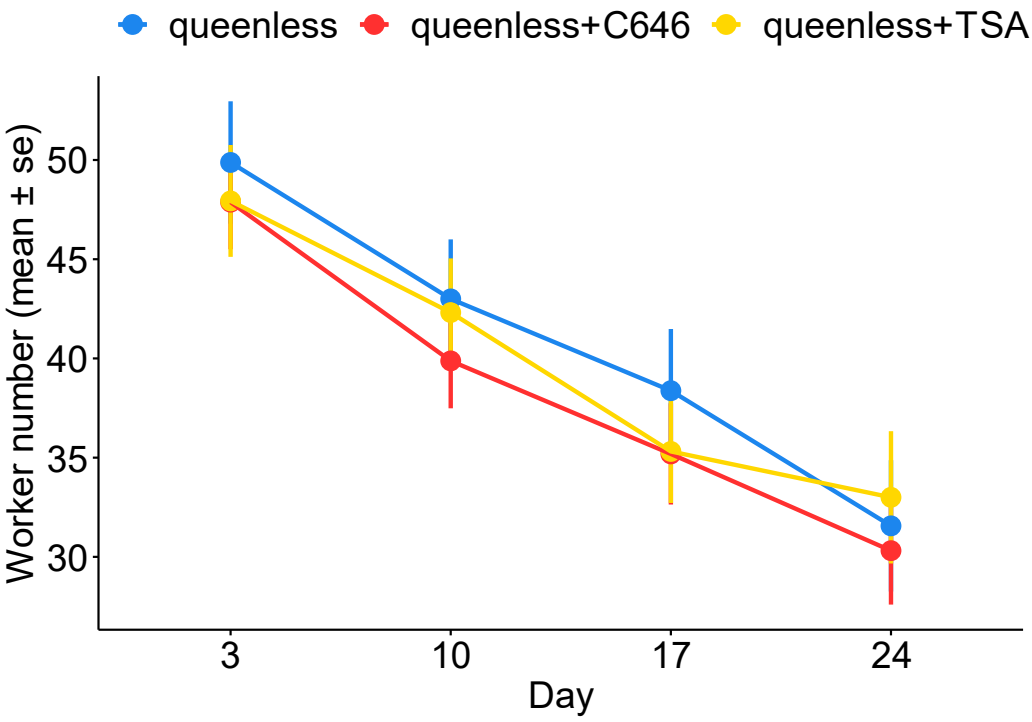

Figure S3. Changes in worker number over time in queenless (blue, control), queenless+C646 (red), and queenless+TSA (yellow) colonies.

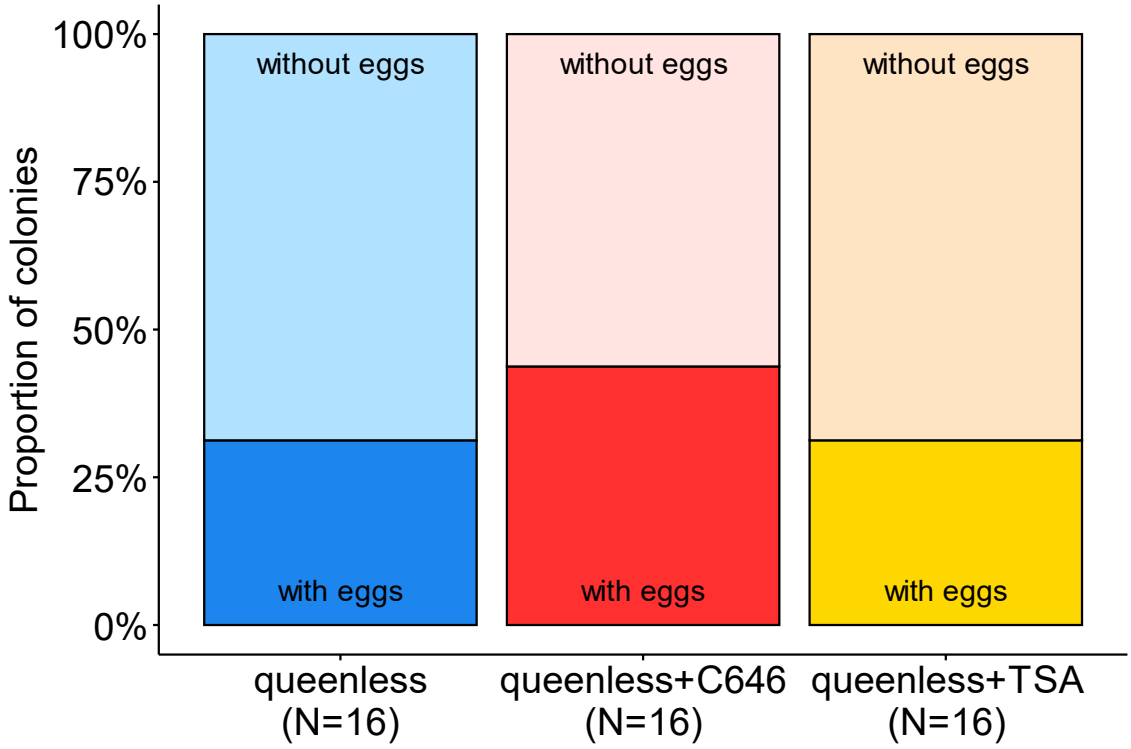

Figure S4. Proportion of colonies with eggs at the end of the experiment (Day 24) depending on the treatment.

CHAPTER 4

Experimentally increased fecundity in ant queens might be facilitated by histone acetylation

Marina Choppin, Barbara Feldmeyer, and Susanne Foitzik

Unpublished data

Abstract

Not only can social insect queens live for decades, but they also remain highly fecund throughout their long lives. This positive association between fecundity and longevity in social insects has been linked to diverse proximate mechanisms such as reduced extrinsic mortality or oxidative stress resistance. Queen fecundity can also be experimentally increased with manipulations like egg removal, and this plastic increase in fecundity is associated with the upregulation of genes involved in reproduction and lifespan. Because epigenetic mechanisms are known to be key players in the regulation of phenotypic plasticity in social insects, we decided to investigate the role of histone acetylation in the regulation of queen fecundity in the ant *Temnothorax rugatulus*. We removed newly-laid eggs in colonies weekly, while feeding chemical inhibitors of histone (de)acetylation to the ants. We found that egg removal increased egg production as expected, but not in colonies fed with the chemical inhibitors of histone (de)acetylation. This finding suggests that the upregulation of fecundity genes associated with the plastic increase in queen fecundity might require the activity of histone acetyltransferases and deacetylases. Our study enriches existing knowledge about the role of epigenetic mechanisms in the regulation of social insect extraordinary phenotypic plasticity.

Introduction

Social insect queens have always fascinated the scientific community because they live extremely long lives (a couple of decades) compared to solitary insects (Keller and Genoud 1997; Korb 2016). Even more impressive, whereas most living organisms show a decline in reproductive function with age (Partridge and Barton 1993), social insect queens retain high reproductive potential throughout their long lives, and fecundity is thus positively associated with longevity (Schrempf et al. 2005, 2017; Heinze and Schrempf 2008, 2012; Negroni et al. 2016). Termite queens even show non-linear senescence with a sudden physiological disruption at older ages right before they die (Monroy Kuhn et al. 2021). It has been hypothesized that the absence of selection shadow in older queens could be due to late-life fitness benefits obtained via the production of sexuals (Jaimes Nino et al. 2021). Besides, the extreme longevity of social insect reproductives has been linked to sociality since group-living provides fitness benefits and decreases extrinsic mortality (Carey 2001; Korb 2016; Lucas and Keller 2020; Korb and Heinze 2021). Supporting this theory, other social, non-insect organisms such as the naked mole-rat display unusually long lifespans as well, compared to their solitary relatives (Schmidt et al. 2013). Furthermore, older ant queens display strong anti-aging mechanisms as they invest more in antioxidant production with age (Negroni et al. 2019). The long lifespans of honey bee queens have been associated with various proximate mechanisms like nutrition (Kunugi and Ali 2019), oxidative stress resistance (Corona et al. 2007), or the rewiring of endocrine pathways (Rüppell et al. 2016), and gene expression patterns are associated with longevity in *Apis mellifera* (Corona et al. 2005). Similarly, older social insect queens do not show typical aging signals in their transcriptome compared to older flies (Von Wyschetzki et al. 2015), but rather transcriptomic signatures of high fecundity and long lifespans (Harrison et al. 2021; Lin

et al. 2021). Despite the existence of taxon-specific transcriptomic changes with age, a comparative analysis revealed the common, important role of juvenile hormone and other downstream components of target of rapamycin (TOR) and insulin/insulin-like growth factor 1 signaling (IIS) pathways in the regulation of aging and fecundity in social insects (Korb et al. 2021).

Other than not being traded off with lifespan, queen reproduction also appears to be rather plastic in social insects. Indeed, egg removal can increase queen fecundity in the ant *Cardiocondyla obscurior* (Schrempf et al. 2017) and our study species *Temnothorax rugatulus* (Negroni et al. 2021a). In the latter, the experimentally-induced increase in fecundity is associated with the upregulation of genes that have fecundity and body maintenance functions (Negroni et al. 2021a). The role of epigenetics in the regulation of insect life-history traits has been discussed in an increasing number of reviews over the past years (Bonasio 2012, 2014; Herb 2014; Yan et al. 2014, 2015; Vaiserman 2015; Maleszka 2016; Vaiserman et al. 2018). Although the epigenetic basis of aging has received more attention, empirical studies point to the role of histone modification in the regulation of reproduction as well. For example, the inhibition of histone (de)acetylation affects multiple traits like development, fertility, and longevity in the pea aphid (Kirfel et al. 2020). In the planthopper *Nilaparvate lugens*, multiple histone deacetylases are crucial for both male and female fertility (Zhang et al. 2018). Besides, our previous study using the ant *T. rugatulus* suggests a role of histone acetylation in the regulation of genes associated with worker reproduction in queenless colonies (Choppin et al. 2021a). These examples attest to the relevance of investigating epigenetic processes to better understand the molecular underpinnings of social insect queen fecundity.

Hence, we conducted an experiment using the ant *T. rugatulus* to examine whether the plastic increase in queen fecundity caused by egg removal might require the

regulation of fecundity genes using epigenetic mechanisms like histone modification. We frequently removed newly-laid eggs from colonies while feeding the ants with inhibitors of histone acetylation (C646), deacetylation (Trichostatin A; TSA), a combination of both, or the solvent (DMSO) only. After six weeks, we dissected queen ovaries for fecundity measures. If histone acetylation does play a role in regulating queen fecundity, we expected queens to be more fecund in colonies where the eggs were removed, as previously seen in this species (Negroni et al. 2021a), but not in colonies treated with the chemical inhibitors.

Methods

This experiment was conducted in parallel with a similar experiment using workers (Choppin et al. 2021). *Temnothorax rugatulus* colonies were collected and maintained as indicated in that study. We selected 18 polygynous (source) colonies with at least five queens and 250 workers, and we divided them into five monogynous (fragment) colonies with one queen, 50 workers, and five larvae. Before starting the experiment, colonies were randomly assigned to one of five experimental groups (Table 1) and moved to a climate chamber at 25°C and 70% humidity with a 12:12 light:dark cycle for two weeks to increase their activity. On the first day of the experiment, we removed all eggs, pupae, and males from the colonies. For six weeks, colonies were fed with the chemical inhibitors of histone (de)acetylation C646 and TSA as described in Choppin et al. 2021 (Table 1). Once a week, colonies in the egg removal groups were anesthetized using CO_2, and their eggs were removed and counted. Egg removal can increase queen fecundity (Schrempf et al. 2017; Negroni et al. 2021a). Colonies where the eggs were not removed experienced a similar disturbance with CO_2, but their eggs were only counted. Once every two weeks,

Table 1. Experimental group name, manipulation, treatment, and sample size for each experimental group. The egg removal control was always used as a reference in the comparisons with other experimental groups because it differs only in single factors (egg removal or inhibitor treatment).

Group name	**Manipulation**	**Treatment**	**Colonies**
No egg removal	No egg removal	DMSO	N = 18
Egg removal	Egg removal	DMSO	N = 18
Egg removal + C646	Egg removal	DMSO + C646	N = 18
Egg removal + TSA	Egg removal	DMSO + TSA	N = 18
Egg removal + C646/TSA	Egg removal	DMSO + C646/TSA	N = 18

we counted all colony members (queens, workers, eggs, larvae, and pupae). After six weeks, a subset of colonies was selected for dissections as per Choppin et al. (2021), and queens from these colonies were dissected in ice-cold, sterile saline solution under a Leica stereomicroscope. We cleaned the ovaries and took pictures for fecundity measures. Queen tissues were collected in empty tubes (ovaries and heads with thoraces) or tubes containing 50 µl of TRIzol (fat bodies), flash-frozen using liquid nitrogen, and stored at -80°C until further processing. Using the ovary pictures, we measured ovariole length and counted the number of eggs in development (white eggs) and yellow bodies in the ovaries using the Leica software LAS v4.5.

The effects of egg removal and treatment on worker number over time were analyzed using two separate linear mixed-effects models (LMMs). For each week, we calculated the number of new eggs in the colonies as follows: new eggs = number of eggs counted (egg removal group) and new eggs = number of eggs counted – number of eggs counted the week before (no egg removal group). We analyzed the effects of egg removal and treatment on the number of new eggs in the colonies at week 6 using two separate generalized linear-mixed effects models (GLMMs, Poisson family). In the no egg removal group, numbers of new eggs < 0 at week 6 were adjusted to 0 to facilitate the analysis. We tested for egg removal and treatment effects on the number of white eggs and the number of yellow bodies using four separate GLMMs (Poisson family). Model fit was assessed by visually inspecting the residual distribution for the LMMs and using the package DHARMa for the GLMMs (Hartig 2020). Both source and fragment colony identification (ID) were used as random factors to account for inter-colony variability when relevant. Statistical analyses were conducted in R v4.1.0 (R Core Team 2020).

Results

Changes in worker number over time were neither affected by egg removal (X_1^2 = 0.037, p = 0.847), nor by treatment (X_3^2 = 5.548, p = 0.136). Colonies in the egg removal group had more new eggs at week six compared to colonies in the no egg removal group (X_1^2 = 24.457, p < 0.001; Figure 1A), but not when treated with the chemical inhibitors of histone (de)acetylation (X_3^2 = 22.995, p < 0.001; Figure 1B). Egg removal did not affect ovariole length (X_1^2 = 0.141, p = 0.708; Figure 2A), the number of white eggs (X_1^2 = 0.607, p = 0.806; Figure 2B), or yellow bodies (X_1^2 = 0.027, p = 0.870; Figure 2C) in the ovaries. Queens subjected to egg removal in colonies fed with inhibitors of histone (de)acetylation did not differ in ovariole length (X_3^2 = 2.358, p = 0.502; Figure 3A) or number of yellow bodies (X_3^2 = 1.082, p = 0.611; Figure 3C) compared to queens in control colonies. However, they had fewer white eggs in the ovaries (X_3^2 = 2.187, p = 0.027; Figure 3B).

Discussion

In this study, we investigated the role of histone (de)acetylation in the regulation of queen fecundity by experimentally increasing queen fecundity while feeding colonies chemical inhibitors of histone (de)acetylation. Egg removal did not induce changes in queen ovaries in terms of ovariole length, eggs in development, or yellow bodies. However, egg production was higher in colonies where eggs were removed, as expected, but this increase in the number of new eggs produced was absent in colonies fed with chemical inhibitors of histone (de)acetylation. Queens from inhibitor-treated colonies also had fewer white eggs in the ovaries compared to queens from control colonies. Worker number remained constant over time in all colonies indicating that neither egg removal nor the chemical inhibitor treatment negatively affected the ants.

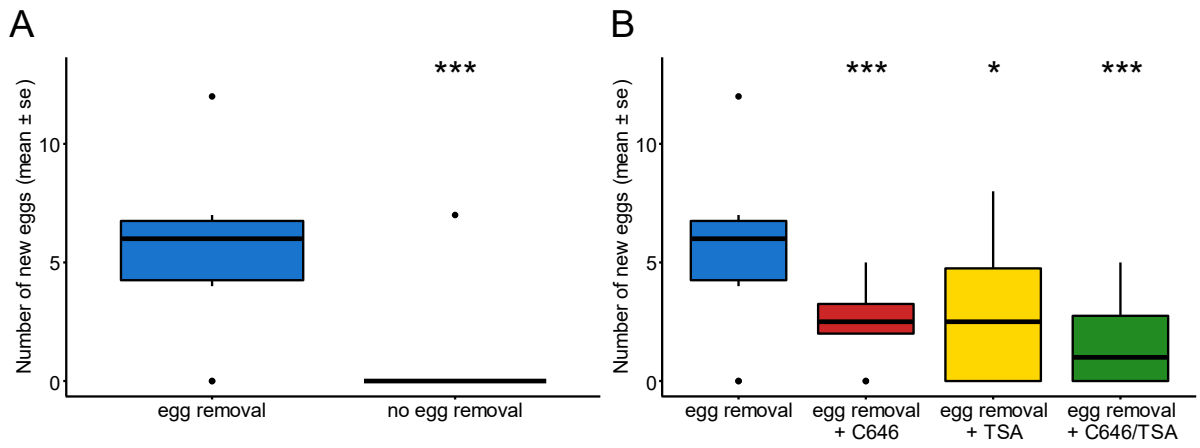

Figure 1. Number of new eggs in the colonies at week 6 depending on **(A)** the manipulation (egg removal or not) and **(B)** the treatment (solvent or chemical inhibitors). Levels of significance always refer to comparisons with the egg removal control and are indicated as follows: * $p < 0.05$ and *** $p < 0.001$.

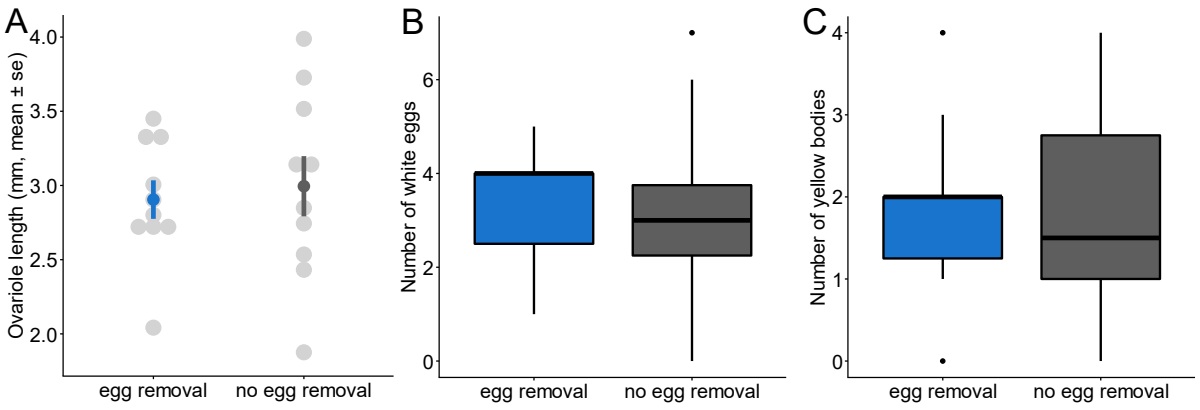

Figure 2. Effect of removal on **(A)** ovariole length, **(B)** the number of white eggs, and **(C)** the number of yellow bodies in the ovaries.

Previous work using the same ant species *Temnothorax rugatulus* showed that egg removal enhances queen fecundity and causes the upregulation of fecundity genes in the fat body of ant queens (Negroni et al. 2021a). Moreover, we have evidence that worker reproductive potential is associated with histone acetylation (Choppin et al. 2021a). Thus, the plastic increase in queen fecundity might require changes in the acetylation of histone-associated fecundity genes. The role of epigenetic mechanisms in the regulation of the impressive phenotypic plasticity displayed by social insects has been confirmed in several empirical studies (Bonasio et al. 2010; Spannhoff et al. 2011; Simola et al. 2013, 2016; Glastad et al. 2019; Choppin et al. 2021a). Moreover, a link between fertility and histone (de)acetylation has been established in two solitary insects (Zhang et al. 2018; Kirfel et al. 2020) but also mammals like mice (Ma et al. 2012) and humans (Wang et al. 2019). We can thus speculate that this epigenetic modification might regulate the reproductive output of queens by facilitating changes in the expression of specific fecundity genes.

In *T. rugatulus*, workers in queenless colonies start to develop their ovaries, live longer, and show transcriptomic changes in the fat body (Negroni et al. 2020, 2021b; Choppin et al. 2021a; Majoe et al. 2021). Moreover, histone acetylation seems to play a role in worker plastic reproduction by regulating the expression of genes with fecundity and longevity functions (Choppin et al. 2021a). Workers and queens differ in multiple traits and queens are by far the most fecund individuals in the colony (Hölldobler and Wilson 1990, 2009). Nevertheless, when comparing our findings from the worker and queen experiments, it seems that similar regulatory mechanisms might be at play when it comes to increasing individuals' fecundity, regardless of their caste. This would not be surprising considering the broad spectrum of action of epigenetic modifications (Goldberg et al. 2007; Kouzarides 2007).

