The molecular basis of behavioural and life-history strategies and parasite-host coevolution in ants

Dissertation

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"Until recently, genetic considerations have predominated in discussions of evolution and selection. Compared to the enormous progress made in genetics, there has been relatively little systematic effort to analyze environmental effects on the phenotype, and their evolutionary consequences. The plastic phenotype, stigmatized by poorly understood environmental influences and the ghost of Lamarck, has sometimes been lost from view as the focus of selection." - Mary Jane West-Eberhard (1989)

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Zusammenfassung

In fluktuierenden, aber vorhersehbaren Umgebungen stellt die phänotypische Plastizität eine wichtige Strategie für einen Organismus dar, um sich an diese Schwankungen anzupassen. In Kolonien sozialer Insekten kann Plastizität, insbesondere bei Verhaltensmerkmalen, das Überleben einer Kolonie sicherstellen, wenn sich das soziale Umfeld ändert. Dies ist zum Beispiel der Fall, wenn eine Königin oder ein großer Teil der Arbeiterinnen verloren geht oder wenn eine Kolonie von Parasiten befallen wird. Phänotypische Plastizität kann zwar zur erfolgreichen Abwehr von Parasiten beitragen, sie kann aber auch von Parasiten zu ihrem eigenen Vorteil ausgenutzt werden.

In der folgenden Dissertation untersuche ich am Beispiel von Ameisen der Gattung *Temnothorax* den Einfluss des sozialen Umfelds auf die Genexpression, insbesondere im Gehirn, dem Zentrum der Verhaltenskontrolle. Darüber hinaus nutze ich vergleichende Transkriptomanalysen, um zu klären, ob phänotypische Plastizität eine treibende Kraft in der Evolution des Sozialparasitismus bei Ameisen sein könnte und ob Endoparasiten in der Lage sind, die Genexpression von plastischen, kastenspezifischen Gene zu verändern, um deren Übertragung zu erleichtern.

Meine Arbeit zeigt, dass experimentelle Manipulationen des sozialen Umfelds nicht nur das Verhalten, sondern vor allem die Genexpression im Gehirn beeinflussen, und dass die Funktion dieser Gene Aufschluss über andere biologische Merkmale, einschließlich Veränderungen der Immunität, geben kann (**Kapitel 1 und 2**). Ebenso wirkte sich die Anwesenheit sozialer Parasiten sowohl auf der Populations- als auch auf Kolonieebene spezifisch auf das Transkriptom des Arbeiterinnengehirns aus, was zeigt, dass es den Parasiten nicht vollständig gelingt, ihre Wirte zu täuschen (**Kapitel 4 und 5**). Als Nächstes untersuchte ich, ob Sozialparasiten und ihre Wirte homologe Gene für dieselben Verhaltensweisen, Kasten, Geschlechter und Entwicklungsstadien

verwenden, was die Evolution des Sozialparasitismus vermutlich erleichtert hätte (**Kapitel 3, 6 und 7**). Meine Ergebnisse legen nahe, dass vergleichbaren Phänotypen von Sozialparasiten und ihren Wirten trotz ihrer engen phylogenetischen Verwandtschaft unterschiedliche Gene zugrunde liegen. Schließlich zeige ich in **Kapitel 8**, dass die verlängerte Lebensdauer von *Temnothorax*-Ameisen, die mit einer Bandwurmzestode infiziert sind, nicht durch die Expression von Genen mit plastischer Expression zustande kommt, die typischerweise in Königinnen überexprimiert werden. Insgesamt zeigen meine Ergebnisse, dass sich Verhaltensplastizität als Reaktion auf Veränderungen in der sozialen Umwelt direkt in der Genexpression widerspiegelt und dass die hier untersuchten Parasiten jedoch diese Plastizität ihrer Wirte nicht zu nutzen scheinen.

Summary

In fluctuating but predictable environments, phenotypic plasticity represents an important strategy for an organism to adapt to these fluctuations. In colonies of social insects, plasticity, especially in behavioural traits, can ensure the survival of a colony when the social environment changes. This is the case, for example, when a queen or a large proportion of workers are lost or when a colony is attacked by parasites. However, while phenotypic plasticity can contribute to successfully defending against parasites, it can also be exploited by parasites to their own advantage.

In the following thesis by using ants of the genus *Temnothorax* as models, I investigate the influence of the social environment on gene expression, particularly in the brain, the centre of behavioural control. In addition, I make use of comparative transcriptomics to investigate whether phenotypic plasticity is a driving force in the evolution of social parasitism in ants and whether endoparasites are able to alter gene expression of plastic, caste-specific genes to facilitate transmission.