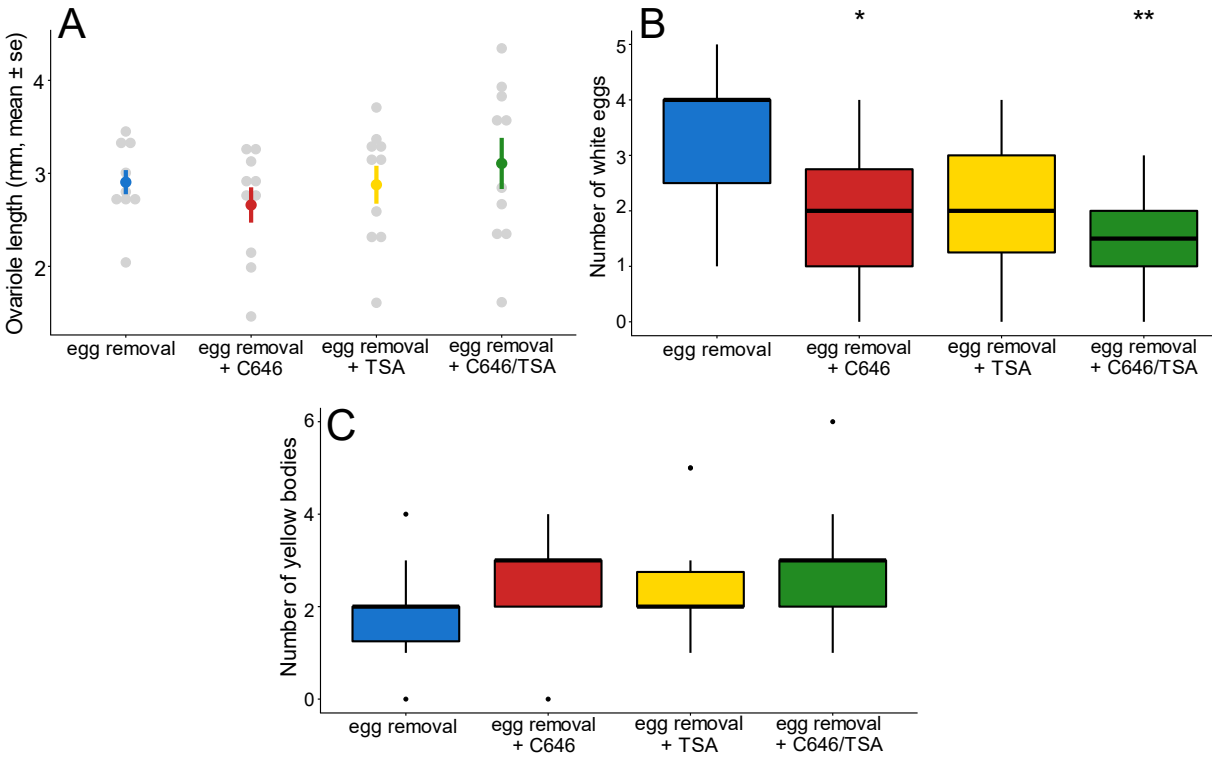

Figure 3. Treatment effect on **(A)** ovariole length, **(B)** the number of white eggs, and **(C)** the number of yellow bodies in the ovaries. Levels of significance always refer to comparisons with the egg removal control and are indicated as follows: * $p < 0.05$ and ** $p < 0.01$.

Whereas egg removal boosted egg production, this manipulation did not increase the number of eggs in development in queen ovaries, contrary to what was found in Negroni et al. 2021a. Although both experiments lasted similarly long, queen fecundity was overall lower in our experiment (3.2 developing eggs in the ovaries of our control queens on average versus 7.5 in the study mentioned above). Thus, the effect of egg removal on queen ovaries might have been more subtle. Variation in queen fecundity between studies could be due to season-induced differences, as seen in other ant species (Oi 2021). We can also speculate that the effect of egg removal on ovary development might have been stronger in monogynous queens since polygynous queens – like we used in this experiment – are known to be less fecund (Vargo and Fletcher 1989; Schrempf et al. 2011).

Our findings give preliminary evidence for a role of histone acetylation in the upregulation of fecundity genes associated with experimentally increased fecundity in ant queens. The results we found also point to a similarity in the regulatory mechanisms of both worker and queen reproduction. Of course, further analysis of gene expression and chromatin profiling data should strengthen these speculations. Nevertheless, our study reinforces the idea that epigenetic mechanisms are of major importance for the regulation of social insect phenotypic plasticity and highlights once again the relevance of these organisms to study evolutionary questions.

Author contributions

Ant colonies were collected by MC, BF, and SF. The three authors designed the study. MC collected and analyzed the data, and wrote the first draft of the manuscript. All three authors contributed to the improvement of the manuscript.

CHAPTER 5

Functional validation of fecundity and longevity genes associated with ant worker reproduction

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Unpublished data

Abstract

Social insects like ants, bees, and termites, are known to circumvent one important life-history trade-off, as queens retain extremely high fecundity throughout their long lives, while short-lived workers are mostly sterile. However, the molecular underpinnings of this fecundity-longevity reversal are not fully unraveled yet. Studies have suggested a physiological uncoupling of gene networks like the association of insulin insulin-like growth factor 1 signaling (IIS) pathway with downstream juvenile hormone (JH) and the yolk protein vitellogenin. While the focus has mostly been on social insect queens, we decided to look at shorter-lived workers that, in some species, can plastically reproduce in absence of their queen, live longer as a consequence, and show transcriptomic changes in the fat body. We intended to use gene silencing to confirm the role of specific genes in worker reproduction. To do so, we induced worker fecundity using queen removal while feeding queenless colonies with dicer-substrate small interfering RNA (dsiRNA) targeting three candidate genes with known longevity and fecundity functions in various organisms, that were previously found upregulated in fertile *Temnothorax rugatulus* ant workers. Unfortunately, no significant effects of gene silencing on worker fecundity or longevity could be found. Besides, the low expression of the reproductive gene Vitellogenin 1 in the positive control compared to the negative control tends to suggest a detrimental effect of siRNAs themselves on worker fecundity. Studies have shown that RNAi-mediated gene silencing can trigger immune reactions. Our results thus contribute to existing knowledge about the challenges of using this molecular intervention in insects.

Introduction

Most living organisms face a resource allocation trade-off between somatic maintenance (lifespan) and germline production (reproduction) (Kirkwood 1977). This trade-off is often associated with other life-history traits like body size, as larger animals tend to live longer and have fewer offspring, compared to smaller, short-lived ones that are highly fecund (Stearns 1992; Speakman 2005). On the molecular level, this trade-off has been associated with several gene networks. For example, the insulin/insulin-like growth factor 1 signaling (IIS) and target of rapamycin (TOR) pathways can sense nutrients, and in turn affect the downstream production of lipid-like juvenile hormone (JH), an important regulator of various biological traits including lifespan (Tatar et al. 2003; Flatt et al. 2005; Yamamoto et al. 2013). In parallel, JH titers influence the production of vitellogenin, a yolk protein involved in egg maturation, thus associated with reproduction (Hartfelder 2000; Sheng et al. 2011; Libbrecht et al. 2013a; Zhu et al. 2021). In the solitary insect *Drosophila melanogaster*, elevated IIS activity benefits fecundity, but shortens lifespan due to pleiotropic effects (Toivonen and Partridge 2009), highlighting the versatility of JH in insects.

In social insects, the trade-off between longevity and fecundity appears to be missing or even reversed, as large queens monopolize reproduction throughout their long lives, while small, short-lived workers are mostly sterile (Parker 2010; Korb 2016; Negroni et al. 2016; Oettler and Schrempf 2016; Schrempf et al. 2017). Hence, a reshaping of the signaling pathways has been suggested (Monroy Kuhn and Korb 2016), potentially throughout the physiological uncoupling of the fecundity-longevity trade-off observed in solitary organisms (Rodrigues and Flatt 2016; Rüppell et al. 2016). Moreover, a recent transcriptomic analysis suggests that downstream components of signaling pathways might have been overlooked when it comes to the regulation of reproduction and lifespan

in social insects (Korb et al. 2021). The authors proposed the TI-J-LiFe (TOR/IIS – JH – Lifespan and Fecundity) network as a foundation to gain insights into the proximate mechanisms of social insect aging and fecundity. The TI-J-LiFe network encompasses many genes originally listed in *Drosophila* (Flatt et al. 2005; Yamamoto et al. 2013). Such a list allows comparisons with gene expression studies that focus on the extraordinary lifespan of social insect queens for example (Negroni et al. 2019; Lin et al. 2021). Although queens are central elements of social insect studies on the fecundity-longevity reversal, the fact that they can live up to three decades makes shorter-lived workers a particularly interesting alternative. Besides, in many social insect species, workers start laying haploid, male-destined eggs following the loss of their queen (Miller and Ratnieks 2001; Helanterä and Sundström 2005; Heinze 2008; Lopes et al. 2020; Giehr et al. 2020b; Princen et al. 2020). Such plastic worker reproduction also occurs in our model species *Temnothorax rugatulus*, where workers in queenless colonies do not only become fertile but also live longer and show transcriptomic changes in the fat body (Negroni et al. 2020, 2021b; Choppin et al. 2021a; Majoe et al. 2021). Considering workers' shorter lives and plastic ability to reproduce, it seems particularly advantageous to look at worker reproduction and associated lifespan extension to understand the molecular underpinnings of longevity and fecundity in social insects. Hence, we intended to confirm the role of specific genes involved in worker reproduction using gene silencing.

We were interested in genes with known longevity and fecundity functions from previous studies. Our first candidate gene was "alpha-tocopherol transfer-protein" (Alpha-Tocopherol) that we found upregulated in fertile *T. rugatulus* workers in three different studies conducted in our laboratory (Negroni et al. 2020, 2021b; Choppin et al. 2021a). Alpha-tocopherol is a natural form of vitamin E that reduces oxidative stress in *Drosophila* (Bahadorani et al. 2008; Casani et al. 2013), extends lifespan in rotifers

(Enesco and Verdone-smith 1980) and *Caenorhabditis elegans* (Harrington and Harley 1988), and increases reproduction in the Colorado potato beetle (Zwołińska-Śniatałowa 1976) and the parasitoid *Agria affinis* (House 1966). Alpha-tocopherol transfer-protein binds alpha-tocopherol, facilitates its transfer between separate membranes, and stimulates its release from liver cells (Manor and Morley 2007). Our second candidate gene was "transketolase protein 2" (Transketolase) that we consistently found upregulated in fertile workers compared to three groups of infertile workers in our former study Choppin et al. (2021a). Transketolases are enzymes of the pentose phosphate pathway that possess strong antioxidant properties (Xu et al. 2016; Kovarova et al. 2018). Our third and last candidate was "vitellogenin 1" (Vitellogenin 1), also found upregulated in fertile workers in the above-mentioned study. Vitellogenins are storage proteins and yolk precursors with multiple functions like oxidative stress resistance (Seehuus et al. 2006) and caste differentiation (Amdam et al. 2003; Feldmeyer et al. 2014; Morandin et al. 2014; Corona et al. 2016; Kohlmeier et al. 2018).

We aimed to functionally validate the role of Alpha-Tocopherol, Transketolase, and Vitellogenin 1 in worker reproduction. To do so, we removed queens from their colony to induce worker fecundity while feeding workers dicer-substrate small interfering RNA (dsiRNA) targeting our candidate genes. We wanted to see whether the forced downregulation of these genes would disrupt worker reproduction in queenless colonies. In case our candidate genes were indeed playing a role in the regulation of worker longevity and fecundity, we expected worker lifespan and reproduction to be altered in dsiRNA-treated colonies compared to control colonies following queen removal.

Methods

Ant collection and maintenance

The ant species *Temnothorax rugatulus* can be found in the western part of North America. Colonies live inside or under rocks of forests at high elevations. Two queen morphs occur in this species (large macrogynes and small microgynes) and differ in their reproductive strategies (Rüppell et al. 1998, 2001a; Choppin et al. 2021b; Negroni et al. 2021c). We collected our ant colonies in the Chiricahua Mountains (Arizona, USA) in August 2018. Ant colonies were kept in climate chambers at 21°C and 70% humidity with a 12:12 light:dark cycle. Colonies were kept individually in three-chambered, squared boxes with plastered floors. The nests were made of plastic inserts between two glass slides, forming cavities for the ants to live in. Upper glass slides were covered with red foils to shield off the light. We fed the ants with honey and crickets and provided them with *ad libitum* water.

Candidate gene sequences, qPCR primers, and dsiRNA probes

Among the genes that we found upregulated in fertile workers in our previous studies Negroni et al. (2020, 2021a) and Choppin et al. (2021a), we selected three candidates with known fecundity and/or longevity functions to knock down in workers from queenless colonies for functional validation. The three candidate genes were "alpha-tocopherol transfer-protein" (Alpha-Tocopherol), "transketolase protein 2" (Transketolase), and "vitellogenin 1" (Vitellogenin 1). Gene nucleotide sequences were retrieved from the transcriptome obtained with the genome-guided assembly performed in Choppin et al. (2021).

We designed quantitative polymerase chain reaction (qPCR) primers for our three candidate genes using Primer3 v4.1.0 (Koressaar and Remm 2007; Untergasser et al. 2012) with the following parameters: %GC minimum 50, optimum 58, and maximum 65,

and product size range from 80 to 120bp. We selected five primer pairs produced by Primer3. To test for primer specificity, we first blasted the primer sequences against the transcriptome mentioned previously using the option "blastn" from BLAST+ v2.2.29 (Altschul et al. 1990). Primer pairs were considered good when they targeted the other transcript isoforms belonging to the same gene, and three good primer pairs were selected for each gene. Then, we blasted the selected primer sequences against the draft genome of *Temnothorax rugatulus* (Jongepier et al. 2021) using the same parameters as described above to obtain their scaffold positions and ensure that they mapped to a single position only. The primer positions were then inspected using an integrative genomics viewer (IGV; Thorvaldsdóttir et al. 2013), and the two best primer pairs (i.e., pairs that spanned introns preferably) were chosen and ordered from Biolegio. We additionally ordered one primer pair targeting the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

To knock down our three candidate genes in workers from queenless colonies, we used dsiRNA from IDTDNA (https://eu.idtdna.com/pages). We designed the dsiRNA probes using their online tool with our gene nucleotide sequences. We visualized the probe positions in the genome using IGV and selected two probes per candidate gene that were preferably close to the first exon and/or matching the position of the corresponding primer pair. We additionally designed a nonsense probe by generating random nucleotide sequences with appropriate GC contents that we blasted against the *T. rugatulus* genome, as described above, to make sure that no hits were found. Probes were ordered at a concentration of 10 nmol.

Primer efficiencies

We calculated qPCR primer efficiencies using a standard curve based on a 1:5 dilution series of four points. To do so, we first collected and pooled ten fat bodies of workers from queenless colonies in TRIzol (ThermoFisher) and extracted RNA using the RNeasy Mini Kit from QIAGEN (see Protocol 1). We used the RNA to produce cDNA with the QuantiTect Reverse Transcription kit from QIAGEN (see Protocol 2). Then, we performed three 1:5 dilutions of the cDNA from queenless workers (cDNA concentrations: 1, 0.2, 0.04, and 0.008) to use as template. We tested one primer pair for each candidate gene and the housekeeping gene GAPDH as well. Primer pairs were diluted (1:10) before use. Finally, we prepared the qPCR master mixes using the SensiFASTTM SYBR® Hi-ROX kit from QIAGEN (see Protocol 3), and conducted the qPCR on a MicPCR machine with the following cycling parameters: hold 95°C for 2 minutes, and cycling 95°C for 5 seconds and 58°C for 20 seconds. We used two technical replicates for both the housekeeping gene and the three candidate genes. Ct values were obtained from the MicPCR software and corrected with the baseline algorithm "LinRegPCR". Primer efficiencies were calculated in Excel using the template available at https://toptipbio.com/calculate-primer-efficiencies/. The obtained primer efficiencies (E) were as follows: E_{GAPDH} = 95.16, $E_{Alpha\text{-}Tocopherol}$ = 111.05, $E_{Transketolase}$ = 115.10, and $E_{Vitellogenin\ 1}$ = 99.79.

Confirmation of RNA-seq results

Before starting our main experiment, we wanted to confirm using qPCR that our three candidate genes were upregulated in workers from queenless colonies compared to workers from queenright colonies, as found in our RNA-seq studies Negroni et al. (2020, 2021a) and Choppin et al. (2021a). To do so, we collected and pooled ten worker fat bodies from three queenless and three queenright colonies in TRIzol, extracted the RNA,

converted the RNA into cDNA, and conducted qPCR assays using the same protocols and with the same parameters as described in the previous section "Primer efficiencies". We used two technical replicates for the housekeeping gene GAPDH and three technical replicates per candidate gene. We discarded one technical replicate (queenless sample, vitellogenin 1) because the total variation between technical replicates was > 0.25 (we removed the replicate that was most different from the middle one). We first assessed the expression of the housekeeping gene GAPDH across samples and used a t-test to make sure that no differences were found between queenless and queenright samples. We then calculated the relative expression of our three candidate genes using GAPDH expression values and the Pfaffl equation as described at https://toptipbio.com/pfaffl-method-qpcr/. For each candidate gene, we used t-tests to determine whether the relative expression differed between workers from queenless colonies compared to workers from queenright colonies. The gene expression analysis was conducted in R v4.1.0 (R Core Team 2020).

Main experiment

We selected 30 single-queen colonies with 50 to 200 workers and reduced worker number to 90 maximum. Colonies were randomly assigned to one of five experimental groups (Table 1), provided with fresh nests and food, and kept under the same conditions in a climate cabinet (21°C and 70% humidity with a 12:12 light:dark cycle) for one week. Then, colonies were starved for ten days to stimulate their appetite in preparation for the experiment. One day before queen removal, colonies were fed with 11 µl of their respective solution: sucrose solution (0.102 g/ml) with water, one probe of nonsense dsiRNA, or two probes of dsiRNA targeting our candidate genes (Table 1). The dsiRNA concentration in the sucrose solution was 0.05 ug/ml. Afterward, colonies were fed every

Table 1. Treatment solution and sample size for each experimental group.

Experimental group	**Treatment solution**	**Colonies**
none	sucrose solution with water	N = 6
nonsense	sucrose solution with one fragment of nonsense dsiRNA	N = 6
dsiRNA alpha-tocopherol	sucrose solution with two fragments of dsiRNA targeting Alpha-Tocopherol	N = 6
dsiRNA transketolase	sucrose solution with two fragments of dsiRNA targeting Transketolase	N = 6
dsiRNA vitellogenin 1	sucrose solution with two fragments of dsiRNA targeting Vitellogenin 1	N = 6

other day for four weeks with their respective solution. On Day 0 of the experiment, colonies were anesthetized with CO2 so that the queens could be removed from experimental colonies and returned to their source colony. Workers were counted on that day and every following week. Three collection points were established on Day 1, Day 8, and Day 29 during which six workers located on the brood pile were collected and dissected in ice-cold PBS under a Leica stereomicroscope. Workers on the brood pile are usually younger and more likely to develop their ovaries following queen removal (Pamminger et al. 2014; Kohlmeier et al. 2017). Their fat bodies were pooled in 100 µl of TRIzol. Additionally, ovary pictures were taken for the last two workers and the heads of these workers were collected. The full experimental timeline can be found in Figure S1. For each worker, we counted the number of eggs in development in the ovaries and measured the egg surface, ovariole length, and head width. We also calculated the proportion of fertile workers in each colony (we considered workers as fertile when they had at least one egg in development in the ovaries). Apart from head width, for each one of the phenotypic measures (worker number, ovariole length, number of eggs in development in the ovaries, proportion of fertile workers, and egg surface) we first compared our negative control (none) with our positive control (nonsense) to make sure that no differences originated from feeding dsiRNA probes on itself. Then we compared our three treatment groups (dsiRNA alpha-tocopherol, dsiRNA transketolase, and dsiRNA vitellogenin 1) with our positive control to test for treatment effects on worker longevity and fecundity. Details on the models can be found in Table 2.

Plotting our data revealed that fecundity measures remained constant or decreased in the dsiRNA vitellogenin group (non-significant visual pattern) so we chose to only validate the dsiRNA-mediated knockdown of Vitellogenin 1 at Day 29 in our two controls (none and nonsense), the group dsiRNA alpha-tocopherol, and the group dsiRNA

Table 2. Description of the models used in the statistical analyses. Two models were used for each response variable: one to compare the negative control (none) and positive control (nonsense) and another one to compare the treatment groups (dsiRNA alpha-tocopherol, dsiRNA transketolase, and dsiRNA vitellogenin 1) to the positive control. Model fit was assessed using visual inspection of the residual distribution for the LMMs and using the package DHARMa for the GLMMs (Hartig 2020). The "bobyqa" optimizer (Nash 2014) was used to help GLMMs converge and prevent singular fits when necessary. Day was analyzed as a continuous factor in all models. LMM: linear mixed-effects model. GLMM: generalized linear mixed-effects model.

Response variable	**Explanatory variables**	**Models**	**Covariate**	**Random factor**
worker number	day treatment day:treatment	LMMs	cohort	colony ID
ovariole length	day treatment day:treatment	LMMs	cohort	colony ID
number of eggs in development	day treatment day:treatment	GLMMs (Poisson)	cohort	colony ID
proportion of fertile workers	day treatment day:treatment	GLMMs (Binomial)	cohort	colony ID
egg surface	day treatment day:treatment	LMMs	cohort	colony ID

vitellogenin using qPCR to confirm that Vitellogenin 1 was indeed downregulated in the latest. Hence, we only extracted the RNA of samples from these four groups (N = 24). RNA extractions, cDNA conversions, and qPCR assays were conducted using the same protocols and with the same parameters as mentioned in the previous section "Primer efficiencies". We used three technical replicates for both the housekeeping gene GAPDH and Vitellogenin 1 and discarded one technical replicate as indicated in the previous section "Primer efficiencies" (none sample, GAPDH). Two samples (one nonsense and one dsiRNA alpha-tocopherol) only had two replicates for both GAPDH and Vitellogenin 1 due to a technical error. We first tested the treatment effect on GAPDH expression using an LMM. We then calculated Vitellogenin 1 relative expression as described in the previous section "Confirmation of RNA-seq results". And we also used an LMM to test the treatment effect on the relative expression of Vitellogenin 1. Both statistical and gene expression analyses were conducted in R v4.1.0 (R Core Team 2020).

Results

Confirmation of RNA-seq results

As expected, GAPDH expression did not significantly differ across samples (t = 0.493, df = 2.974, p = 0.656; Figure S2). However, we also did not find significant relative expression differences between queenright and queenless samples for any of the three candidate genes (Figure S3): Alpha-Tocopherol (t = -1.637, df = 3.035, p-value = 0.199), Transketolase (t = -1.070, df = 3.921, p-value = 0.346), and Vitellogenin 1 (t = -0.656, df = 3.368, p-value = 0.554).

Worker number and fecundity

Changes in worker number over time did not differ between the negative and positive controls (day:treatment: X_1^2 = 1.364, p = 0.243; Figure 1A), and only tended to differ between the positive control and treatment groups (day:treatment: X_3^2 = 7.662, p = 0.054; Figure 2A). Similarly, no differences were found between the two controls in terms of ovariole length (day:treatment: X_1^2 = 2.123, p = 0.145; Figure 1B), eggs in development in the ovaries (day:treatment: X_1^2 = 0.235 , p = 0.628; Figure 1C), proportion of fertile workers (day:treatment: X_1^2 = 0.956, p = 0.328. Figure 1D), and egg surface (day:treatment: X_1^2 = 0.159, p = 0.690; Figure 1E). And again, no differences were found between the positive control and treatment groups in terms of ovariole length (day:treatment: X_3^2 = 0.737, p = 0.864. Figure 2B), eggs in development in the ovaries (day:treatment: X_3^2 = 1.130, p = 0.770. Figure 2C), proportion of fertile workers (day:treatment: X_3^2 = 4.083 , p = 0.253; Figure 2D), and egg surface (day:treatment: X_3^2 = 4.230, p = 0.238; Figure 2E). Nevertheless, we did find a significant correlation between worker head width (a proxy for body size) and ovariole length (r = 0.194, p < 0.001; Figure S4), and fertile workers had longer ovarioles (X_1^2 = 16.012, p < 0.001; Figure S5).

qPCR validation of dsiRNA-mediated Vitellogenin 1 knockdown

GAPDH expression did not significantly differ across samples ($F_{3,20}$ = 0.359, p = 0.783; Figure S6). As shown by Figure 2B-E, fecundity measures remained constant or decreased in the dsiRNA vitellogenin treatment group (non-significant visual pattern). Hence, we only quantified the relative expression of Vitellogenin 1 and found significant differences between treatment groups ($F_{3,20}$ = 3.546, p = 0.033; Figure 3). Surprisingly, compared to the negative control, Vitellogenin 1 was downregulated in the positive control (t = -2.965,

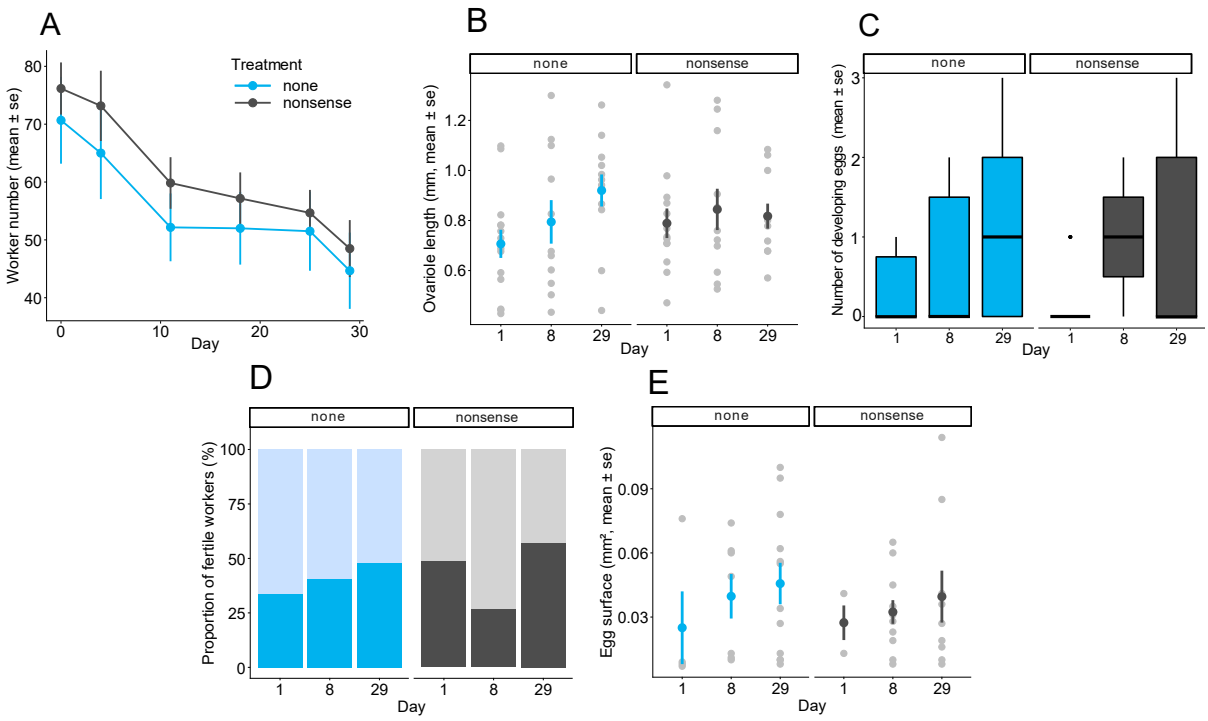

Figure 1. Comparison of the negative (none) and positive (nonsense) controls for all phenotypic measures: **(A)** worker number, **(B)** worker ovariole length, **(C)** number of developing eggs in worker ovaries, **(D)** the proportion of fertile workers per colony, and **(E)** egg surface.

p = 0.008), the group treated with dsiRNA vitellogenin 1 (t = -2.458, p = 0.023), and the group treated with dsiRNA alpha-tocopherol (t = -2.430, p = 0.025).

Discussion

In our previous studies Negroni et al. (2020, 2021a) and Choppin et al. (2021a), we identified several genes upregulated in fertile *Temnothorax rugatulus* workers from queenless colonies, with known longevity and fecundity functions in other organisms. In this study, we selected three of these genes to functionally validate their role in worker reproduction using dsiRNA-mediated knockdowns. First, the confirmatory qPCR assays we conducted could not confirm the upregulation of the three candidate genes in workers from queenless colonies compared to workers from queenright colonies. In our main experiment, worker number (used as a proxy for longevity) and fecundity were not significantly affected by the feeding of dsiRNA targeting our different candidate genes. Nevertheless, fecundity measures remained constant or decreased in workers treated with dsiRNA vitellogenin, whereas they increased in control workers (non-significant visual patterns). Surprisingly, when looking at the relative expression of Vitellogenin 1 in different treatment groups, we found that this fecundity-associated gene was downregulated in workers from colonies treated with dsiRNA vitellogenin 1 as expected, but also in the positive control (nonsense), and the dsiRNA alpha-tocopherol treatment, compared to the negative control (none). Finally, we did find a correlation between worker ovariole length and head width (used as a proxy for body size), and fertile workers had longer ovarioles.

The correlation that we see between worker ovariole length and head width was to be expected as body size is correlated with fecundity in insects (Honek 1993). Besides, workers with at least one egg in development in the ovaries had longer ovarioles. In some

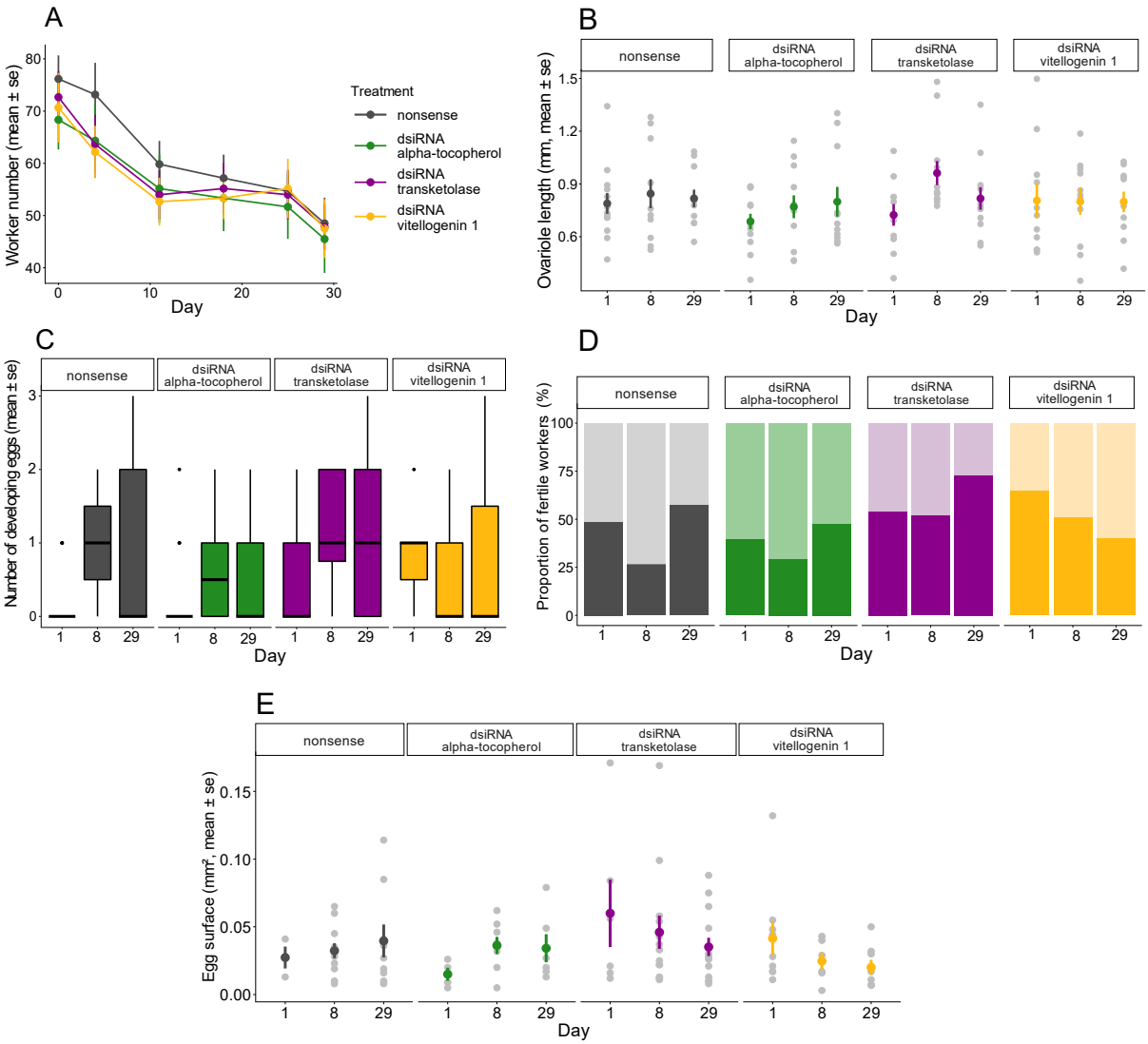

Figure 2. Comparison of the positive control (nonsense) with the treatment groups (dsiRNA alpha-tocopherol, dsiRNA transketolase, and dsiRNA vitellogenin 1) for all phenotypic measures: **(A)** worker number, **(B)** worker ovariole length, **(C)** number of developing eggs in worker ovaries, **(D)** the proportion of fertile workers per colony, and **(E)** egg surface.

ant species, having more ovarioles leads to a reproductive advantage (Heinze et al. 1997). These findings raise the question of whether the largest workers in the colony are necessarily the ones that will take over reproduction in absence of the queen. This is not the case in the monogynous slave-making ant *Harpagoxenus sublaevis*, as dominance is associated with ovarian development, trophallaxis' frequency, and time spent in the nest but not body size in this species (Bourke 1988). Many other cues than body size might influence the establishment of novel reproductive hierarchies among workers in queenless colonies, like dominance behaviors (Monnin and Peeters 1999; Miller and Ratnieks 2001; Oldroyd et al. 2001; Helanterä and Sundström 2005; Heinze 2008; Princen et al. 2020) or changes in chemical signatures (Peeters et al. 1999; Liebig et al. 2000; Heinze et al. 2002). These plastic changes displayed by fertile workers can be reflected on the molecular level in terms of differential gene expression (Wurm et al. 2010; Feldmeyer et al. 2014; Choppin et al. 2021a; Negroni et al. 2021b).

Although Alpha-Tocopherol, Transketolase, and Vitellogenin 1 were all upregulated in fertile workers compared to infertile workers in our previous RNA-seq studies Negroni et al. (2020, 2021a) and Choppin et al. (2021a), we did not retrieve this pattern with our confirmatory qPCR assays. The lack of repeatability of our previous results could stem from using different methods. Indeed, we used qPCR in this study versus RNA-sequencing previously, and both methods are known to not always lead to the same outcome (Everaert et al. 2017). An alternative explanation for our divergent results could be the different timespans that workers spent in queenless colonies before being sampled. In Choppin et al. (2021a), we sampled workers six weeks after queen removal, while the colonies in this study were queenless for many months or even up to three years. We can speculate that fecundity and longevity genes might be upregulated in workers during the first days/weeks following the loss of their queen, during which they

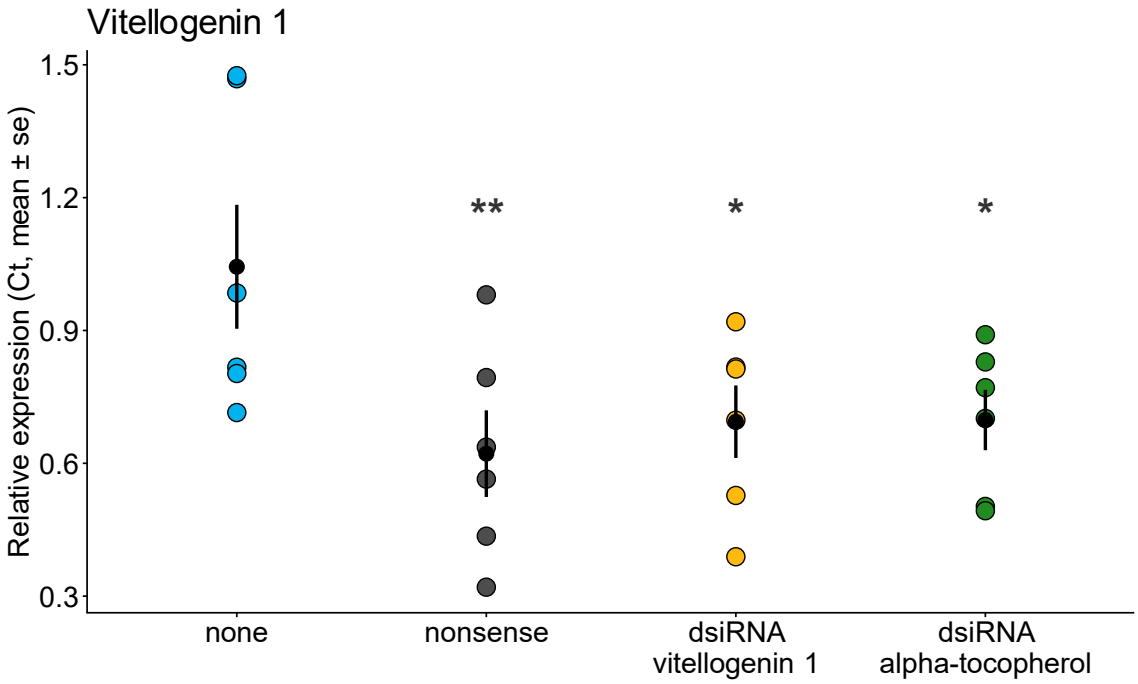

Figure 3. Relative expression of Vitellogenin 1 in the negative control (none), the positive control (nonsense), the group treated with dsiRNA vitellogenin 1, and the group treated with dsiRNA alpha-tocopherol. Relative expression values were calculated using the GAPDH expression values and the Pfaffl equation. Levels of significance are indicated as follows: * $p < 0.05$, ** $p < 0.01$.

establish their novel reproductive hierarchy and start laying eggs, but the expression of those key genes might return to basal levels after some time.

The downregulation of Vitellogenin 1 in the positive control of the main experiment suggests a detrimental effect of dsiRNA probes on the ants. This idea is reinforced by the downregulation of Vitellogenin 1 in the dsiRNA alpha-tocopherol treatment as well, where only Alpha-Tocopherol was targeted. Hence, we cannot confirm that the knockdown of Vitellogenin 1 technically worked since low levels of Vitellogenin 1 in the workers fed with dsiRNA targeting Vitellogenin 1 could result from toxicity-induced decreased worker fecundity. Indeed, vitellogenins are yolk proteins that have been associated with reproduction in social insects (Amdam et al. 2003; Negroni et al. 2020) and we can speculate that workers challenged with such an invasive method do not need to upregulate this gene, as they do not engage in egg production as much as healthy workers, and rather invest in immunity. This hypothesis is reflected, to some extent, by the fecundity results. Although not significant, p-values associated with the interactive effect of day and treatment on fecundity measures were always smaller when comparing the negative and positive controls than when comparing the positive control with the dsiRNA treatment groups. Hence, these results might suggest that workers from the negative control are more fecund than workers from the positive control, as visible in Figure 1 (non-significant visual pattern). Moreover, siRNAs are known to trigger immune responses (Marques and Williams 2005; Schlee et al. 2006). Altogether, it seems that our results could partially be explained by an adverse reaction of the ants to ingesting dsiRNA.

RNA interference (RNAi) is receiving increased attention in insect science and the efficiency of RNAi-mediated gene silencing depends on many factors such as the mode of admission, dosage, the genes being targeted, or the model organism, making the use of this technology very tedious (Scott et al. 2013). Despite the challenges and potential

adverse effects of this molecular intervention, studies using various insect species have shown successful downregulation of genes using RNAi-mediated gene silencing and associated phenotypic consequences. For example, the downregulation of vitellogenin-like-A in the closely related species *Temnothorax longispinosus* induced a transition from brood care to nestmate care in workers (Kohlmeier et al. 2018). In the fire ant *Solenopsis invicta*, silencing a vitellogenin receptor in virgin queens impaired egg formation (Lu et al. 2009). And in the field of pest control, the use of gene silencing has exploded in the past years and seems to be a promising technology (Baum and Roberts 2014; Mamta and Rajam 2017).

To conclude, our gene expression results do not allow us to disentangle whether the low relative expression of Vitellogenin 1 was induced by the knockdown using dsiRNA targeting Vitellogenin 1, or associated with reduced worker fecundity potentially caused by side effects of dsiRNA probes themselves. Although we could not functionally validate the role of our candidate genes in worker reproduction, our study enriches existing knowledge about the potential side effects of experimental manipulations using foreign nucleotide sequences and highlights the relevance of negative controls in RNAi studies.

Author contributions

MC, BF, and SF collected the ant colonies. MC and BF designed the study, with the input of SF. MC collected and analyzed the data, with the guidance of BF. MC wrote the first draft of the manuscript and all three authors contributed to its improvement.

Acknowledgments

Many thanks to Yongqiang Wu and Marion Kever for teaching me how to do qPCR, to Dr. Joe Colgan for his help with the gene expression analysis, and to Dr. Romain Libbrecht for his feedback on the interpretation of our results.

Supplementary material

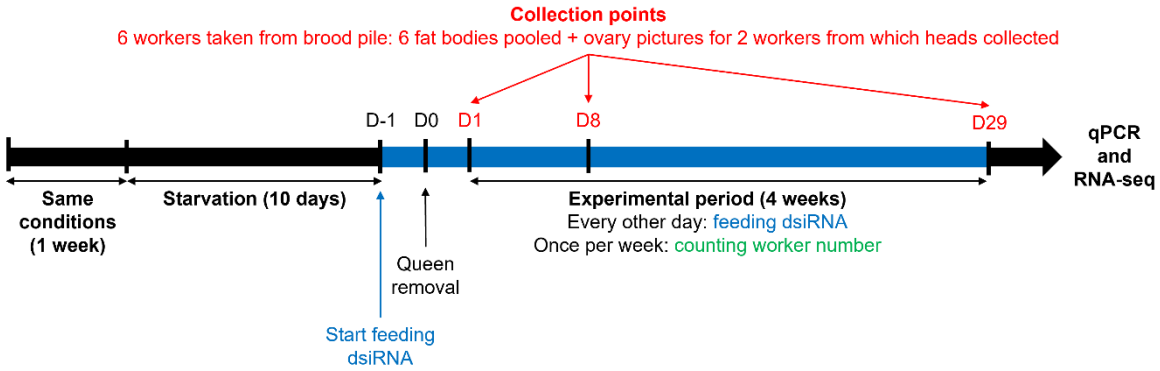

Figure S1. Experimental timeline.