My work demonstrates that experimental manipulations of the social environment affect not only behaviour but, more importantly, gene expression in the brain, and that the function of these genes can provide information about other life-history traits, including changes in immunity (**Chapters 1 and 2**). Similarly, the presence of social parasites, either at the population or colony level, specifically affected the transcriptomic signature in the worker's brain, demonstrating that parasites do not completely succeed in deceiving their hosts (**Chapters 4 and 5**). Next, I investigated whether social parasites and their hosts share homologous genes for the same behaviours, castes, sexes, and developmental stages, which would likely have facilitated the evolution of social parasitism (**Chapters 3, 6, and 7**). My results suggest that different genes underlie comparable phenotypes of social parasites and their hosts despite their close phylogenetic relationship. Finally, I show in **Chapter 8** that the

extended lifespan of *Temnothorax* ants infected with a tapeworm cestode is not realized through the expression of genes with variable expression, typically overexpressed in queens. Overall, my results show that behavioural plasticity in response to changes in the social environment is directly reflected in gene expression and that the parasites studied here, however, do not appear to exploit this plasticity in their hosts.

General Introduction

Phenotypic plasticity

The ability of an organism to adapt to its environment directly affects its fitness. Already over 160 years ago Darwin described in "On the Origin of Species" how beak shape in finches likely represents an adaptation towards their habitats and the food they consume (Darwin, 1859). Similar examples of adaptations to different habitats are found across all living systems, some prokaryotes adapted to extreme salinity, plants such as succulents to periods of drought, and blind mole rats to hypoxia (Madern *et al*, 2000; Griffiths and Males, 2017; Nevo, 2013). When species are faced with environmental changes, they can employ different strategies to deal with those fluctuations, which ultimately depend on the variability and predictability of the environment (Xue *et al*, 2019). Genetic adaptations are mutations of the DNA sequence, which are beneficial for an organism's fitness and thus become fixed throughout the population (Orr, 2005). Bet-hedging on the other hand is a strategy where phenotypes with less within-generation fitness are expressed to avoid zero fitness in extreme and less predictable environments (Simons, 2011). One such strategy is the diversification of phenotypes across the population thus spreading the risk. As at least one of the phenotypes is likely to be adapted to the new environment this strategy might still increase the mean fitness of the genotype across generations, especially in environments that change constantly and unpredictably (Veening *et al.*, 2008; Hopper, 1999; Mahony and Thumm, 2002; Grimbergen *et al.*, 2015). The third strategy is phenotypic plasticity. Phenotypic plasticity represents the interaction of the environment with the genotype resulting in the regulation of gene expression thus creating diverse phenotypes (West-Eberhard, 1989). This can either result in continuous phenotypes correlating with the environmental cue (Tollrian, 1993; Neufeld, 2012) or in discrete ones, so-called polyphenisms (Yang and Pospisilik, 2019). Especially in predictably changing environments, the strategy of phenotypic plasticity

allows organisms to flexibly respond to their surroundings (Chevin and Hoffmann, 2017). In that respect, polyphenisms are often already triggered during an individual's development, providing less plasticity throughout the adult stage. Juvenile clownfish for example adapt the white stripe formation of their adult stage to the respective anemone species they live in during development (Salis *et al.*, 2021). Another famous example is the water flea *Daphnia* which during development adjusts to a high predator density by developing specialized neck teeth (Krueger and Dodson, 1981). Due to the speed of climate change, phenotypic plasticity as a means of dealing with environmental fluctuations might gain importance for species to avoid extinction (Urban, 2015; Wiens, 2016; Lewis *et al.*, 2018; Chown *et al.*, 2010; Crozier *et al.*, 2008; Merilä and Hendry, 2014). But whether phenotypic plasticity is a driver of genetic adaptations or hinders them, is still highly debated (Price *et al.*, 2003; Oostra *et al.*, 2018; Fierst, 2011; Crispo, 2007; Pfennig *et al.*, 2010; West-Eberhard, 1989).

Plastic traits

A multitude of traits of an organism is affected by its environment. These include life-history traits such as body size, reproduction, and longevity as these are directly related to an organism's fitness, making adaptations necessary (Ashton *et al.*, 2000; Winemiller, 1989; Capdevila *et al.*, 2020; Healy *et al.*, 2014). If organisms are faced with different environmental cues, they might adapt to this variability using phenotypic plasticity in these life-history traits (Nylin and Gotthard, 1998). For example, in crickets, the timing of hatching determines the growth rate and adult body size (Carrière *et al.*, 1996), while water spiders maintain their reproductive potential at the cost of longevity in food-restricted environments (Kaitala, 1991). Changes in life-history traits might moreover influence other traits such as behaviour. For example, the evolution of parental care is known to be influenced by life-history traits including egg death rate