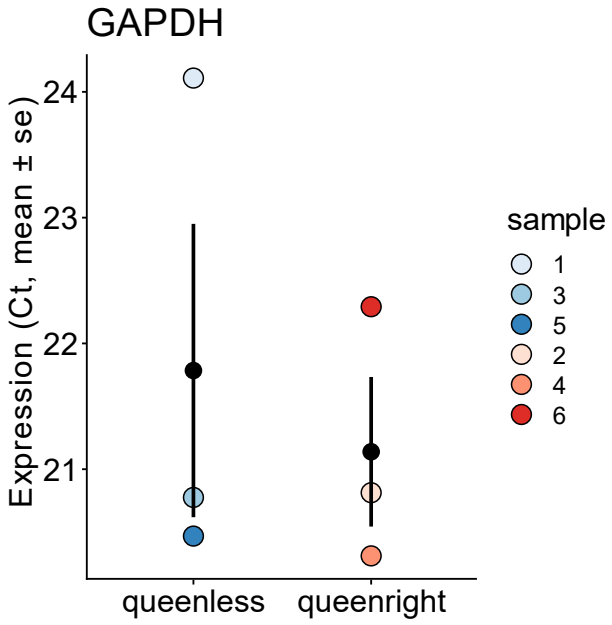

Figure S2. GAPDH expression across queenless and queenright samples.

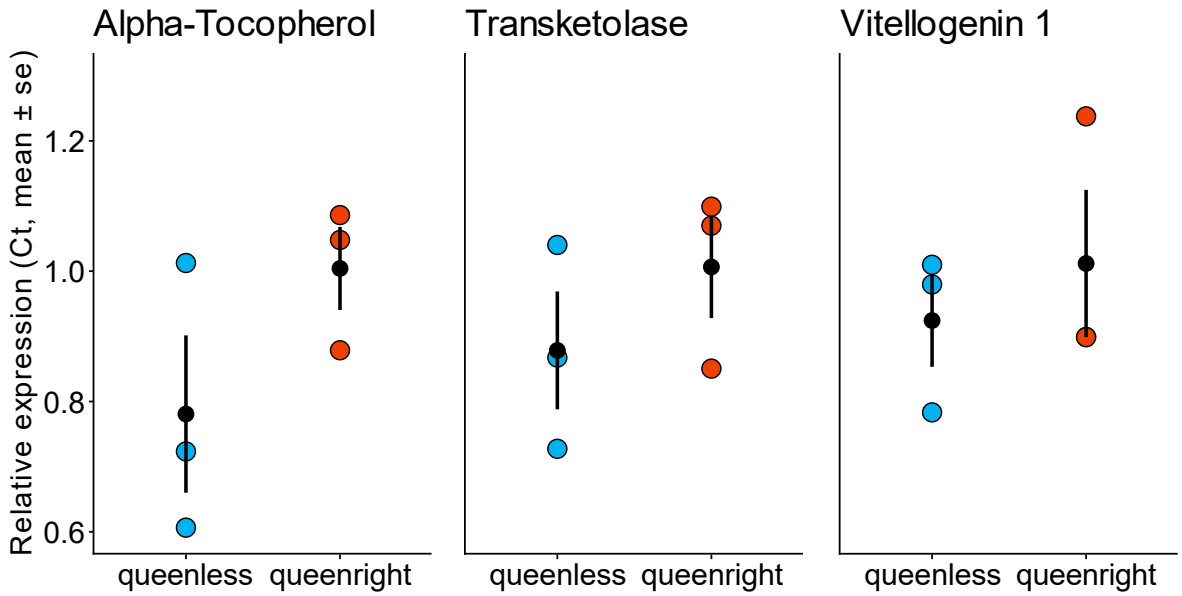

Figure S3. Relative expression of Alpha-Tocopherol, Transketolase, and Vitellogenin 1 across samples. Relative expression values were calculated using the GAPDH expression values and the Pfaffl equation.

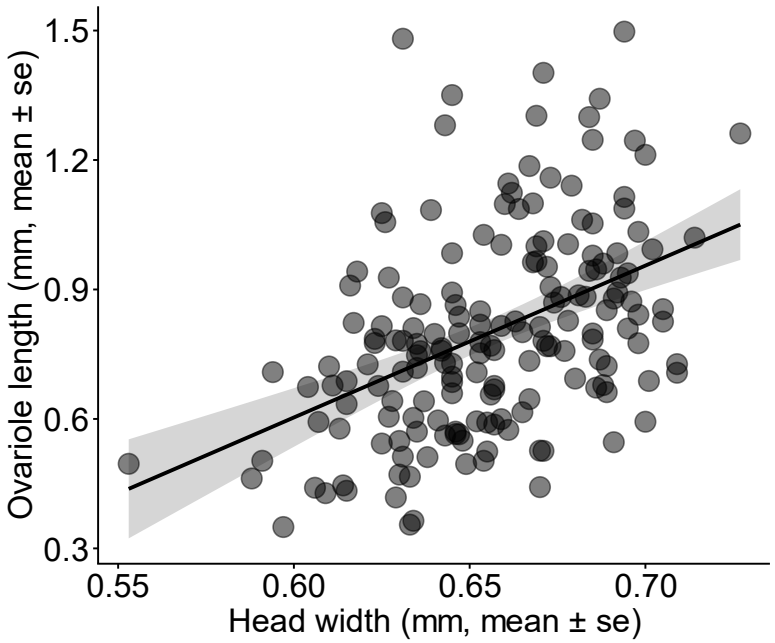

Figure S4. Correlation between worker ovariole length and head width.

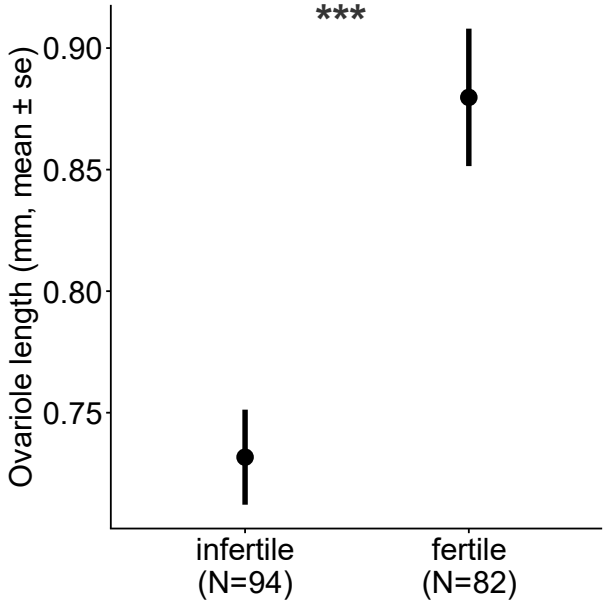

Figure S5. Ovariole length of infertile and fertile workers. Workers were considered fertile when they had at least one egg in development in the ovaries. The level of significance is indicated as follows: *** $p < 0.001$.

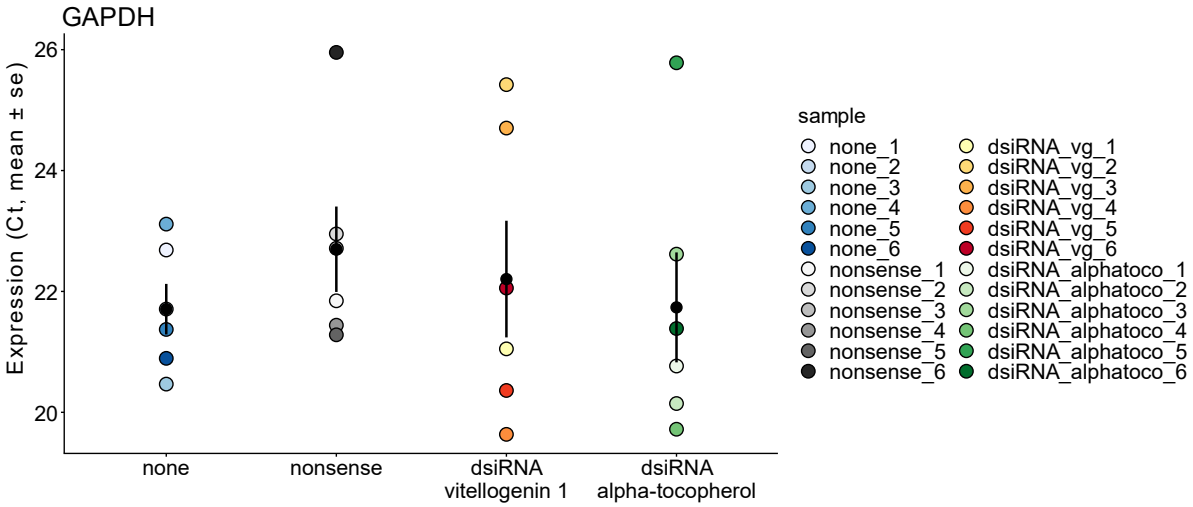

Figure S6. GAPDH expression across samples.

Protocol 1. RNA extraction

Kit: RNeasy Mini from QIAGEN

Note: before starting cool down the centrifuge to 4°C

1. Clean all surfaces with 70% Ethanol and RNase away
2. Take samples out of the -80°C and place on ice
3. Crush fat bodies with small pestles
4. Add 400 µl of TRIzol under the hood
5. Flick the tube
6. Add 100 µl (i.e. 1/5 volume) of Chloroform:Isoamyl alcohol 24:1
7. Flick the tube → leave 5 minutes on ice
8. Centrifuge at 12000 xg and 4°C for 15 minutes
9. Transfer upper phase with gel loading tips into new tube on ice
Note: angle the tube to not take any interphase or TRIzol
10. Add 1/2 of the upper phase volume of ice-cold 100% Ethanol
11. Flick the tube and mix with pipette
12. Transfer to column → leave 1 minute on wet ice
13. Centrifuge at 11000 xg and 4°C for 1 minute
14. Wash with 400 µl of Buffer RPE mixed with ethanol
15. Centrifuge at 11000 xg and 4°C for 1 minute
16. Wash with 300 µl of Buffer RPE
17. Centrifuge at 11000 xg and 4°C for 1 minute
18. Place the column into collection tube
19. Centrifuge at 11000 xg and 4°C for 1 minute to dry the membrane
20. Place the column into new tube
21. Add 30 µl of RNase-free water right on the column membrane
22. Leave 1 minute at room temperature
23. Centrifuge at 11000 xg and 4°C for 1 minute to elute the RNA
Note: tubes must be placed in the centrifugation direction
24. Remove column, check labels, put parafilm around the lid, and store at -80°C

Protocol 2. cDNA synthesis

Kit: QuantiTect Reverse Transcription from QIAGEN

(!) 1 µl of RNA to get 20 µl of cDNA per biological sample

Note: before starting, set one heating block at 42°C and the other at 95°C

A. Prepare the Master Mixes (prepare for N + 2 samples)
A.1. Wipeout Master Mix (WMM)
► for *one* sample: 11 µl of RNAse free water + 2 µl of Wipeout Buffer → vortex
A.2. Reverse Transcription Master Mix (RTMM)
► for *one* sample: 4 µl of RT Buffer + 1 µl of Primer Mix + 1 µl of RT enzyme → vortex

B. Wipeout step
In new tube, mix 13 µl of WMM with 1 µl of RNA → 2 minutes at 42°C on heating block

C. Reverse transcription step
Add 6 µl of RTMM to samples (14 µl from B.) and mix → 15 minutes at 42°C on heating block

D. End of the reaction
D.1. Place the cDNA samples for 3 minutes at 95°C on heating block
D.2. Store samples at -20°C

Protocol 3. qPCR

Kit: SensiFAST™ SYBR® Hi-ROX from QIAGEN

Note: here one sample = one well = one biological sample and associated technical replicate

1. Prepare SYBR mix

► Add 35 µl of ROX Additive to the 1 ml tube of Blue S'Green → vortex

Note: when done, mark the Blue S'Green's tube with a cross

2. Prepare diluted primer solutions

► Dilute 1:10 the stock primer solutions (100 µM) with RNAse free water

3. Prepare Master Mixes for each gene

► For *one* sample: 10 µl SYBR mix + 6.4 µl RNAse free water + 0.8 µl forward and 0.8 µl reverse diluted primer solutions (10 µM) → vortex

Note: prepare for N + 1 biological sample

4. Pipetting in qPCR plate on ice (!)

Note: To avoid pipetting errors use a template with 6 x 8 cells, use one pipette tip per cell and pay attention to the plate orientation based on letters/numbers

► Vortex and quick centrifuge Master Mix → pipette 18 µl for each gene

Note: pipette right on top of the gel against the tube wall

► Vortex and quick centrifuge cDNA → add 2 µl for each sample

Note: pipette slightly below the master mix surface and avoid making bubbles

5. Close each column of four tubes with four lids using the clamp → put them into the MicPCR machine in the correct order (!) → click "run" in MicPCR software

6. Save Ct values corrected with baseline algorithm "LinRegPCR"

CHAPTER 6

The influence of diet composition on worker phenotype in the ant *Temnothorax rugatulus*

Marina Choppin, Miriam Schall, Barbara Feldmeyer, and Susanne Foitzik

Will be submitted as: Marina Choppin, Miriam Schall, Barbara Feldmeyer, and Susanne Foitzik. Protein-rich diet negatively affects survival in ant workers.

Abstract

Aging is associated with many processes such as the accumulation of oxidative damage, the decrease in immunocompetence, or the increase in epigenetic abnormalities, mutations, and inflammations. Many of these molecular mechanisms underlying aging are linked to nutrient-sensing pathways like target of rapamycin (TOR) or insulin/IGF-1 signaling, suggesting that nutrients play a critical role in the aging process itself. In particular, the protein content in the diet is a key determinant of the relationship between nutrition and longevity. In many organisms, such as mice and *Drosophila*, a high-protein diet shortens lifespan. This is explained by a trade-off in energy allocation since resources are either invested in soma maintenance (lifespan) or germline production (reproduction). In this study, we used the ant *Temnothorax rugatulus* to examine the effect of dietary protein content on the longevity, fecundity, behavior, and fatty acids of workers in queenless colonies. We induced fecundity in workers by removing their queen and fed colonies either a high-protein or a high-carbohydrate diet. For twelve weeks, we monitored worker survival, foraging activity, and egg production. We then reported the location and behavior of young workers (callows), measured their ovariole length, and collected two workers per colony to analyze their fatty acids. The high-protein diet decreased worker survival but did not affect callow fecundity, foraging activity, or the quantity or composition of fatty acids in workers. However, callows from colonies fed a carbohydrate-rich diet tended to perform more brood and nestmate care. We conclude that a high-protein diet negatively affects the fitness of ant workers, similar to what has been demonstrated in solitary organisms.

Introduction

Aging is a process of intrinsic deterioration. Macromolecules such as the DNA accumulate damages and mutations over time, causing malfunctions in the organism that ultimately lead to severe organ failure (Partridge and Gems 2002). Despite the varying lifespans of organisms, the basic molecular mechanisms responsible for aging appear to be shared from yeast to humans (Fontana et al. 2010). These highly conserved processes associated with aging include the accumulation of oxidative damage, the decrease in immunocompetence, and the increase of epigenetic abnormalities, mutations, and inflammations (Holliday 2006; Santoro et al. 2014). Thus, how long an organism lives depends largely on the efficiency of its somatic maintenance (Holliday 2006). In nature, however, internal maintenance does not play a major role for most organisms because they are subject to high extrinsic mortality due to predation, starvation, or diseases. Moreover, somatic maintenance is very costly (Holliday 2006) and smaller animals facing higher extrinsic mortality risks do not benefit from investing heavily in such function; they should rather invest in reproduction (Kirkwood 1977). In fact, most living organisms face a fundamental trade-off between investing in somatic maintenance (lifespan) or germline production (reproduction) (Kirkwood 1977). Nevertheless, the investment in one or the other function can be plastically adjusted by an organism depending on the environmental constraints it faces, like food deprivation (Holliday 2006; Negroni et al. 2019). Indeed, the nutritional state of an organism plays an important role in aging.

Nutrition is a process by which organisms acquire energy for growth, metabolism, and repair. Food usually consists of macronutrients i.e., proteins, carbohydrates, and fats, and micronutrients that are essential vitamins and minerals (Dato et al. 2016). Carbohydrates are the main source of energy, while lipids are used to store energy that is available when the organism runs low in carbohydrates. Proteins can be broken down

into amino acids, which are molecules involved in important biological functions such as cell signaling, regulation of gene expression, and protein phosphorylation cascades. However, amino acids in large amounts can be harmful to organisms, as they and their metabolites are associated with neurological disorders and the accumulation of oxidative stress, one of the main causes of aging (Wu 2009; Dato et al. 2016). Amino-acid imbalance also affects longevity and fecundity (Grandison et al. 2009; Arganda et al. 2017). Many biological pathways can sense nutrients and respond to calorie availability. The nutrient-sensing pathways TOR and insulin/IGF-I signaling are major regulators of metabolism, growth, and development (Fontana and Partridge 2015). For example, a decrease in the concentration of IGF-1 serum induced by protein restriction protects against cancer and slowdowns aging in rodents (Fontana et al. 2008). These pathways also respond to nutritional changes by regulating downstream genes with antioxidant, antimicrobial, or metabolic functions, and as a consequence also regulate lifespan (Kenyon 2005). Studies on diverse organisms such as yeast, *Drosophila*, or mice, have shown that dietary interventions such as caloric restriction increase resistance to oxidative stress and reduce macromolecular damage, extending lifespan sometimes up to 50% (Fontana et al. 2010; Fontana and Partridge 2015). Hence, it has been hypothesized that an increase in proteins might fuel nutrient-sensing pathways like TOR or insulin/IGF-I signaling leading to accelerated senescence. Experimental work confirmed that high-protein diets were detrimental to the lifespans of mice (Fontana and Partridge 2015), *Drosophila* (Mair et al. 2005), workers of the ants *Lasius niger* (Dussutour and Simpson 2012) and *Linepithema humile* (Arganda et al. 2017), and caged honey bees (Pirk et al. 2010).

Interestingly, the reversed pattern was observed in another study using the ant *Platythyrea punctata*, where a high-protein diet was beneficial for longevity and fecundity (Bernadou et al. in prep). This finding could be explained by the particular lifestyle of

eusocial insects. Indeed, ants, termites, and honey bees exhibit a reproductive division of labor wherein fecundity and longevity are positively associated (Monroy Kuhn and Korb 2016; Negroni et al. 2016). One to a few long-lived females usually called the queens monopolize reproduction, while the short-lived workers perform various tasks in the colony such as brood care or foraging (Hölldobler and Wilson 1990). Thus, although they share the same genetic background, females from the reproductive and non-reproductive castes strongly differ in life-history traits, attesting to the importance of phenotypic plasticity in eusocial insects (Keller and Ross 1998; Libbrecht et al. 2013a). Here again, the quantity and quality of food received during larval development often play a major role in caste determination (Weaver 1966; Smith et al. 2008a; Slater et al. 2020). However, although the queen dominates reproduction, workers are often not completely sterile. In many social insect species, workers can begin to develop their ovaries and lay haploid, male-destined eggs after the loss of the queen, or sometimes even in colonies that do have a queen (Monnin and Peeters 1999; Beekman and Oldroyd 2008; Heinze 2008; Giehr et al. 2020a, b). And more importantly, these fertile workers can live longer (Kohlmeier et al. 2017; Lopes et al. 2020; Negroni et al. 2020; Majoe et al. 2021), showing once again the positive association between fecundity and longevity in eusocial insects. However, the role of diet in the plastic reproduction of workers and associated lifespan extension is understudied.

We used the ant *Temnothorax rugatulus* to investigate the effect of protein content in the diet on the longevity, fecundity, behavior, and fatty acids of workers in queenless colonies. We conducted an experiment during which we fed ant colonies a high-carbohydrate or a high-protein diet. We removed queens from their colony to induce worker fecundity and monitored worker survival, foraging activity, and egg production for twelve weeks. In this species, workers in queenless colonies show a general increase

in lifespan (Negroni et al. 2020, 2021b). After those twelve weeks, we reported the location and behavior of young workers (callows), assessed their fecundity by dissecting their ovaries, and extracted the fatty acids of two workers per colony. We predicted that ants fed with the high-carbohydrate diet would be more active, as shown in other ants (Grover et al. 2007; Dussutour et al. 2016). As dietary components influence fatty acid content and composition in insects (Stanley-Samuelson et al. 1988; Canavoso et al. 2001), we expected differences in fatty acids between our treatment groups. Finally, based on the reversed fecundity-longevity trade-off that ants display (Monroy Kuhn and Korb 2016; Blacher et al. 2017), we predicted that the high-protein diet would actually be beneficial for worker reproduction and survival following the loss of their queen.

Methods

Ant collection and maintenance

For our study, we used the small Myrmicine ant *Temnothorax rugatulus*, which is widely distributed throughout the western part of North America. Colonies of this ant species reside in small crevices or under rocks in high elevation forests. Two queens morphs occur in this species and are associated with alternative reproductive strategies (Rüppell et al. 1998, 2001a; Choppin et al. 2021b). We collected the ant colonies in the Chiricahua Mountains (Arizona, USA) in August 2018. We then kept the colonies in the laboratory in climate chambers at 21°C with 70% humidity and a 12:12 light:dark cycle. Colonies were kept separately in nests consisting of plastic inserts forming a cavity between two glass slides and placed in three-chambered boxes with plastered floors. Nests were covered with red foils to darken the cavity.

Pilot experiment

We first conducted a pilot experiment to ensure that ants were accepting the artificial food with manipulated protein to carbohydrate (P:C) ratios. For this experiment, we selected 20 queenless colonies with 14 to 78 workers. Half of the colonies were fed with a high-carbohydrate diet (1:2) and the other half with a high-protein diet (2:1) for four weeks *ad libitum*. Diet recipes were taken from Dussutour & Simpson (2008) and the exact diet compositions are shown in Table 1. The food was additionally colored with green dye (Dr. Oetker) to visually confirm food intake by the ants. After four weeks, two workers of each colony were dissected to assess the presence of green food in the gut, confirming that the ants ate the food since they do not have green guts under normal conditions (own observation). In both treatment groups, 75% of the dissected workers had green guts (Figure 1), demonstrating that the ants had ingested the artificial food.

Long-term experiment

In a longer-term experiment, we removed queens from their colony to induce worker fecundity and examined whether workers showed differences in fecundity and longevity depending on the protein content of their diet. In this experiment, we marked young workers after they hatched from their pupae (callows) to know their exact age.

We selected 15 polygynous source colonies with two queens and at least 120 workers, which we observed for four weeks to mark callows with metal wires (0.2 mm Elektrisola), whose colors were associated with the week of callows' hatching. After marking 10 to 16 individuals per source colony, we divided each source colony into two queenless fragment colonies, each containing 60 workers and 30 larvae. The fragment colonies were fed either a high-carbohydrate (1:2) or high-protein (2:1) diet, as in the

Table 1. Amounts and types of proteins and carbohydrates composing the two artificial diets used in both the pilot and the long-term experiments.

P:C	Whey Protein (g)	Calcium Caseinate (g)	Whole Egg Powder (g)	Sucrose (g)	Water (mL)	Agar (g)
1:2	7.3	6.7	16	40	300	5
2:1	19.3	17.7	16	20	300	5

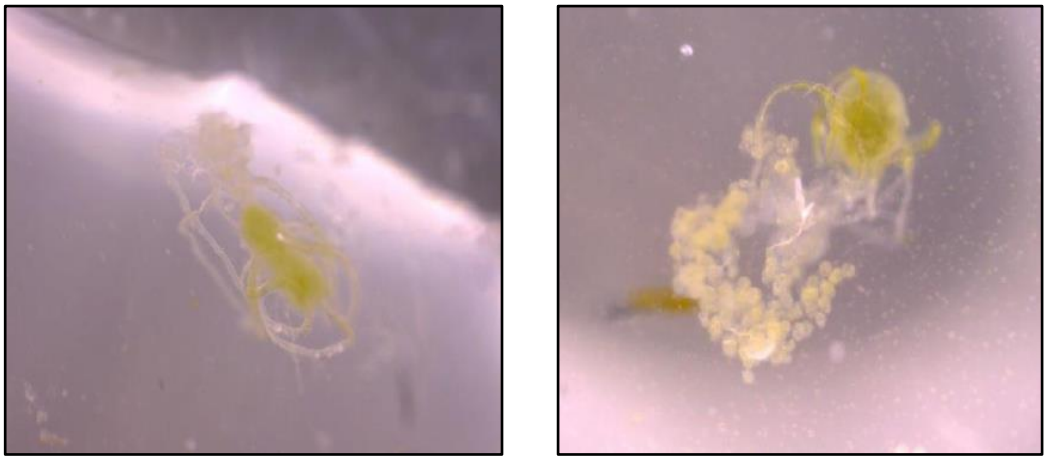

Figure 1. Green-colored guts of workers dissected at the end of the pilot experiment during which colonies were fed with the artificial food dyed in green.

pilot experiment. They were fed four times per week for twelve weeks. Each week we counted the number of workers and brood items and reported how many workers were foraging outside the colony. Every four weeks, each colony was anesthetized with CO_2, and newly-laid eggs were removed and counted. Every two weeks, all new pupae were removed so that marked callows remained the youngest individuals in the colony during the experimental period. During the last week of the experiment, behavioral scans of each colony were performed. The location and behavior of each marked callow were recorded (Table 2). Following the behavioral scans, the marked callows were dissected on ice. Their fat bodies were put in TRIzol (Thermo Fisher), frozen with liquid nitrogen, and stored at -80°C. Ovaries were photographed using a Leica stereomicroscope. In addition, we froze two adult workers for further fatty acid analysis using gas chromatography-mass spectrometry (GC-MS). First, the two adult workers were thawed individually and generously covered with chloroform:methanol 2:1 in a glass vial (Folch solution). After 24 hours, the ants were removed from the glass vial and the solution was evaporated under a nitrogen stream. After evaporation, 250 µl of dichloromethane:methanol (DCM/MeOH) 2:1 solution was pipetted into the glass vial. We added 10 µl of the internal standard (C19:0 dissolved in DCM/MeOH 2:1, 0.2 mg/ml) and vortexed the solution. We transferred 30 µl of the solution into 1.5 ml glass vials with micro-inlets and evaporated the solution under a nitrogen stream again. Finally, we added 20 µl of DMSH (Sigma-Aldrich) and vortexed the solution before injecting the samples into the GC-MS machine. As young workers were emerging at slightly different times in the different colonies, the 30 source colonies were divided into two cohorts. The second cohort started to be fed with the manipulated diet two weeks after the first cohort. The full experimental pipeline can be found in Figure 2. Because the mortality of the first cohort was higher than

Table 2. Lists of callows' locations and behaviors (with corresponding categories in italics) that were reported during the behavioral scans.

Location	Behavior
In the nest on the brood In the nest near the brood (0-1 cm) In the nest away from the brood In the nest at the entrance Outside the nest	*Non-interactive behaviors* Walking Self-grooming
	Resting Resting
	Brood care Antennating larvae Antennating pupae Carrying larvae Carrying pupae Grooming larvae Grooming pupae Trophallaxis larvae
	Nestmate care Antennating worker Carrying worker Grooming worker Trophallaxis worker
	Aggressiveness Mandible opening Antennal boxing

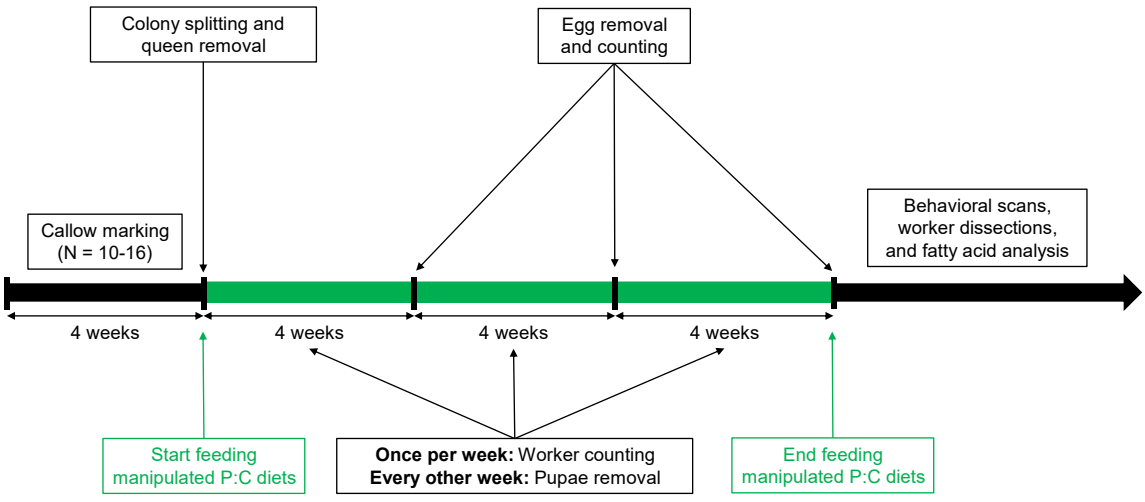

Figure 2. Experimental timeline of the long-term experiment.

expected, we decided to stop the monitoring of the second cohort after nine weeks instead of twelve and to only analyze this data.

We conducted a Cox survival analysis using the packages "survival" (Therneau 2021) and "coxme" (Therneau 2020) to test the effect of diet on worker number over time. With a generalized mixed-effects model (GLMM; binomial family) we analyzed the effect of diet on egg production using a binary variable (presence/absence of eggs at week 8). We used a linear mixed-effects model (LMM) to test the effect of diet on foraging activity. Because all behaviors were codependent, we decided to group them into five different variables for the behavioral analysis: non-interactive behaviors, resting, brood care, nestmate care, and aggressiveness (Table 2). We tested for diet effect on these five variables using principal component analyses (PCAs) only keeping PCs explaining at least 10% of variance for the LMMs. We then measured ovariole length using the Leica software LAS v4.5 and tested the effect of diet on ovariole length using an LMM. The GC-MS data were integrated from minute 8 to 30 using the software MSD ChemStation E02.02 (Agilent 2008) and manually aligned using Microsoft Excel. The retention times were used to identify the different fatty acids. They were classified as saturated, monounsaturated, diunsaturated, polyunsaturated, or unknown fatty acids. The correlation areas resulting from the manual integration were used to determine the total fatty acid content of each sample, using the internal standard as reference. We used LMMs to test the effect of diet on the absolute quantity of fatty acids and the relative proportion of the three sorts of fatty acids. We additionally used PCAs to investigate how the two different diets might affect the proportion of different fatty acids and again only kept PCs explaining more than 10% of variance for the LMMs. Finally, we used the add-on package PERMANOVA+ v1.0.4 (Anderson et al. 2008) of the software PRIMER v6 (Clarke and Gorley 2006) to test for diet effects on the ant fatty acid composition. The assessment of models' fit was performed

via visual inspection of the residual distribution for the LMMs and using the package "DHARMa" for the GLMM (Hartig 2020). We used both source and fragment colony identification (ID) as random factors in all linear models, except in the LMMs used for the behavioral analysis and the analysis of fatty acid composition using PERMANOVA+, where only source colony ID was used as random factor. Statistical analyses were conducted in R v3.5.1 (R Core Team 2020).

Results

Long-term experiment

More workers died over time in colonies fed with the high-protein diet (X^2 = 7.202, df =1, p = 0.007; Figure 3). Diet affected neither the egg production (X^2 = 0.409, df = 1, p = 0.523; Figure S1), nor the foraging activity (X^2 = 0.145, df = 1, p = 0.703). We plotted a Principal Component Analysis (PCA) using callows' location (Figure S2) but did not test for diet effects on specific PCs because of the absence of pattern in the PCA. We also used a PCA to analyze the effect of diet on callows' behavior (Figure 4A) and found that callows from colonies fed with the high-protein diet tended to perform less brood and nestmate care compared to callows from colonies fed with the high-carbohydrate diet (X^2 = 3.708, df = 1, p = 0.078; Figure 4B). The protein content in the diet did not affect callows' ovary development (X^2 = 1.106, df = 1, p = 0.293). And we did not find significant effects of diet on the absolute quantity of fatty acids (X^2 = 0.033, df = 1, p = 0.855) or the proportion of saturated (X^2 = 0.625, df = 1, p = 0.430), monounsaturated (X^2 = 0.005, df = 1, p = 0.946), and diunsaturated (X^2 = 1.548, df = 1, p = 0.213) fatty acids. We additionally plotted a PCA using the proportions of different fatty acids (Figure S3) and although PC1 explained 69.7% of variance, we did not find a significant effect of diet on this PC (X = 0.017, df = 1,

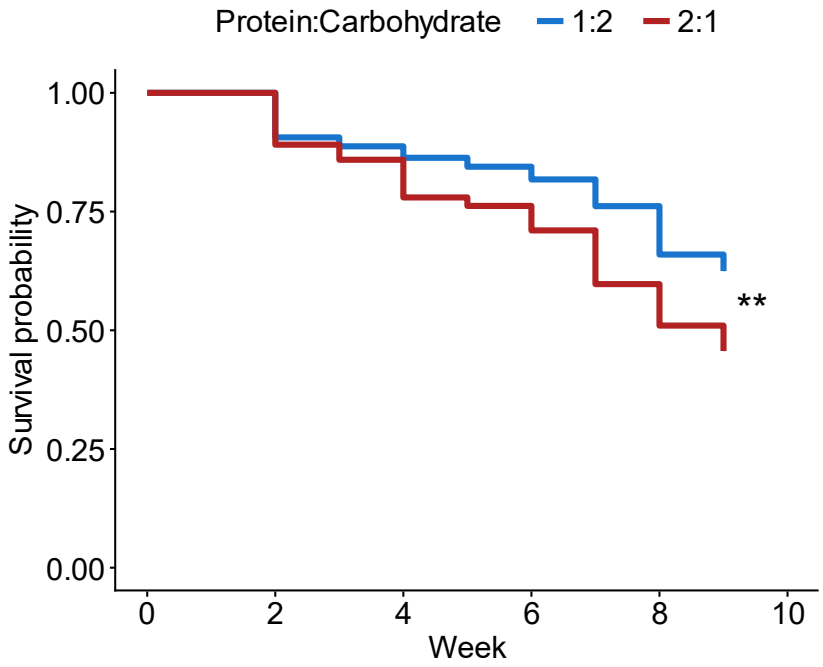

Figure 3. Diet effect on worker survival probability over time. The level of significance is indicated as follows: ** $p < 0.01$.

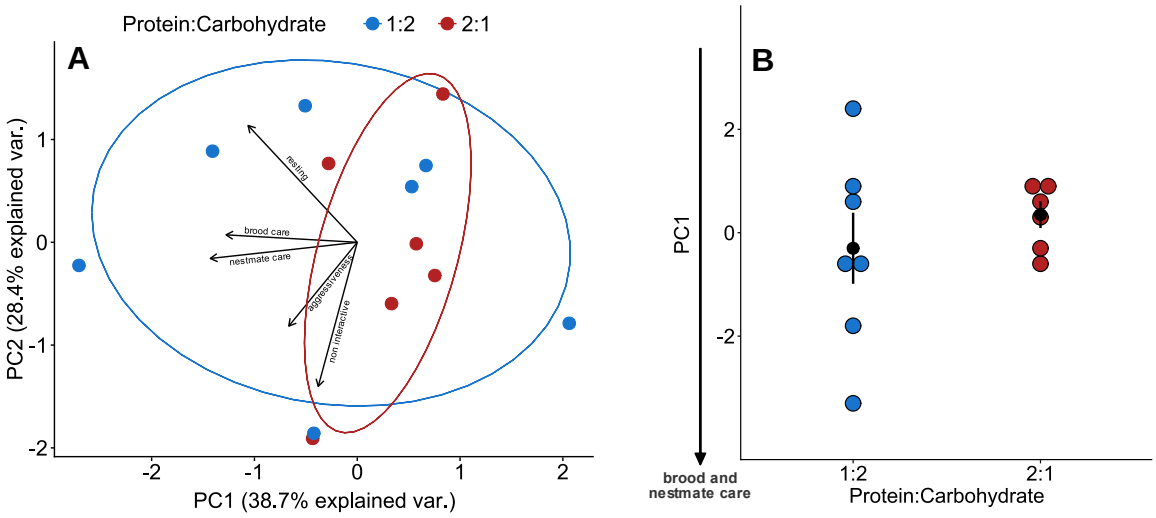

Figure 4. (A) Principal Component Analysis (PCA) showing the five different categories of callows' behaviors reported during the behavioral scans and the sample clustering per diet. **(B)** Diet effect on PC1: lower values are associated with more brood and nestmate care.

p = 0.896). Finally, we could not detect an effect of diet on the composition of worker fatty acids using a PERMANOVA (p = 0.218).

Discussion

Diet composition affects life-history traits across the tree of life (Aron et al. 2001; Naya et al. 2007; Anagnostou et al. 2010; Harvey et al. 2012; Bryndová et al. 2020). Here we conducted an experiment to investigate whether ant worker lifespan, fecundity, behavior, and fatty acids were altered by different ratios of proteins to carbohydrates in the diet. Our results provide evidence for a detrimental effect of proteins on worker survival, while egg production, foraging activity, and worker fecundity and fatty acid amount and composition were not affected. We also observed that workers in colonies fed with the high-carbohydrate diet tended to do more brood and nestmate care.

The high-protein diet negatively affected worker survival. This is especially interesting since *Temnothorax rugatulus* ants show extraordinary resistance to starvation (Rüppell and Kirkman 2005). Consistent with our finding, high-protein diets shorten the lifespan of both solitary insects like the German cockroach (Cooper and Schal 1992) and social insects like the Argentine ants *Linepithema humile* (Arganda et al. 2017), black garden ants *Lasius niger* (Dussutour and Simpson 2012; Poissonnier et al. 2014), and honey bees (Pirk et al. 2010; Paoli et al. 2014). Authors from these studies suggest that the digestion of high amounts of proteins could be detrimental because of its cost, the production of toxic nitrogen waste, and the overstimulation of nutrient-sensing pathways such as TOR. Moreover, large amounts of proteins come with large amounts of amino acids which can alter longevity (Wu 2009; Dato et al. 2016), especially when imbalanced (Grandison et al. 2009; Arganda et al. 2017). Besides, it has been experimentally demonstrated in many organisms including yeast, mice, or other insects, that the

inhibition of TOR signaling by amino acid depletion improves resistance to heat and oxidative stress, therefore slowing down aging (Mair et al. 2005; Powers III et al. 2006; Fontana and Partridge 2015). Altogether, these findings indicate that protein intake should be regulated to optimize survival, as shown in a study using the trap-jaw ant *Odontomachus hastatus*, wherein complementary diets minimized mortality in the context of nutritional challenges (Bazazi et al. 2016).

However, we did not find diet effects on egg production or worker ovariole length. The overall worker mortality was relatively high throughout the experiment, potentially because ant colonies were freshly out of hibernation (pers. comm. Susanne Foitzik) and/or due to the repetitive use of CO_2 to remove pupae (Nicolas and Sillans 1989). Hence, callows might have been too weak to develop their ovaries properly following queen removal. Indeed, the average callow ovariole length was 0.35 mm in our experiment, versus 0.97 mm and 0.66 mm for queenless workers from two other experiments conducted in our lab using the same species (Choppin et al. 2021a). Nevertheless, we know that ants rely on proteins for egg-laying (Hölldobler and Wilson 2009). For instance, queens of the ant *Camponotus floridanus* produced more eggs when their colony received increased amounts of proteins (Nonacs 1991), and colonies fed with a high-protein diet produced more sexuals in *Linepithema humile* (Aron et al. 2001). Thus, there seems to be a correlation between fecundity and nutrient intake, which we might have missed here due to the mortality-induced low sample size mentioned in the methods section. The lack of diet effect on fecundity in our study could also be due to differential effects of proteins on queens and workers. In most social insects, castes are not genetically determined but develop through phenotypic plasticity (Corona et al. 2016), and gene expression differences are stronger between castes of the same species than within the same caste of two closely related species (Hunt et al. 2011). Also, fertile worker gene expression is more

similar to sterile worker gene expression than queen gene expression (Feldmeyer et al. 2014). Therefore, the diet-induced changes observed in ant queens might not apply to ant workers and vice versa. Finally, the ratios of proteins to carbohydrates that we used (1:2 and 2:1) may be too similar to detect diet effects on fertility.

It has been shown in many ant species that nutritional imbalances alter behavior. For example, in the black garden ant *Lasius niger*, aggression levels were increased by both an egg-white diet (Poissonnier et al. 2014) and a high-carbohydrate diet (Dussutour et al. 2016). A similar carbohydrate-induced increase in aggression was found in colonies of the Argentine ant *Linepithema humile* (Grover et al. 2007). In our experiment, we found that workers fed with the high-carbohydrate diet tended to perform more brood and nestmate care, which is consistent with the above-mentioned studies and could be explained by the increased energy uptake from the carbohydrate-rich diet. Nevertheless, worker foraging activity was not affected by the different ratios of proteins to carbohydrates in the diet. This finding goes against what was found in other ant species like *Lasius niger*, *Linepithema humile*, or *Rhytidoponera metallica*, where foragers recruited in larger numbers and collected more food when colonies were fed with a high-protein diet (Dussutour and Simpson 2008, 2012; Arganda et al. 2014). Ants use carbohydrates as an energy source and when the carbohydrate content in their food is low, foragers will collect more food to compensate, leading to an overconsumption of proteins on the colony level (Csata and Dussutour 2019). Conversely, another study using *Lasius niger* found that foragers recruited more intensively for carbohydrates than for proteins (Portha et al. 2002). *Temnothorax* ants are known to have low foraging rates compared to other ant species and workers forage mainly solitarily (Deyrup 2017). However, ants from *Temnothorax* colonies where the density rises show greater foraging and scouting rates than established colonies with low densities (Cao 2013). We can thus

speculate that foraging activity in *T. rugatulus* might be rather linked to colony size and composition, than dietary alterations.