and juvenile survival (Klug and Bonsall, 2010). But behaviour itself is a very plastic trait too and strongly influenced by the environment. As definitions differ, I here refer to behaviour as "the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and/or external stimuli, excluding responses more easily understood as developmental changes" (Levitis *et al.*, 2009). It is considered a labile trait as it can change over the lifetime of an individual and even be reversible, allowing adaptations to be regulated during the adult stage and not only during development (Scheiner, 1993). Behaviours that represent plastic responses include, amongst others, aggression, foraging, and mating behaviour (Bhat *et al.*, 2015; Kause *et al.*, 1999; Forsgren *et al.*, 2004). As these behaviours influence an organism's fitness, natural selection is acting on them. Learning for example has also been shown to directly influence an organism's fitness demonstrating that this behaviour, like others, is subject to natural selection (Dukas and Duan, 2000). Moreover, plasticity in behaviour is often a prerequisite for genetic adaptations (West-Eberhard, 1989). By changing the behaviour and transmitting it to other conspecifics, species suddenly experience novel selection pressures, and this could drive the adaptation in other traits such as morphology or physiology (Wyles *et al.*, 1983). Dim-light foraging behaviour in *Apis mellifera* for example preceded the evolution of other morphological and physiological changes associated with low-light environments (Wcislo and Tierney, 2009). The feeding behaviour of humans to consume milk drove the fixation of genes involved in lactose digestion (Gerbault *et al.*, 2011). And the morphological and physiological differentiation between workers and queens in social wasps originated from solitary species which had to adjust their behaviour into a reproductive and ovarian depletion phase (West-Eberhard, 1987). Overall behaviour can not only be used to deal with changing environments, but it might also facilitate adaptations in

other traits including life-history traits and new behaviours that may arise due to natural selection.