We also did not find differences in the absolute amount or composition of fatty acids between workers fed with the two different diets. Many studies have found diet effects on fatty acid composition across different taxa (Brett et al. 2006; Mccue et al. 2009) including insects (Stanley-Samuelson et al. 1988). It is also established that insects can synthesize fatty acids from sugars (Canavoso et al. 2001; Cook et al. 2010). We can speculate that the amounts of carbohydrates in both diets were sufficient for the ants to synthesize all types of fatty acids needed, and thus no differences between the two groups could be detected. Also, larvae and workers might have different digestion and metabolism abilities, and diet-induced changes in fatty acid composition could have been present in the larvae, but not in the workers. And again, the composition of the two diets might not be different enough to observe changes in the ant fatty acids.

In summary, feeding ant workers different ratios of proteins to carbohydrates greatly altered their survival and, to a lesser extent, their behavior. Although the complex molecular mechanisms underlying aging have not been fully unraveled yet, our study provides novel information on the role of diet in the deterioration of living organisms.

Author contributions

Ant colonies were collected by the three authors. MS collected the data that MC analyzed. MC wrote the first draft of the manuscript and improved it with the help of BF and SF.

Acknowledgments

Thank you to Dr. Florian Menzel for his help with the GC-MS and the fatty acid analysis and to Marion Kever for preparing the wire loops used to mark the callows.

Supplementary material

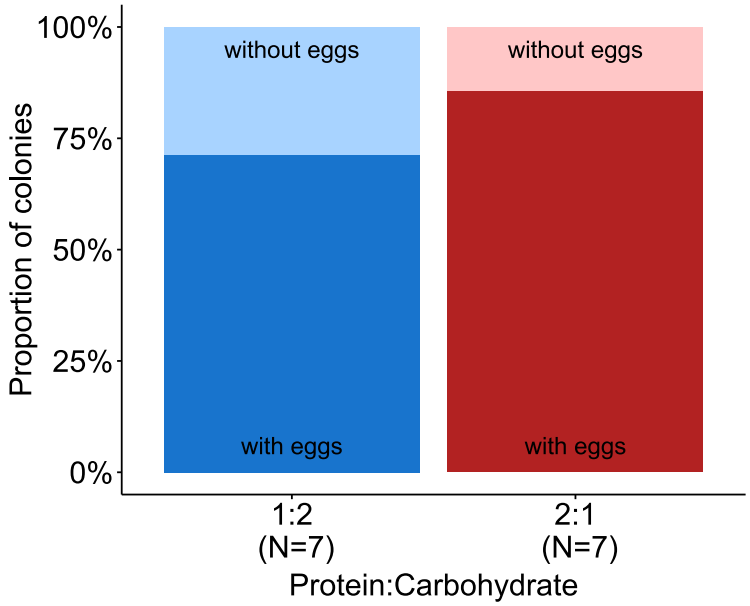

Figure S1. Proportion of colonies with eggs at the end of the experiment depending on the diet.

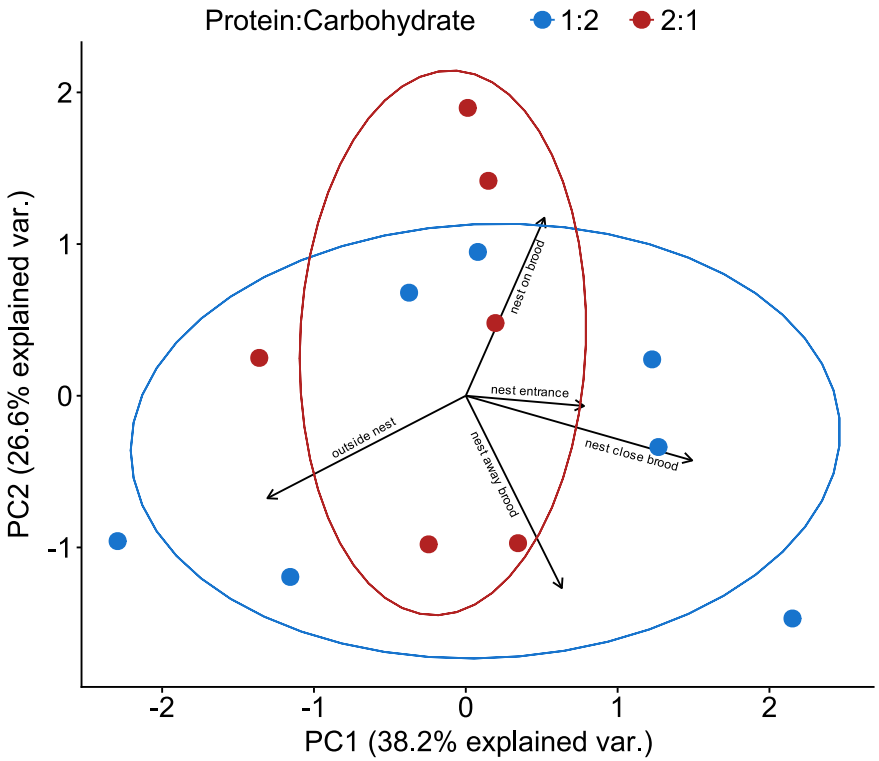

Figure S2. Principal Component Analysis (PCA) showing the five different callows' locations reported during the behavioral scans and the sample clustering per diet.

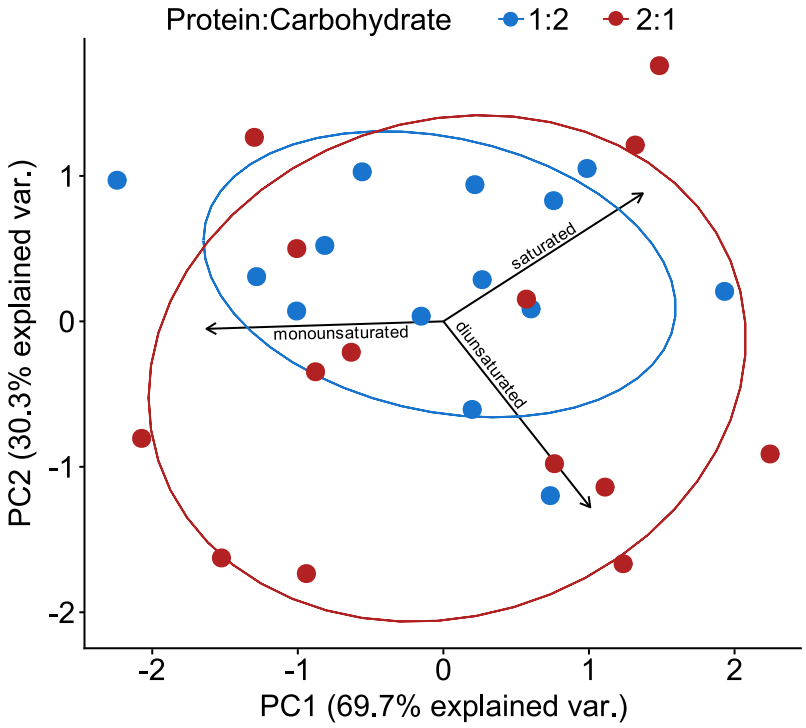

Figure S3. Principal Component Analysis (PCA) showing the three different sorts of worker fatty acids and the sample clustering per diet.

CHAPTER 7

Caste-associated gene expression in the fat body of ant workers

Marina Choppin, Megha Majoe, Matteo Negroni, Barbara Feldmeyer, and Susanne Foitzik

Will be submitted as: Marina Choppin, Megha Majoe, Matteo Negroni, Barbara Feldmeyer, and Susanne Foitzik. Caste-associated gene expression in the fat body of ant workers.

Abstract

Social insects specialize in the task they perform inside and outside the colony, and this division of labor contributes to their ecological success. Not only do reproductives (usually queens) and non-reproductives (workers) differ in a plethora of traits, but chores are also divided between different groups of individuals within the worker caste. In many species, this worker polyethism is associated with morphology, experience, and age, as older workers take over dangerous tasks outside the colony. Studies focusing on characterizing the molecular underpinnings of such phenotypic plasticity revealed both conserved and species-specific genes and pathways. Most of these studies investigated transcriptional activity in workers' brains to establish a link with their behavior. In contrast, we were more interested in the underlying physiology of worker division of labor and therefore investigated fat body gene expression differences between nurses and foragers using the ant *Temnothorax rugatulus*. The fat body is not only involved in fat storage but also fulfills functions in immunity, body maintenance, and fecundity. Many genes associated with stress-response and immunity were differentially expressed between the two worker groups, which we argue is due to foragers having a riskier lifestyle and being older. Moreover, we enrich existing knowledge about the role of histone acetylation in the regulation of foraging behavior in ants, as we found several histone-associated genes upregulated in foragers, but none in nurses. Our findings thus contribute to the characterization of ant worker polyethism and its molecular underpinnings.

Introduction

Ants, termites, and some bees and wasps form complex societies that are highly successful ecologically (Wilson and Otto 1990), mostly due to an efficient division of labor. Indeed, insect societies are characterized by task specialization among colony members. Some individuals (usually called queens) monopolize reproduction, while workers take over various tasks from brood and nestmate care to guarding and foraging (Hölldobler and Wilson 1990; Mersch et al. 2013). Since being outside the colony increases the risk of extrinsic mortality, older workers that have shorter lifespans ahead of them usually take over nest defense or food collection, while younger workers remain inside the nest to care for brood and nestmates (Wilson 1971). This age-related division of labor can be observed in most insect societies. Aside from age, other factors have been associated with the plastic task transition performed by workers such as resource availability, experience, or population density (Beshers and Fewell 2001; Libbrecht et al. 2013b). Moreover, the response threshold model states that internal individual thresholds regulate how workers respond to task-associated stimuli and select tasks as a consequence (Bonabeau et al. 1998; Beshers and Fewell 2001). Although switching tasks can be costly in some cases (Goldsby et al. 2012; Leighton et al. 2017), workers have the potential to be flexible in the task they perform when needed (Robinson et al. 1992, 2009; Giehr et al. 2017). For example, removing younger workers coerced older workers into taking care of the brood in the ant *Temnothorax longispinosus*, although the reversal was not true (Kohlmeier et al. 2018). Task flexibility might be facilitated in species with lower levels of sociality like bumblebees (O'Donnell et al. 2000). Finally, an increasing number of studies demonstrate that worker polyethism can be experimentally manipulated (Simola et al. 2016; Kohlmeier et al. 2018; Libbrecht et al. 2020).

With the rapid advances in molecular techniques and bioinformatic tools in the past decades, researchers have increasingly focused on characterizing the molecular underpinnings of insect societies (see reviews; Toth and Robinson 2007, Smith et al. 2008, Libbrecht et al. 2013, and Corona et al. 2016). Some studies revealed specific behavior-associated genes shared among lineages of social insects that have evolved independently (Toth et al. 2010; Feldmeyer et al. 2021), while others suggest that the evolution of task division involves species-specific mechanisms, albeit connected to common molecular pathways (Morandin et al. 2016; Araujo and Arias 2021). Surprisingly, some genes that regulate behavioral phenotypes in the solitary fruit fly *Drosophila* are also associated with behavior in the ant *Pogonomyrmex occidentalis*, such as *foraging* (Ingram et al. 2011) and circadian-clock genes (Ingram et al. 2009). In the honey bee, the age-related transition from nursing to foraging is associated with changes in the expression of both *foraging* (Ben-Shahar et al. 2002) and *Amfor* (Heylen et al. 2008). On a physiological level, *vitellogenin* genes ancestrally encoding egg-yolk proteins have undergone diversification and neofunctionalization, and are now associated with foraging behavior (Nelson et al. 2007; Marco Antonio et al. 2008), reproduction and brood care behavior (Feldmeyer et al. 2014; Kohlmeier et al. 2018), and belong to endocrine signaling pathways (Sheng et al. 2011; Zhu et al. 2021). Downstream components of these pathways like juvenile hormone regulate polyphenism in ants through maternal effects (Libbrecht et al. 2013a). Altogether, task division appears to be a complex characteristic of insect societies encompassing many components including individuals' age and correlating with the expression of multiple genes in various tissues, that may or may not be lineage-specific.

Many of the studies investigating the transcriptomic signatures of worker polyethism focused on whole body and/or brain gene expression. Here we chose to analyze fat body transcriptomes because this tissue does not only serve as energy storage

but is also very active physiologically (Corona et al. 2007). Previous studies on fat body gene expression in our study species *Temnothorax rugatulus* revealed many interesting genes coding for proteins that have longevity, fecundity, and immunity functions (Negroni et al. 2019; Choppin et al. 2021a). To investigate the transcriptomic signatures of worker polyethism in the ant *T. rugatulus*, we performed gene expression, enrichment, and word search analyses on fat body samples from six nurses (brood carers) and six foragers (outside workers). Based on the literature, we expected to detect age-associated and/or task-associated gene expression differences between the two worker groups.

Methods

Temnothorax rugatulus colonies were collected in 2015 (Chiricahua Mountains, Arizona), transported to our laboratory, and kept in a climate chamber at 22°C under 12:12 light/dark conditions. Colonies were fed crickets and honey twice a week and were provided with water *ad libitum*. Here we analyzed the fat body gene expression of six nurses and six foragers of the ant *T. rugatulus* using a genome-guided transcriptome assembly. Before obtaining the *T. rugatulus* genome, these same fat body samples of nurses and foragers were analyzed using a de-novo transcriptome assembly. Results from the principal component analysis (PCA) and associated enrichment analysis are reported in the study by Korb et al. (2021). Hence, further information on sample collection and sequencing parameters can be found in the aforementioned study.

Raw reads and associated adapters were trimmed using fastp v0.2 (Chen et al. 2018a) with a minimum length of 70 bp and the quality of trimmed reads was assessed using FastQC v0.11.7 (Andrew 2010). Trimmed reads were then mapped against the draft genome of *T. rugatulus* (Jongepier et al. 2021) using HISAT2 v2.1.0 (Kim et al. 2015). Mapping rates were > 90% for all samples (Table S1). We then converted and sorted the

output files with SAMtools v1.10 (Li et al. 2009) and generated a quality report using Qualimap v2.2.1 (Okonechnikov et al. 2016). We created a genome-guided transcriptome assembly with StringTie v2.1.5 (Pertea et al. 2015) and transcripts were extracted using GffRead v0.12.6. We assessed the quality of our transcriptome using TransRate v1.0.3 (Smith-Unna et al. 2016) and removed transcripts with an open reading frame < 100 bp. Finally, we generated the gene count table using the Python script "prepDE.py" from the online StringTie Manual.

We filtered the gene count table so at least five samples had ten or more reads for each gene. We used the filtered gene count table to perform a differential expression analysis with DESeq2 (Love et al. 2014). We plotted a principal component analysis (PCA; Figure S1) with "ggplot2" (Wickham 2010) using all genes to assess the clustering of our samples per group and to detect putative outliers. We also created a heatmap with the package "pheatmap" (Kolder 2012) using the differentially expressed genes (DEGs) only to assess expression differences between samples and sample clustering. Transcripts were annotated by (i) conducting a BlastX homology search with BLAST v2.11.0+ (Altschul et al. 1990) using the non-redundant invertebrate protein database from NCBI (April 2021) and (ii) including additional information on gene function in the organisms *Apis mellifera*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens* retrieved from the UniProt database (https://www.uniprot.org/). We also used TransDecoder v5.5.0 (Haas et al. 2013) to translate nucleotide into amino-acid sequences and ran InterProScan v5.51-84.0 (Quevillon et al. 2005) to get gene ontology (GO) term annotations. We then conducted a GO enrichment analysis (biological processes) with the R package "topGO" (Alexa and Rahnenführer 2020) using the "weight01" algorithm (see Choppin et al. 2021 for details) and created bar plots to visualize enriched GO terms. Finally, we used the package "quanteda" (Benoit et al. 2018) to perform a word search for

immunity terms (see additional method) associated with gene annotations and compared the proportion of upregulated genes containing at least one immunity term in their annotation between nurses and foragers. For both the enrichment and the word search analyses, statistical significance was obtained from Fischer exact tests.

Results

We found 208 differentially expressed genes between nurses and foragers, of which 80 were upregulated in nurses and 128 were upregulated in foragers. The heatmap based on differentially expressed genes (DEGs) separated the two worker groups well, except for one nurse and one forager sample which clustered with the other worker group, and one forager sample which was an outgroup (Figure 1). Among the 10 most upregulated genes in foragers, we found the immune gene "lysozyme-like" (Ragland and Criss 2017) (Table S2) and two "retrovirus-related Pol polyprotein from transposon TNT 1-94" (Table S2). The stress-associated gene "corticotropin-releasing factor-binding protein" (Ragland and Criss 2017) was also present in this list (Table S2). In addition to the top 10 we found several histone genes upregulated in foragers (4/128), but none in nurses (0/80), and among them we found the gene "histone H3-like" ($p = 0.015$) that was also upregulated in workers treated with C646 (inhibitor of histone acetyltransferase p300/CBP) in our previous study Choppin et al. (2021). The enrichment analysis revealed the over-representation of six and seven biological processes associated with genes upregulated in nurses and foragers respectively (Figure 2 and Table S3). The function "DNA integration" was significantly more represented than expected in both nurses (7 genes, $p < 0.001$; Figure 2 and Table S3) and foragers (4 genes, $p = 0.028$; Figure 2 and Table S3). In foragers, two functions related to muscle development were over-represented "striated muscle tissue development" (1 gene, $p = 0.011$; Figure 2 and Table S3) and "actomyosin

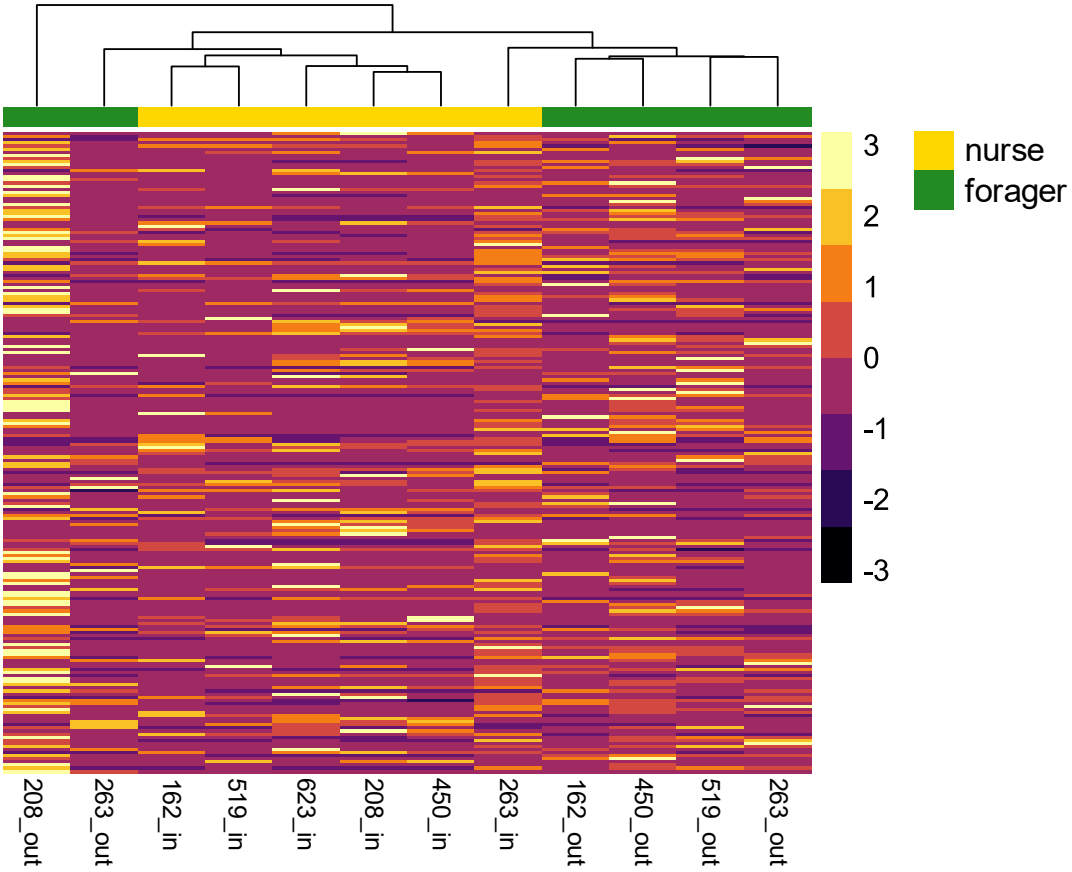

Figure 1. Heatmap showing the expression levels of differentially expressed genes between nurses (yellow) and foragers (green), and the clustering of samples per group. The letters "in" and "out" in sample names stand for "inside" and "outside" referring to worker location at the time of collection.

structure organization" (1 gene, p = 0.022; Figure 2 and Table S3). Interestingly, the function "social behavior" was over-represented in foragers as well (1 gene, p = 0.043; Figure 2 and Table S3) and the gene associated with the corresponding GO term was annotated as "pheromone-binding protein Gp-9-like" (p = 0.031) in the closely related species *Temnothorax curvispinosus*. Finally, based on the word search analysis, foragers upregulated more genes with an immunity function compared to nurses (p = 0.049; Figure 3), which is consistent with the differential gene expression results.