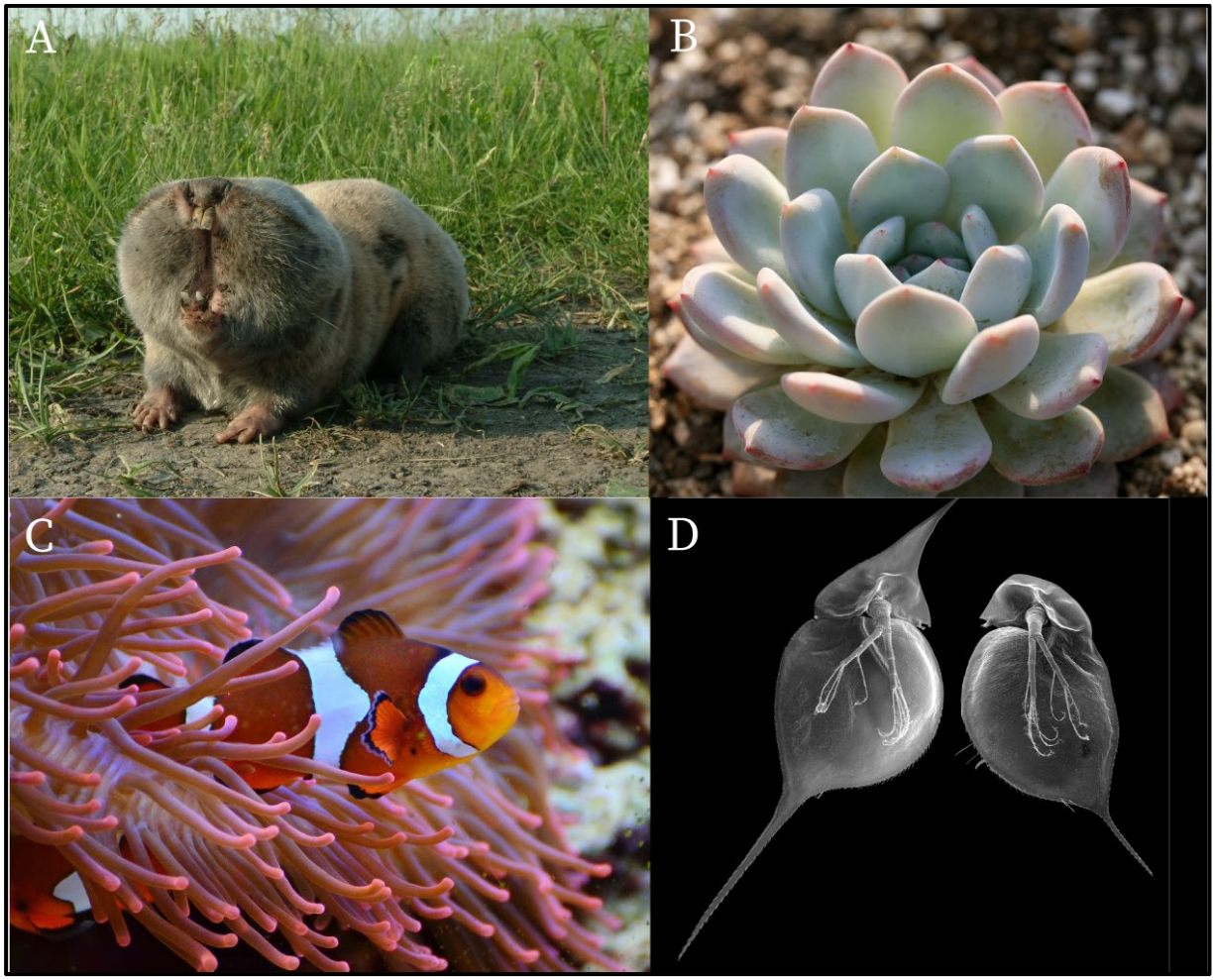

Figure 0-1 Examples of genetic adaptation and phenotypic plasticity. (**A)** The blind mole rat adapted to extreme environmental conditions in underground burrows and has a high tolerance to hypoxia. (**B)** Succulents adapted to dry environments by storing more water inside. (**C)** The white stripes of clownfish are plastically regulated depending on the anemone they live in. (**D)** The formation of a helmet is induced via the presence of predators in the environment in *Daphnia* water fleas. Photo credit: Gábor Csorba (**A**), Pixabay (**B, C**), and Anurag A. Agrawal (**D**).

How the social environment shapes traits

Social behaviour can be summarized as all biological actions which involve at least two individuals of the same species. These behaviours range from the usage of pheromones to attract or dispel others and visual or acoustic signals to communicate to physical interactions including parental care or aggression (Dweck *et al.*, 2015; Khannoon *et al.*, 2011; Vannoni and McElligott, 2008; Setchell *et al.*, 2006; Clutton-

Brock, 1991; Otronen, 1988). The sum of all these behaviours an organism experiences at a certain time point can be described as its social environment. The social environment has been shown to influence both behaviour and life-history traits in various species. Sexual behaviours for example are influenced by social interactions in *Drosophila melanogaster* males (Krupp *et al.*, 2008) and guppies flexibly adjust their mating strategy to the current sex ratio (Karlsson *et al.*, 2010). Similarly, a complete lack of interactions was shown to cause altered behaviour as well as changes in physiology and life-history in several species including fish, rodents, monkeys, and insects (Shams *et al.*, 2018; Harlow *et al.*, 1965; Zhao *et al.*, 2009; Boulay, 1999). Due to the high number of interactions, especially social species are strongly influenced by changes in their social network including eusocial insects. One of the more pronounced examples of how changes in the social environment affect social insects is the presence of the queen: After the removal of queens from colonies, worker fecundity and behaviour are altered strongly (Kohlmeier *et al.*, 2017; Almond *et al.*, 2019; Heinze *et al.*, 1997; Friend and Bourke, 2014). Due to its strong impact on multiple phenotypic levels, the social environment can facilitate the emergence of new behaviours within a species. Brood-mixing for example probably evolved from individuals providing parental care close to others, increasing the number of interactions (Wong *et al.*, 2013). While in some cases this might even be beneficial (Eberhard, 1986), in others this might lead towards the evolution of brood parasitism. Females of the dung beetle *Onthophagus taurus* for example exchange eggs laid by other females in food-provisioning brood balls with their own (Moczek and Cochrane, 2006). Another famous example for brood parasitism is the cuckoo which lays its eggs into the nest of another species (Davies, 2000). It has been suggested that this form of interspecific parasitism evolved from intraspecific parasitism and that it is more likely to arise in species that provide parental care to their offspring (Lyon and Eadie, 1991;

Yamauchi, 1995; Robert and Sorci, 2001). These examples highlight how the social environment can shape the evolution of novel traits including parasitism.

(Social) Parasitism

Parasites are characterized by the exploitation of their host's resources for their benefit and are found all over the tree of life comprising the majority of species (Windsor, 1998). This comes at a cost for the host, often affecting its life-history traits such as fecundity and longevity (Agnew *et al.*, 2000; Baudoin, 1975; Morand and Harvey, 2000). Especially obligate parasites, those that cannot survive without their host, have adapted to their lifestyle by increasing the efficiency and frequency of host infection, in turn inducing host defence counteradaptations (Dawkins and Krebs, 1979; Britton *et al.*, 2007). Parasites with complex life cycles often use behavioural manipulation of hosts to facilitate transmission to their finite host and to circumvent any host defence (Moore, 2013). One inexpensive way to manipulate the host's behaviour is to make use of already existing phenotypic plasticity and to redirect it for its purpose. Wasps parasitized by a parasitoid for example showed shifts in brain gene expression resembling those of gynes, suggesting that the parasite might exploit the already present gene-regulatory network which regulates caste differentiation in wasps for its benefit (Geffre *et al.*, 2017). But behaviours of hosts are not exclusively altered by endoparasites. Parasites that exploit the social behaviour of their hosts are known from the birds where they are termed brood parasites and from the social insects as social parasites (Davies, 2000; Buschinger, 1970). Especially the many parallels between both phylogenetically distant systems have made them interesting subjects to study the convergent evolution of coevolutionary dynamics between parasite and host (Kilner and Langmore, 2011). Social insects provide a broad variety of forms of social parasitism: Xenobiosis, temporary parasitism, inquilinism, and dulosis (Buschinger,