Discussion

Social insect colonies operate following a division of labor that involves the repartition of tasks among different groups of workers and is influenced by diverse factors including age (Hölldobler and Wilson 1990). Using the ant *Temnothorax rugatulus*, we compared fat body gene expression of nurses and foragers to characterize the molecular underpinnings of worker polyethism. Around 200 genes were differentially expressed between the two worker groups. Foragers upregulated multiple genes associated with immunity, stress-response, muscle development, and gene regulation (histones) compared to nurses. We also found a protein-coding gene resembling the pheromone binding protein Gp-9 associated with the enriched biological process "social behavior", which was over-represented in foragers.

The first explanation we give for the upregulation of immunity and stress-response genes in foragers is that worker task alters gene expression. Immune genes upregulated in foragers included genes coding for lysozymes, which are enzymes considered cornerstones of innate immunity (Ragland and Criss 2017). For example, in the Lepidopteran *Bombyx mori* C-lysozymes participate in antiviral defenses against nucleopolyhedrovirus infection (Chen et al. 2018b). Additionally, we found a gene coding

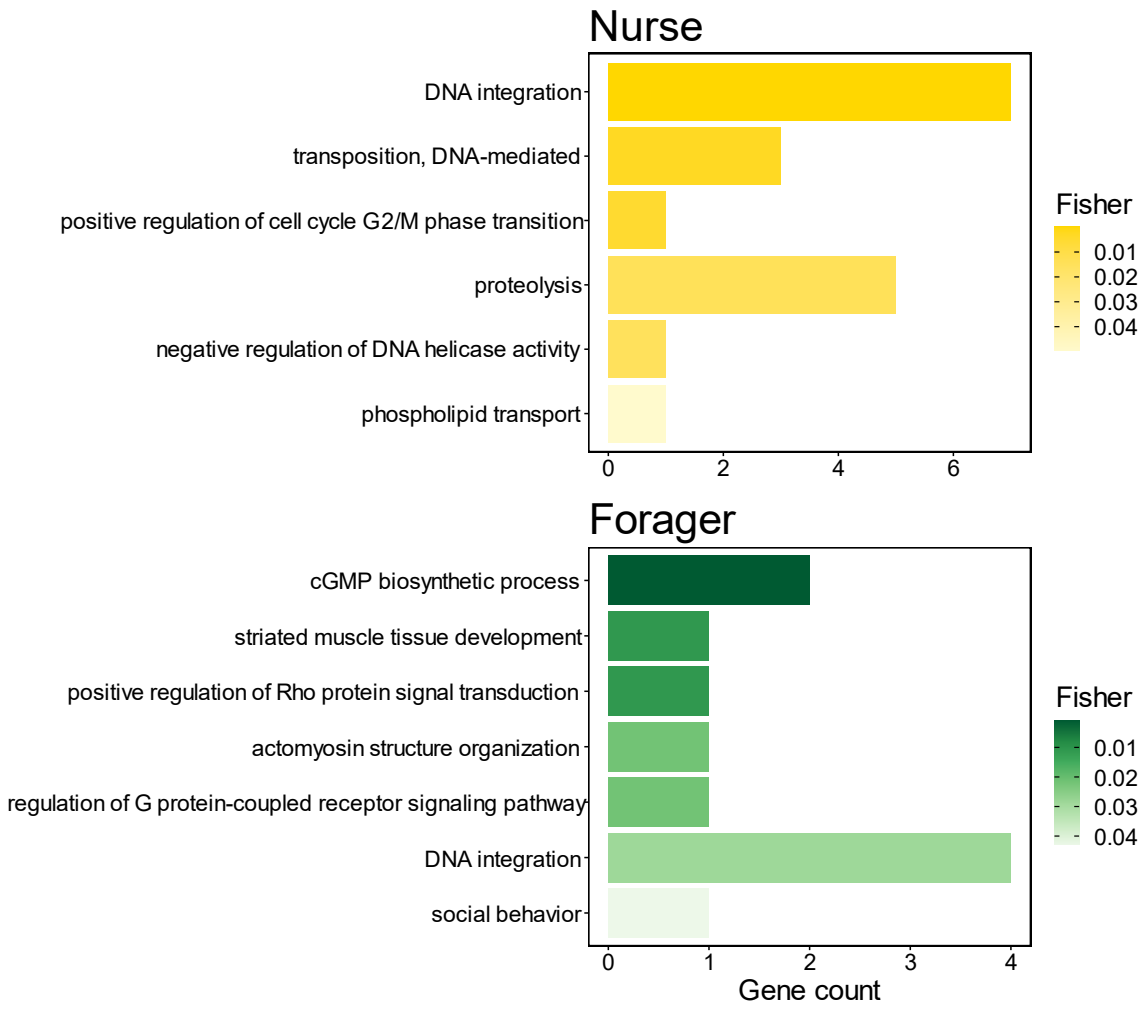

Figure 2. Bar plots showing the significantly over-represented GO terms (biological processes) associated with upregulated genes in nurses (top, yellow) and foragers (bottom, green). Both color darkness and order of terms are proportional to p-value significance. P-values were obtained from Fisher exact tests. Gene counts on the x-axis correspond to the number of significant genes associated with each GO term.

for corticotropin-releasing hormone-binding protein (CRH-BP) upregulated in foragers. This highly conserved glycoprotein coordinates neuroendocrine responses to stress in many organisms (Westphal and Seasholtz 2006), including invertebrates like the honey bee (Liu et al. 2011; Even et al. 2012). Hence, although little is known about foraging-induced stress in social insects aside from the honey bee, we can speculate that the foragers we collected upregulated many immunity and stress-related genes due to the challenges that come with spending time outside the colony. In the wild, individuals that forage for food are indeed required to leave the protected environment provided by their nest and to expose themselves to higher threats of extrinsic mortality such as pesticides, predators, pathogens, or temperature hazards. Consequently, foraging behavior is associated with oxidative stress in different bird species (Glucs et al. 2020; Koyama et al. 2021) and flying produces high levels of reactive oxygen species (ROS) in honey bee flight muscles (Williams et al. 2008). ROS are fundamental elements of the oxidative stress theory of aging (Sohal et al. 2002). Similarly, brain levels of stress-related biogenic amines are higher during the summer months when honey bee colony foraging is peaking (Harris and Woodring 1992). And honey bee foragers upregulate more genes with a immune function (Vannette et al. 2015), which is consistent with our results. Based on this first hypothesis, gene expression would be strongly associated with worker task, similar to what Kohlmeier et al. (2019) found in the closely related ant species *Temnothorax longispinosus*.

Nevertheless, another explanation for the differential expression of immunity and stress-response genes between nurses and foragers could be their age difference. Newly-hatched workers care significantly more for the brood than older workers in *T. longispinosus* (Kohlmeier et al. 2018). Thus, we can assume that the foragers we collected

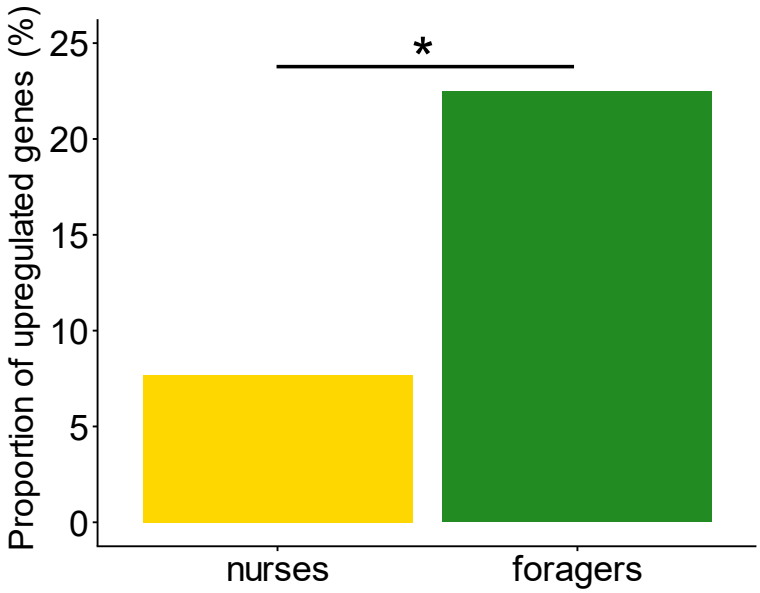

Figure 3. Comparison of the proportion of upregulated genes that had at least one immunity term in their annotation, based on the word search analysis, between nurses and foragers. The level of significance is indicated as follows: * $p < 0.05$.

were on average older than the nurses and consequently showed age-related gene expression differences, similar to what has been found in bees (Colgan et al. 2011; Séguret et al. 2021). Besides, foragers upregulated many genes coding for Pol polyproteins which are characteristic of retroviruses (Varmus and June 1988). Foragers of *T. longispinosus* also upregulate genes related to retroviruses (Kohlmeier et al. 2019). Retroviruses are common in ant genomes (Flynn and Moreau 2019) and respond to stress levels in humans (Cho et al. 2008). These viral elements can also act as transposons (Nelson et al. 2004). And in termites, old workers upregulate many genes that are related to transposable elements (Elsner et al. 2018), suggesting a link between longevity and transposon activity in social insects. Moreover, the GO term "DNA integration" that is related to transposon activity was significantly enriched in both nurses and foragers, raising the question of whether genetic elements are integrated similarly in the DNA of young nurses and old foragers. This term was also found enriched in both queens and workers of another *Temnothorax* species (Gstöttl et al. 2020). Finally, lysozymes (also upregulated in foragers as mentioned above) do not only contribute to innate immunity, but they also operate within lysosomes on top of which mTORC1 (target of rapamycin complex 1) is active (Puertollano 2014; Carmona-Gutierrez et al. 2016). mTOR is considered a key regulator of aging (Stanfel et al. 2009). To summarize, the gene expression differences that we found between nurses and foragers could not only be associated with worker task, but also the age difference between the two worker groups. Both hypotheses are not mutually exclusive and would be difficult to disentangle anyway. In brief, the challenges that foragers face outside the colony might automatically lead to the upregulation genes associated with immunity and stress response, and foragers' defenses might be weaker than those of nurses because of their older age, in line with the disposable soma theory (Kirkwood 1977).

Interestingly, the function "social behavior" was over-represented in foragers. We found that the gene associated with this GO term was coding for a protein resembling the pheromone binding protein Gp-9. This protein regulates social organization in fire ants (Ross and Keller 1998; Krieger 2005). Gp-9 was found downregulated in workers collected in the foraging area compared to workers inside the nest in *Solenopsis invicta* (Lucas et al. 2015). The fact that we found opposite results is consistent with other studies that shed light on how similar traits can be expressed in a species-specific manner in social insects (Feldmeyer et al. 2017; Kohlmeier et al. 2019; Korb et al. 2021).

We also found two functions associated with upregulated genes in foragers that were related to muscle development. This result seems intuitive since foragers take over regular trips outside the colony to collect food and are consequently more active, compared to nurses that spend most of their time inside the nest. Following the same logic, cestode-infected ants show muscular deformations due to their inactivity (Feldmeyer et al. 2016). Changes in muscle gene expression patterns have been attributed both to age (Roberts and Elekonich 2005) and behavior (Margotta et al. 2013) in the honey bee. Besides, termite soldiers show biases in the expression of gene transcripts encoding for muscle proteins (Scharf et al. 2003). To our knowledge, we are the first study to report expression changes of genes related to muscle development in ant foragers.

Finally, four histone genes were upregulated in foragers, but none in nurses. In *Camponotus floridanus*, major and minor workers show behavioral differences, namely in foraging and scouting behaviors, that are epigenetically regulated by a combination of CBP-mediated acetylation and HDAC-mediated deacetylation of histones in the brain (Simola et al. 2016). Moreover, inhibiting the histone acetyltransferases p300/CBP using the selective inhibitor C646 altered foraging behavior in *T. longispinosus* (Libbrecht et al. 2020) and led to the upregulation of "histone H3-like" in *T. rugatulus* workers (Choppin

et al. 2021a), a gene that foragers upregulated in this study. Taken together, these results shed light on the role of histone acetylation in the regulation of ant division of labor.

To conclude, the expression of immunity and stress-related genes was stronger in foragers potentially because they take over riskier chores, but also due to the decline of defense mechanisms with age. Genes associated with muscle development were upregulated in foragers as a result of their active lifestyle. And we also found evidence for an epigenetic component to worker foraging behavior, consistent with results from previous studies in ants. Our study contributes to the characterization of the molecular basis of worker polyethism in social insects.

Author contributions

Ant colonies were collected by MC, MN, BF, and SF. MN collected the samples. MM provided and prepared the data set for the analysis. MC analyzed the data and wrote the first draft of the manuscript. MC, MM, BF, and SF contributed to the improvement of the manuscript.

Acknowledgments

I am grateful to Marah Stoldt for her assistance with the gene expression analysis and to Maide Macit for helping to develop the script for the word search analysis.

Supplementary material

Table S1. Overall alignment rates after mapping for each sample. The letters "in" and "out" in sample names stand for "inside" and "outside" referring to worker location at the time of collection.

Sample	Overall alignment rate
162_out	91.64%
162_in	91.22%
208_out	91.69%
208_in	93.45%
263_out	91.28%
263_in	91.90%

Sample	Overall alignment rate
450_out	91.45%
450_in	92.92%
519_out	93.34%
519_in	92.02%
623_out	92.19%
623_in	90.83%

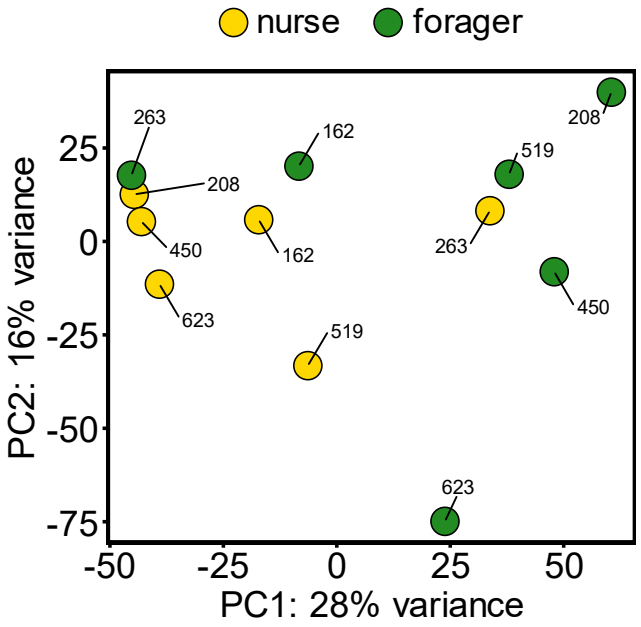

Figure S1. Principal Component Analysis (PCA) using all genes showing the clustering of samples from nurses (yellow) and foragers (green). One dot is one sample and the associated number is the colony ID.

Table S2. Top 10 most upregulated genes in nurses and foragers based on adjusted p-value. We did not include genes that contained the terms “predicted", "low quality", or "uncharacterized" in their blast hit (N = 13 for nurses and N = 5 for foragers).

Caste	Blast hit	Species	Adjusted p-value
Nurse	DNA replication licensing factor Mcm2	*Temnothorax curvispinosus*	0.00068
	trichohyalin	*Temnothorax curvispinosus*	0.00088
	protein NYNRIN-like	*Temnothorax curvispinosus*	0.00182
	leucine-rich repeat-containing protein 24-like	*Temnothorax curvispinosus*	0.00306
	alpha-(1,6)-fucosyltransferase-like	*Temnothorax curvispinosus*	0.00306
	F-box/LRR-repeat protein 4-like	*Temnothorax curvispinosus*	0.00330
	nose resistant to fluoxetine protein 6-like	*Temnothorax curvispinosus*	0.00349
	protein enabled isoform X2	*Temnothorax curvispinosus*	0.00356
	protein artichoke-like	*Temnothorax curvispinosus*	0.00356
	putative nuclease HARBI1	*Temnothorax curvispinosus*	0.00356
Forager	retrovirus-related Pol polyprotein from transposon TNT 1-94	*Trichinella spiralis*	< 0.00001
	lysozyme-like	*Temnothorax curvispinosus*	0.00004
	corticotropin-releasing factor-binding protein	*Temnothorax curvispinosus*	0.00013
	retrovirus-related Pol polyprotein from transposon TNT 1-94	*Trichinella spiralis*	0.00027
	lysozyme 2-like	*Temnothorax curvispinosus*	0.00034
	guanylate cyclase soluble subunit beta-1	*Temnothorax curvispinosus*	0.00035
	histone H1E-like	*Temnothorax curvispinosus*	0.00040
	peptidyl-alpha-hydroxyglycine alpha-amidating lyase 2-lik	*Temnothorax curvispinosus*	0.00046
	POU domain, class 6, transcription factor 2 isoform X2	*Temnothorax curvispinosus*	0.00051
	fatty acyl-CoA reductase wat-like	*Temnothorax curvispinosus*	0.00058

Table S3. GO IDs with corresponding GO terms and associated numbers of genes that were annotated in the universe (Annotated), that were expected to be found among the differentially expressed genes (Expected), and that were indeed found among the DEGs (Significant). P-values were obtained from Fisher exact tests.

Caste	GO ID	GO term	Annotated	Expected	Significant	P-value
Nurse	GO:0015074	DNA integration	108	0.54	7	< 0.00001
	GO:0006313	transposition, DNA-mediated	59	0.30	3	0.003
	GO:1902751	positive regulation of cell cycle G2/M phase transition	1	0.01	1	0.005
	GO:0006508	proteolysis	299	1.51	5	0.014
	GO:1905775	negative regulation of DNA helicase activity	3	0.02	1	0.015
	GO:0015914	phospholipid transport	10	0.05	1	0.049
Forager	GO:0006182	cGMP biosynthetic process	5	0.05	2	0.001
	GO:0014706	striated muscle tissue development	1	0.01	1	0.011
	GO:0035025	positive regulation of Rho protein signal transduction	1	0.01	1	0.011
	GO:0031032	actomyosin structure organization	2	0.02	1	0.022
	GO:0008277	regulation of G protein-coupled receptor signaling pathway	2	0.02	1	0.022
	GO:0015074	DNA integration	108	1.17	4	0.028
	GO:0035176	social behavior	4	0.04	1	0.043

Additional method. Immunity-related terms that were used in the word search analysis (N = 120).

alloimmune
anthelmintics
antibacterial
antibactericidal
antifungal
antifungic
antihelminthics
anti-inflammatory
anti-influenza
antiviral
antiviral-signaling
autoimmune
autoimmunity
autoinflammatory
bacteria
bacteria-associated
bacterial
enteropathogenic
enteropathogens
epithelial-immune
fungi
fungus
helminth
helminths
immune
immune-associated
immune-induced
immune-mediated
immune-responsive
immune-specific
immunity
immunity-associated
immunity-related
immunization
immunized
immunoadhesion
immunoagents
immuno-associated
immunocompetent
immunodeficiency
immunodominant
immunogenic
immunogenicity
immunoglobin
immunoglobulin
immunoglobulin-associated
immunoglobulin-binding
immunoglolin-secreting
immunologic
immunological
immunologically
immunomediatory
immunometabolism
immunomodulating
immunomodulation
immunomodulator
immunomodulatory
immunopathogenesis
immunopathology
immunophilin
immunoproteasome
immunoprotective
immunoreceptor
immunoregulator
immunoregulatory
immunosuppressant
immunosurveillance
immunotherapy
immununity
infected
infecting
infection
infection-dependent
infections
infectious
infectivity
inflammation
inflammation-associated
inflammation-induced
inflammation-related
inflammatory
inflammed
mycobacteria-infected
neuroimmune
neuroinflammation
neuroinflammatory
parasite
parasite-induced
parasites
parasitic
parasitism
parasitize
parasitized
pathogen
pathogen-associated
pathogen-containing
pathogenesis
pathogenesis-related
pathogenic
pathogen-induced
pathogen-mediated
pathogen-recognition
pathogen-responsive
pathogens
pathogen-specific
post-infection
proinflammatory
reinfection
retrovirus
viral
viral-induced
virus
virus-activated
virus-associated
viruses
virus-inactivating
virus-induced
virus-induced-signaling
virus-infected
virus-triggered

General Discussion

Marina Choppin

Summary of findings

The research I conducted for my thesis aimed at better understanding the functional basis of the fecundity-longevity reversal that social insects exhibit, using the ant *Temnothorax rugatulus*. **Chapter 1** reveals that *T. rugatulus* microgynes do not show parasitic tendencies as opposed to smaller queens of other ant species, and I argue that microgynes of this ant species merely are an alternative reproductive morph better adapted to difficult environmental conditions. **Chapter 2** and **Chapter 3** provide novel insights into the modalities of worker reproduction following the loss of their queen. I found that aggressive interactions among individuals and ovarian development, the two main components of worker reproduction, might be differentially regulated. I also suggest a role of histone acetylation in the regulation of longevity and fecundity genes associated with worker reproduction. **Chapter 4** extends this role of histone acetylation in the regulation of ant reproduction to the queen caste, as this epigenetic mark seems to facilitate the experimental increase in queen fecundity induced by egg removal. Although results from **Chapter 5** did not allow the validation of the longevity and fecundity functions associated with the candidate genes we selected, they shed light on challenges that come with RNAi-mediated gene silencing. In **Chapter 6** I demonstrate that a diet with a high protein to carbohydrate ratio has detrimental effects on the survival of reproducing workers, while egg production remains unaffected by this diet alteration. These results are similar to what is found in solitary insects and I discuss why the effect of nutrition on life-history traits might differ between queens and workers. Finally, **Chapter 7** contributes to the characterization of the molecular underpinnings of worker polyethism. I found that foragers upregulated many genes associated with immunity, stress response, and muscle development, and I propose that gene expression differences between nurses and foragers are influenced by both the task they perform and their age difference. Results

from this chapter also add to the growing evidence that histone acetylation participates in the regulation of foraging behavior in ants.