1986). What all have in common is that the parasites occupy a colony of another species and redirect the social behaviours performed by the residing workers towards themselves. Except for the guest ants, which reside inside the colony of another species mostly to be fed by the workers, all other forms of social parasitism occur between closely related species following Emery's rule (Emery, 1909). Despite the close phylogenetic relationship between parasites and hosts their lifestyle differs vastly. Dulotic ants are characterized by annual raids of host colonies during summer and otherwise do not perform any usual tasks such as brood care and foraging during the year (D'Ettorre and Heinze, 2001). These raids allow them to replenish their host worker force to perform all necessary worker chores inside their colony. In the host species *Temnothorax curvispinosus*, which is closely related to the parasite *Temnothorax duloticus*, workers were found to steal brood when close to each other (Wilson, 1975). The close phylogenetic relationship and the fact that raiding behavior can also be observed in host species suggests that the evolution of the parasitic behavior in dulotic ants is facilitated by the reuse of genes present in the non-parasitic ancestor and the host species.

Study system

In the following thesis, I used ants of the *Formicoxenus* (formerly *Formicoxenini*) species group (Blaimer *et al.*, 2018) to study the molecular basis of plastic responses towards different environments and the contribution of phenotypic plasticity to the evolution of parasitism for several reasons which I outline below.

Ants of this subgroup show no morphological differentiation between worker castes and only limited size variation between workers in general, allowing for comparisons between ants performing different behaviours without the confounding factor of different morphologies (Fjerdingstad and Crozier, 2006). The ant *Temnothorax*

longispinosus has already been studied in respect to transcriptomic changes between workers performing brood care and foraging, identifying *Vitellogenin-like A* as a key player (Kohlmeier *et al.*, 2018, 2019). Thus, ants of this subgroup are great models to study the molecular basis of plastic changes in behaviour.

Moreover, the *Formicoxenus* subgroup has previously been described as a hotspot for the emergence of socially parasitic behaviour with at least six independent origins (Beibl *et al.*, 2005). The dulotic ant *Harpagoxenus sublaevis* and its hosts *Leptothorax acervorum* and *Leptothorax muscorum* have been extensively studied in respect to the coevolutionary dynamics (Bauer *et al.*, 2009; Foitzik *et al.*, 2003; Kaib *et al.*, 1993; Heinze *et al.*, 1994). Moreover, in the system of *Temnothorax americanus*, again a dulotic ant from the same tribe, and its host *T. longispinosus* a rebellion of host workers inside parasitic nests has been described as well as a coevolutionary arms race with differing selection pressures depending on the ratio between parasites and hosts (Brandt and Foitzik, 2004; Achenbach and Foitzik, 2009; Brandt *et al.*, 2005a; Jongepier *et al.*, 2015; Kleeberg *et al.*, 2015; Jongepier *et al.*, 2014). Following the loose form of Emery's rule, both parasites are closely related to their respective hosts (Beibl *et al.*, 2005). Therefore, these systems allow studying the plastic responses of hosts towards their parasite in respect to parasite prevalence as well as the evolutionary transition towards parasitism from a non-parasitic ancestor.

Besides social parasitism, this subgroup is rich in other alternative life-history strategies including facultative polygyny. In the ant *Temnothorax rugatulus* the variation in queen number observed between colonies is additionally associated with queen size variation (Rüppell *et al.*, 1998, 2001a; Choppin *et al.*, 2021b). Whether queen size and queen number are genetically determined as in other systems including *Solenopsis invicta* and *Formica selysi* remains to be investigated (Purcell *et al.*, 2014;

Wang *et al.*, 2013). Because the association between both factors is less strict in *T. rugatulus*, this species is an ideal model for experimental manipulation of colonies to study the plastic responses of the different queen morphs towards changes in their social environment.

Finally, at least for some of the ants from this subgroup infection with a tapeworm has been described (Plateaux, 1972; Heinze *et al.*, 1998). In *Temnothorax nylanderi* infection with the cestode stage of *Anomotaenia brevis* induces multiple phenotypic changes including less sclerotization of the cuticle, less activity and flight behaviour, and increased lifespan (Trabalon *et al.*, 2000; Beros *et al.*, 2015, 2021). While the transcriptomic basis of the altered behaviour of infected workers has already been investigated (Feldmeyer *et al.*, 2016), the mechanisms underlying their extended lifespan remain unknown. Comparing the transcriptomes of infected workers with other phenotypes offers the possibility to unravel whether changes induced by the parasite are realized by the expression of genes originally designed to express other phenotypes, for example, related to the queen phenotype.

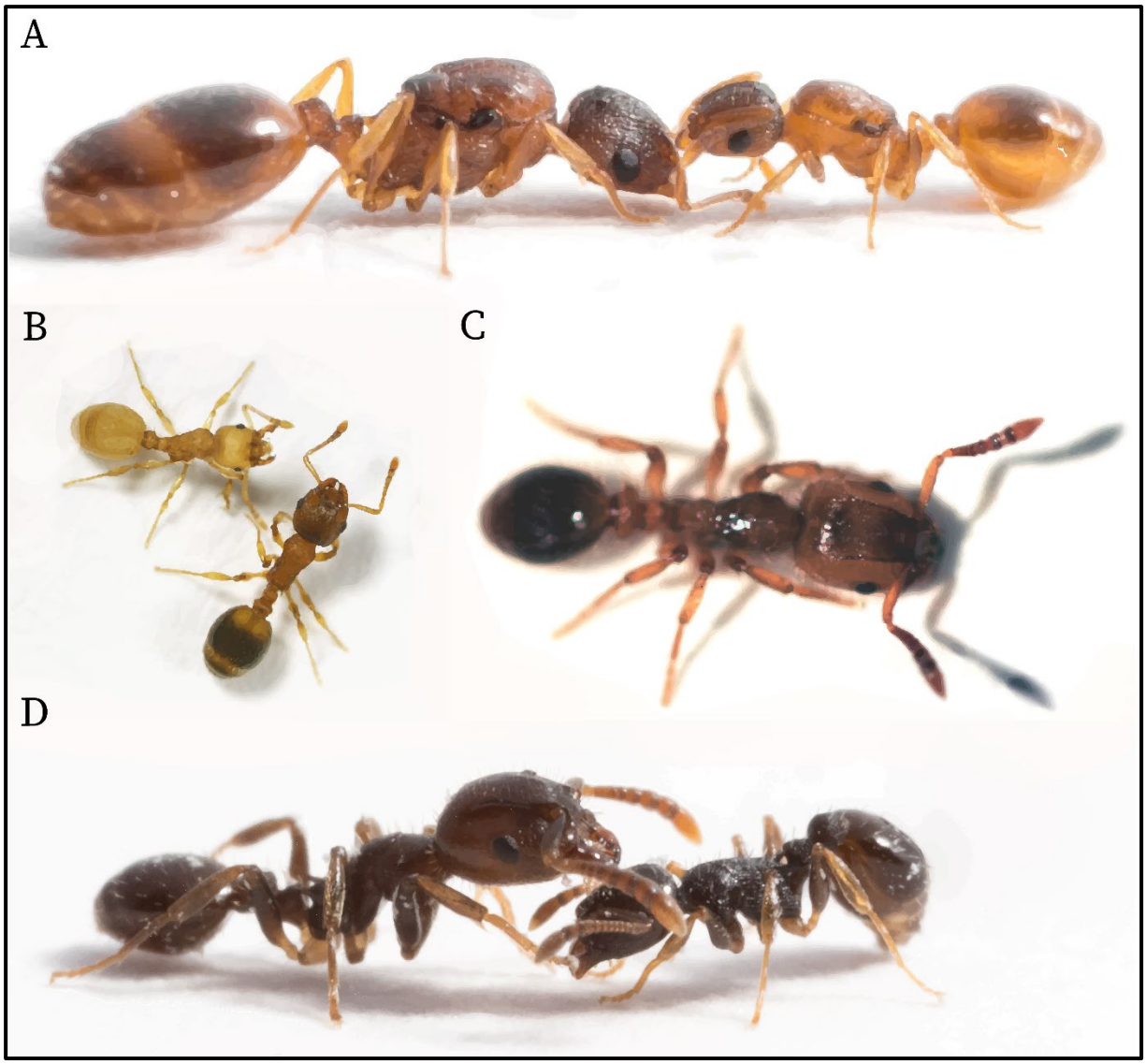

Figure 0-2 Study systems of this dissertation. (**A**) *Temnothorax rugatulus* queens with macrogyne queen morph on the left and microgyne queen morph on the right. (**B**) Workers of *Temnothorax nylanderi* with one worker infected by the tapeworm *Anomotaenia brevis* (upper left) and an uninfected nestmate (lower right). (**C**) *Harpagoxenus sublaevis* worker. (**D**) The dulotic ant *Temnothorax americanus* (left) and a worker of its host species *Temnothorax longispinosus* (right). Photo credit: Romain Libbrecht (**A, D**) and Susanne Foitzik (**B, C**).