Transposons, piRNAs, and the regulation of social insect longevity

Results from **Chapter 7** revealed that foragers upregulated genes associated with retroviruses. Retroviruses are RNA viruses that use reverse transcription to be compatible with the host genome (Varmus and June 1988). Once integrated into the host genome, viral particles can act as transposable elements (TEs) (Nelson et al. 2004), which are nucleotide sequences moving around in the genome. These genomic elements contribute to genome evolution by inducing mutations (Bourque et al. 2018; Schrader and Schmitz 2019). For example, in the ant *Cardiocondyla obscurior* they facilitate adaptation to novel environments (Schrader et al. 2014). However, they can also create instability, interfere with the processing of nucleic acids, and cause cell damage (Bourque et al. 2018). TE activity increases with age in both vertebrates (De Cecco et al. 2013) and invertebrates (Chen et al. 2016), and has been linked to genetic diseases like cancers (Chénais 2013). Moreover, reduced TE activity extends lifespan in *Drosophila* (Wood et al. 2016). For these reasons, regulating TE activity appears to be crucial. Small, non-coding RNAs including piwi-interacting RNAs (piRNAs) play an important role in the regulation of TEs by controlling their expression via RNA silencing pathways (Lewis et al. 2018). Researchers have been increasingly focusing on the activity and regulation of TEs in the context of social insect longevity (Lucas and Keller 2018). Indeed, the recent study from Elsner et al. (2018) revealed that older termite workers have high transposon activity, but low piRNA expression, compared to reproductive queens and kings. These results provide support for the disposable soma theory (Kirkwood 1977), since older workers that are not as valuable as younger ones seem to invest less in defense mechanisms against TEs. Termite

reproductives might achieve incredibly long lifespans in part through the constant upregulation of the piRNA pathway. Besides, the authors quantified piRNA expression in the head of termites, meaning that reproductive queens and kings use this mechanism for somatic maintenance. This finding reinforces the idea that piRNAs do not only protect the germline (Lewis et al. 2018). Overall, the study from Elsner et al. (2018) provides interesting insights into the mechanisms underlying social insect unusual aging patterns, while opening avenues for further investigation of the relationship between TEs and longevity in other social insect species (Lucas and Keller 2018). On this note, one of our collaborative projects recently revealed abundant ping-pong signatures (evidence for piRNA activity) in ant queen ovaries and ongoing work aims at characterizing small RNAs in both young and old workers of our model species, the ant *T. rugatulus*.

Modalities of the longevity-immunity trade-off

The major cost of immune reactions negatively affects lifespan in many organisms (Moret and Schmid-Hempel 2000; Martin et al. 2003; Little and Killick 2007; Fabian et al. 2018; Yamashita et al. 2021). **Chapter 7** revealed that foragers expressed more genes related to immunity compared to nurses and I propose an effect of worker age on gene expression. Indeed, forager transcriptomes might show signs of immune reactions induced by the challenges these workers face outside the colony, but the efficacy of their immune defenses is questionable considering their older age. This speculation is based on other *Temnothorax rugatulus* findings showing that older queens have lower TOLL activity (Negroni et al. 2019). TOLL is an important pathway for the regulation of immunity in insects (Valanne et al. 2011). This suggests the existence of a trade-off between immunity and lifespan in social insects, as in solitary organisms. However, another study using *T. rugatulus* showed that worker transcriptomic response to an immune challenge depends

on their fertility (Negroni et al. 2020). Indeed, when fertile workers were faced with an immune challenge they upregulated more repair mechanisms than infertile workers, along with the immune system regulator "alpha-ketoglutarate". The fact that fertile workers invest more in somatic maintenance could explain the lifespan extension associated with their reproductive activity. Surprisingly, recent experiments using *T. rugatulus* revealed minor changes in queen and worker traits (i.e., behavior, fertility, chemistry, and gut microbiome) following a similar immune challenge (unpublished data). Finally, an experimental increase in *T. rugatulus* queen fecundity led to the upregulation of genes associated with the TOLL pathway (Negroni et al. 2021a). Although it should be mentioned that the colony setting provides individuals with social immunity (Cremer et al. 2007) whereas queens from this study were isolated with five workers only, which might have led them to activate their own immune system. All these studies using the ant *T. rugatulus* deepened our understanding of the association between lifespan and immunity. Nevertheless, the longevity-immunity trade-off appears to be very complex in social insects and intertwined with multiple factors such as the social environment, caste, age, and fertility of individuals, and might even vary between studies using the same species.

Are nutrient-sensing signaling pathways rewired in social insects?

The nutrient-sensing pathway insulin/insulin-like growth factor 1 signaling (IIS) along with downstream components juvenile hormone (JH) and yolk proteins/vitellogenins are of major importance for the regulation of longevity and fecundity in most organisms (Kenyon 2005, 2010; Narasimhan et al. 2009), including social insects (Corona et al. 2016; Kapheim 2017; Weitekamp et al. 2017). In solitary insects such as *Drosophila* fruit flies, the longevity-fecundity trade-off has been partly attributed to the functioning of this

pathway (Flatt and Kawecki 2007; Toivonen and Partridge 2009; Partridge et al. 2011) (Figure 1A). Indeed, the upregulation of IIS is required for the production of downstream yolk proteins, but simultaneously increases JH titers, a versatile hormone that affects immunocompetence and has life-shortening effects (Herman and Tatar 2001; Hodkova 2008; Yamamoto et al. 2013). Interestingly, recent studies have suggested a rewiring of this signaling pathway as an explanation for the positive association between longevity and fecundity exhibited by social insects (Pamminger et al. 2016; Rodrigues and Flatt 2016; Rüppell et al. 2016). This hypothesis hinges on the fact that vitellogenesis does not seem to strictly depend on JH in social insects (Robinson et al. 1991; Hartfelder 2000), meaning that great reproductive activity might not come at the cost of lifespan. In line with this observation, ant queens of a *Diacamma* species are not characterized by elevated JH titers (Sommer et al. 1993) and honey bee queens seem to downregulate IIS while maintaining higher expression levels of vitellogenin than workers (Corona et al. 2007). Besides, throughout social insect evolution, vitellogenins have undergone multiple events of duplication, diversification, and subfunctionalization, and now take over non-reproductive functions like oxidative stress resistance, potentially slowing down aging (Seehuus et al. 2006; Corona et al. 2007; Parker 2010; Flatt et al. 2013).

Results from my thesis along with findings from previous *T. rugatulus* studies provide complementary evidence for the IIS pathway rewiring hypothesis (Figure 1B). Indeed, fertile workers survive better in this ant species (Negroni et al. 2020, 2021b; Majoe et al. 2021) and genes involved in IIS were found upregulated in fertile workers (Negroni et al. 2021b) suggesting that high IIS activity might not result in the usual longevity-fecundity trade-off observed in solitary insects. Similarly, reproducing workers upregulated a gene coding for a conventional vitellogenin and another gene

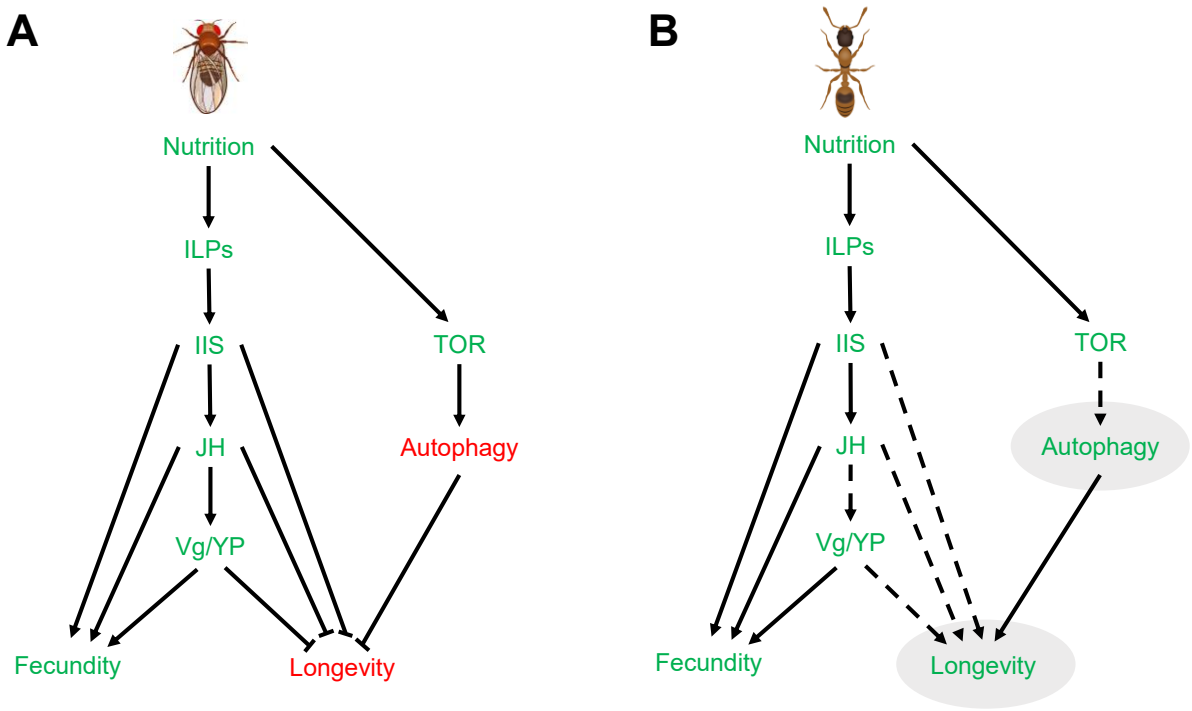

Figure 1. IIS and TOR pathways with downstream components and associated effects on fecundity and longevity in **(A)** the solitary insect *Drosophila* and **(B)** my model species *Temnothorax rugatulus* (hypothetical representation). Full black lines represent known interactions. Dashed black lines and grey ellipses represent parts that are potentially rewired. Upregulated processes are shown in green and downregulated processes are shown in red. Adapted from Lin et al. 2021. Illustrations: iStock (*Drosophila*) and Jenny Fuchs (*Temnothorax rugatulus* queen).

related to JH (**Chapter 2**), while the latest is known to have life-shortening effects in solitary insects, as discussed previously. Target of rapamycin (TOR) is another nutrient-sensing signaling pathway that plays a crucial role in aging (Stanfel et al. 2009; Efeyan et al. 2015). Nutrient intake activates the TOR pathway that negatively regulates autophagy (a cell cleaning process) and decreases lifespan as a result (Hansen et al. 2008; Rubinsztein et al. 2011; Minina et al. 2013; Madeo et al. 2015). We have preliminary evidence that the interaction between TOR and autophagy might work differently in *T. rugatulus*, and other social insect species potentially. Indeed, two autophagy-related genes were found upregulated by fertile *T. rugatulus* workers (Negroni et al. 2021b; **Chapter 2**). We would expect TOR to be downregulated in these fertile workers as they live longer, whereas they upregulated a gene coding for the subunit of mTORC2 (Negroni et al. 2021b). Moreover, dietary restriction negatively affected the expression of the autophagy-related gene "sequestosome-1" in *T. rugatulus* queens suggesting a positive effect of food availability on lifespan through increased autophagy, as opposed to what is found in solitary insects (Negroni et al. 2021a). Hence, we can speculate that fertile workers achieve longer lives partly through elevated autophagy independent of TOR activity (Figure 1B), although there is little evidence for this hypothesis so far. This would resemble what was found in a *Drosophila* study wherein flies that were fed with a TOR selective inhibitor showed increased lifespan but no fertility reduction as a consequence (Mason et al. 2018). However, no evidence for such pathway rewiring was found in the termite species *Cryptotermes secundus* (Lin et al. 2021). Termites belong to the infraorder Isoptera within the Blattodea and might thus differ from social Hymenopterans (ants, and some wasps and bees) on many levels, including the functioning of signaling pathways, due to their different evolutionary trajectory. Moreover, the molecular underpinnings of social insect longevity and fecundity seem to encompass both conserved and species-

specific mechanisms (Korb et al. 2021). Finally, collaborative work within the SoLong research unit provided preliminary evidence for social insects to have several copies of key genes that belong to these signaling pathways (Krüppel homolog 1 for example), while solitary species like *Drosophila* flies would possess fewer or even single copies of these genes. Such gene duplication could allow social insects to circumvent trade-offs that are wired on the molecular level. This work remains ongoing and will be extended to different species of ants (*Temnothorax rugatulus*, *Cardiocondyla obscurior*, *Lasius neglectus*, *Atta colombica*, *and Acromyrmex echinatior*), bees (*Frieseomelitta nigra*, *Anthophora plumipes*, *Bombus terrestris*, *and Euglossa viridissima*), and termites (*Zootermopsis angusticollis* and *Cryptotermes secundus*) to further characterize and compare the functioning of important signaling pathways and associated genes across social insects.

The role of nutrition

Dietary restriction (DR) is the most common experimental manipulation to extend lifespan (Fontana et al. 2010; Fontana and Partridge 2015). For a long time, calories were thought to drive the relationship between nutrition and longevity, but both macronutrients, including proteins and carbohydrates, and micronutrients, such as specific amino acids, have been receiving more attention in the past decades (Moatt et al. 2020). Although the underlying molecular mechanisms of DR-induced lifespan extension are still being discussed (Green et al. 2022), there is a consensus on the importance of nutrient-sensing pathways such as TOR and IIS for the regulation of aging and associated life-history traits (Kenyon 2005, 2010; Narasimhan et al. 2009; Efeyan et al. 2015). Indeed, these pathways underlie the common longevity-fecundity trade-off faced by most living organisms. Many studies have empirically demonstrated that high-protein diets

benefit reproduction while low-protein diets improve lifespan in the solitary *Drosophila* fruit flies (Lee et al. 2008; Lee 2015; Hoffmann Schlesener et al. 2018). However, social insect colonies can be seen as "superorganisms" wherein the germline and soma are divided between queens and workers respectively (Wheeler 1911; Hölldobler and Wilson 2009). Hence, social insects offer great opportunities to investigate the effect of nutrition on life-history traits. Results from **Chapter 6** revealed that a high protein to carbohydrate ratio in the diet (2:1) was highly detrimental to the survival of workers in queenless colonies, while egg production remained unaffected. This result is especially interesting since fertile *Temnothorax rugatulus* workers usually survive better than infertile workers (Negroni et al. 2020, 2021b; Majoe et al. 2021; **Chapter 2**). In queens of *T. rugatulus*, dietary restriction led to lower egg production but did not affect survival (Negroni et al. 2021a). Nonetheless, food availability was not only associated with higher fecundity but also increased somatic maintenance via autophagy in this study. The "superorganism" analogy can be used to make sense of these different effects of nutrition on queens and workers belonging to the same ant species. On one hand, queens that constitute the germline benefit from food intake (especially proteins) for egg-laying, while workers can primarily rely on carbohydrates to conduct their task because they represent the non-reproductive soma and thus do not require high protein intake. However, when workers are forced to ingest high amounts of proteins – like in the experiment from **Chapter 6** – their survival is negatively impacted, while their fecundity remains unaffected, similarly to what is found in solitary insects. On the other hand, queens might not face the life-shortening effects of high protein intake because they constantly upregulate processes like autophagy that benefit their longevity. This last point nicely fits the hypothesis that nutrient-sensing signaling pathways are rewired in social insects, as discussed in the previous section (Figure 1B).

Moreover, the disposable soma theory of aging states that organisms must differentially invest between reproduction and lifespan because resources are limited (Kirkwood 1977). But queens established in mature colonies benefit from theoretically unlimited food provisioning potentially allowing them to overcome such limitation. And the positive effect of food intake on fertility and somatic maintenance uncovered by Negroni et al. (2021a) likely contributes to queen long and fecund lifespans. Results from another *T. rugatulus* study support this hypothesis. Both large macrogynes and small microgynes occur in this ant species and queen morph is associated with alternative reproductive strategies (Rüppell et al. 1998, 2001; Negroni et al. 2021c; **Chapter 1**). Interestingly, small queens that have higher metabolic rates do not differ from large queens in terms of egg-laying or survival (Negroni et al. 2021c). However, small queens actually receive more food than large queens and the authors hypothesize that this higher food intake might allow them to keep up with large queens in terms of fecundity and longevity. Of course, this experiment was conducted under laboratory conditions and the two queen morphs might have different lifespans in the field. This would be particularly interesting to look into since microgynes are most often found in large, polygynous colonies with queens of both morphs and polygynous queens are usually less fecund and shorted lived than monogynous ones (Libbrecht and Kronauer 2014). Conversely, microgynes are more often found at higher elevations (Korb and Heinze 2004; **Chapter 1**) and studies show that ectotherms live longer at higher altitudes (Zhang and Lu 2012). The existence of two queen morphs in *T. rugatulus* vouches for the great potential of this ant species to study the regulation of social insect life history.

Conclusions

The experimental results I gathered for my thesis contribute to a better understanding of the mechanisms underlying the regulation of aging and associated life-history traits in social insects. My work complements existing knowledge about the characteristics of queen morph and associated alternative reproductive strategies in my model species *Temnothorax rugatulus*. I also bring attention to the crucial role of nutrition in the regulation of life-history traits and compare what I found in workers with similar studies in queens and solitary insects. By taking advantage of workers' short lifespans and inducible fecundity I was able to identify many genes contributing to the molecular regulation of worker polyethism. These results shed light on how external factors such as the environment associated with worker task can affect gene expression under the influence of internal factors like worker age. Moreover, my work supports accumulating evidence for the importance of epigenetic mechanisms in the regulation of social insect extraordinary phenotypic plasticity. First, I suggest a novel role of histone acetylation in social insect reproduction, since increased fecundity seems to be facilitated by the regulation of gene expression via this epigenetic mark in both queens and workers. Besides, I propose that worker aggressive behavior and ovarian development following queen loss might be differentially regulated. I also identified many genes that contribute to the regulation of lifespan and fecundity in workers and support the hypothesis that signaling pathways might be rewired in social insects. Second, my results complement those of recent studies that suggest an epigenetic component to worker division of labor by revealing an association between histone acetylation and foraging behavior. These findings open up exciting perspectives of research using advanced chromatin profiling tools to further investigate the epigenetic regulation of social insect fascinating life history.

Research perspectives

Using non-model organisms to investigate the molecular regulation of life-history traits can be challenging. Nevertheless, cutting-edge techniques are being adapted to social insect research and genome assemblies with accurate annotations are being generated for an increasing number of species (Sieriebriennikov et al. 2021). For example, the regulation of phenotypic plasticity via epigenetic mechanisms (mainly histone modification, DNA methylation, and non-coding RNAs) has been extensively discussed in the past decades (Bonasio 2012, 2014; Yan et al. 2014, 2015; Vaiserman 2015; Vaiserman et al. 2018). And researchers have used chromatin profiling techniques like chromatin immunoprecipitation sequencing (ChIP-seq) to experimentally demonstrate the relevance of epigenetics for the regulation of social insect traits (Simola et al. 2013, 2016; Glastad et al. 2019). However, ChIP-seq can be limited due to the high number of input cells it requires (Kidder et al. 2011). And for that reason, newer protocols like cleavage under targets and tagmentation (CUT&TAG) are becoming popular. CUT&TAG requires less input chromatin, lower sequencing depth, and can be done faster (Kaya-Okur et al. 2020). Using chemical inhibitors of histone acetylation (C646) and deacetylation (Trichostatin A) I provided evidence for the role of histone acetylation in regulating reproduction both in queens (**Chapter 4**) and workers (**Chapters 2-3**). Hence, combining transcriptomics with chromatin-based techniques to analyze queen and worker gene expression associated with histone marks would be relevant to complement the results from these chapters. Besides, establishing CUT&TAG within our laboratory would allow additional comparisons between the epigenomes of queens and workers, fertile and infertile workers, or nurses and foragers for example. As of today, no ant studies using CUT&TAG have been published. Another process that can be laborious when working with non-model organisms is the experimental validation of candidate genes. For my

thesis, I aimed at validating the function of candidate genes that might be involved in the regulation of worker fecundity and longevity (alpha-tocopherol, transketolase, and vitellogenin 1) using RNAi-mediated gene silencing. However, results from **Chapter 5** suggest that feeding dicer-substrate small interfering RNA (dsiRNA) to target the candidate genes might have negatively affected workers and resulted in low fecundity. In the ant *Harpegnathos saltator*, injecting siRNA targeting the corazonin receptor confirmed the role of this peptide in the regulation of behavioral transitions between workers and pseudo-queens (Gospocic et al. 2017). It would thus be relevant to repeat the gene silencing experiment I conducted using another technique such as siRNA injection.

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