Objectives

The overall objective of the present work was to study which genes are used for plastic responses in ants especially related to plastic behaviours and how these could be utilized by parasites. For that, I studied how changes in the social environment affect behavioural and life-history strategies (**Chapters 1-2**). In **Chapter 1** I investigated how

ants of the same species but representing two alternative life-history strategies differ in their transcriptomic response towards the social organization of their colony, while in **Chapter 2** I examined the transcriptomic responses of ants along a gradient of social isolation. In **Chapter 3** I introduced insect social parasites with a specific focus on the molecular basis of the evolution of social parasites but also host-parasite coevolution. Next, in **Chapter 4** I studied how ants parasitized by a social parasite and living in the parasitic nest differ from free-living nestmates. Additionally, I investigated which genes are responsible for plastic responses towards the social parasites (**Chapters 4 and 5**). Therefore, in **Chapter 5**, I looked at the transcriptomic response of hosts when facing a social parasite and how it is affected by parasite prevalence in both the host and parasite population. In **Chapters 6-8** I examined whether parasites make use of plastically regulated genes in hosts to their advantage. Therefore, I compared host and parasite transcriptomic signatures during recruitment behaviour (**Chapter 6**) and in different developmental stages, castes, and sexes (**Chapter 7**) to examine whether the evolution of social parasitism was aided by the reuse of genes already present in the non-parasitic ancestor. In **Chapter 8** I investigated whether the parasites make use of existing phenotypic plasticity in the host by inducing the expression of longevity-related genes upregulated in queens to extend host lifespan. With my work, I hope to contribute to the understanding of plastic responses in ants towards their social environment and their contribution to the evolution of novel phenotypes.

Chapter 1

Molecular adaptation to a social niche: Brain transcriptomes reveal different influence of social environment on the two queen morphs of the ant *Temnothorax rugatulus*

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In preparation

Abstract

Social insects form complex societies with a division of labour between reproductive queens and non-reproductive workers. In most species, a single queen heads the colony; in others, multiple queens share the task of reproduction. These different social forms are often associated with distinct life-history strategies and occur in different environments. In the ant *Temnothorax rugatulus*, two queen morphs coexist - macrogynes and microgynes - that are associated with mono- and polygyny, respectively. We analyzed plastic changes in brain transcriptomes in response to the social environment in the two queen morphs as well as their workers. To investigate whether gene expression is affected by queen morph, social environment or their interaction, we manipulated queen number. Brain transcriptomes shifted only moderately in response to our manipulations. In queens, most expression changes were influenced by an interaction between morph and social environment. This reversed influence of the social environment on the two queen morphs indicates a divergent molecular adaptation to their social niche. Similarly, worker transcriptomes were affected by an interaction between behavioural type, i.e., brood carers or foragers, and queen morph. Brood carers differentially regulated genes related to nutrition depending on queen morph, revealing a link between queen morph and the socio-dynamics inside ant colonies. We did not detect morph-specific fixed genetic variants, but allele frequencies of 41 SNPs differed between macrogynes and microgynes, corroborating earlier studies indicating a low heritability of queen morph. Overall, our study sheds light on the dynamic transcriptomic changes of social insects to their social environment.

Keywords: alternative reproductive strategies, polygyny, gene expression, social insects

Introduction

Polyphenisms, i.e., the production of multiple phenotypes from the same genotype, is a common phenomenon in insects, especially in social insects. Alternative adult phenotypes are often regulated via food quality and quantity or temperature during larval development. In other cases, genetic influences were detected (Wheeler *et al.*, 2006; Berens *et al.*, 2015b; Schwander *et al.*, 2010). In ants, the same genomes can give rise to a variety of adult phenotypes varying in morphology, physiology, behaviour and life histories. The reproductive division of labour between female castes represents an excellent example of how divergent these phenotypes can be as ant queens and workers often differ several folds in body size and lifespan (Hölldobler and Wilson, 1990). Other traits remain plastic throughout an ant's lifetime as exemplified by the behavioural division of labour among workers, which results in task specialization often without morphological differentiation (Kohlmeier *et al.*, 2018).

The ancestral queen phenotype is that of a well-provisioned large queen that following nuptial and dispersal flights will found a new colony on her own (Boulay *et al.*, 2014). But the trade-off between the costs and benefits of dispersal probably has led to the evolution of alternative queen phenotypes that vary in their dispersal capabilities (Hakala *et al.*, 2019; Helms, 2018; Rüppell and Heinze, 1999). The second queen morph, which frequently arose during ant evolution, are smaller queens that mate within or close to the mother nest, before seeking re-adoption to reproduce side by side with other queens in polygynous nests. These two alternative reproductive phenotypes are therefore typically linked to the social colony organization with the large independent founding queens residing in monogynous societies and dependent founding, smaller queens in polygynous colonies (Keller and Ross, 1993). Besides different dispersal strategies, the two reproductive phenotypes differ in several life-history traits. Theory

and comparative evidence predict monogynous queens to exhibit longer lifespans (Keller and Genoud, 1997; Nonacs, 1988), which holds also true for polymorphic species (Schrempf *et al.*, 2011). Moreover, queens of polygynous colonies were found to be less fecund (see also Vargo and Fletcher, 1989; Schrempf, Cremer and Heinze, 2011, Clark and Fewell, 2014). Examples of alternative reproductive strategies within the same species among others include the invasive red fire ant *Solenopsis invicta, Formica selysi or* several *Myrmica* species (Sundström, 1995; McInnes and Tschinkel, 1995; DeHeer and Tschinkel, 1998; Howard, 2006; Rosset and Chapuisat, 2007). In these systems, large queens are often referred to as macrogynes, whereas the smaller queens are called microgynes. Next to differences in longevity and fecundity between the two morphs, evidence also suggests that in the fire ant *S. invicta,* workers are the driving force in discriminating between the two queen morphs and social form affects worker traits such as body size and lifespan (Goodisman *et al.*, 1999; Keller and Ross, 1998; Calabi and Porter, 1989). In *S. invicta, F. selysi* and the *Myrmica* species, long non-recombining elements, so-called "social chromosomes" determine queen morph and dispersal strategy (Wang *et al.*, 2013; Yan *et al.*, 2020b; Purcell *et al.*, 2014; Brelsford *et al.*, 2020). Although in the above-mentioned systems queen number and morph are strongly associated, the co-existence of multiple queens in the same colony arose many times independently from a monogyne ancestor and is not always associated with queen dimorphism (Hughes *et al.*, 2008; Boulay *et al.*, 2014).

We focus here on molecular changes associated with queen-size dimorphism linked to alternative dispersal and reproductive strategies. Our focal species, the ant *Temnothorax rugatulus,* is a small facultatively polygynous Myrmicine populating the dry forests of Western North America, where it lives in cervices and under stones. Queens of this species show a clear size dimorphism, which is associated with queen

number, in that macrogynes are typically monogynous and microgynes are generally polygynous, and mixed colonies are only found on rare occasions (Rüppell *et al.*, 1998; Negroni *et al.*, 2021b; Choppin *et al.*, 2021b). A former experimental quantitative trait experiment indicated only low heritability of queen morph and identified multiple variables, such as colony size and composition which affected queen morph (Rüppell *et al.*, 2001b).

In this study, we experimentally disentangled the effect of queen morph and social structure on brain gene expression by using a full-factorial design. We were especially interested in changes in genes with lifespan, fecundity and stress functionalities. Additionally, we investigated genetic differences using the RNA-Seq data from queens by calling variants and testing whether any of them are fixed or at least very in allele frequencies between morphs. This could provide a first indication of whether differences between the morphs can be explained by genetic variation or whether they represent solely plastic responses towards the environment. We also studied the effect of queen morph and behavioural task on worker brain gene expression to see whether *T. rugatulus* workers from macrogynous societies could differ in behaviour and brain activity from those raised and living in microgynous societies.

Material and Methods

Ant collection and maintenance

T. rugatulus colonies were collected in rock crevices and under stones in oak-pine forests of the Chiricahua Mountains, Arizona, USA in August 2015 (coordinates: Supplementary Information Table S1-1). In the lab, ants were kept at 22°C with a 12:12 light: dark cycle and fed with crickets and honey twice weekly and provided with water ad libitum.

Experimental manipulation

Queens were classified into micro- or macrogyne based on the body size index (Rüppell *et al.*, 1998; Negroni *et al.*, 2021b; Choppin *et al.*, 2021b) a measure that is closely linked to their dry weight. A total of 91 polygynous colonies (average colony size: 217.71 ± 169.33 workers), 44 with exclusively macrogyne queens, and 47 with only microgyne queens, were selected for further experiments. To experimentally vary social structure, that is queen number, we split 86 of the 91 colonies. Each experimental colony received 50 workers with a similar proportion of each behavioural type (nurses, guards, foragers), as well as 12 larvae, while all eggs were removed. Monogyne colonies received one queen of the respective queen morph, while polygynous colonies received two. Overall, colonies remained under these experimental conditions for over four months, so that queens and workers could adjust to the experimental conditions, i.e., colony size and queen number. During the experiment, colony composition had stayed consistent across treatments. Neither the number of workers nor the number of queens of the source colonies did differ between the macro- and the microgyne samples (Mann-Whitney-U workers: z-value -0.15, p-value 0.88; queens: z-value 1.23, p-value 0.22), and the same was true between colonies from the mono- and polygynous treatment (Mann-Whitney-U workers: z-value -0.46, p-value 0.65; queens: z-value -0.09, p-value 0.92; see Supplementary Information Table S1-1). At the end of the experiment, the head of one queen from six colonies was decapitated and flash-frozen using liquid nitrogen. The same was done for one nurse and one forager from the polygynous colonies.

RNA extraction and Sequencing

Brains were dissected into 50 µl of Trizol and RNA was extracted using the RNAeasy mini extraction kit (Qiagen) following the standard protocol. Samples were sent to

Beijing Genomics Institute (BGI) Hongkong for sequencing on an Illumina HiSeq 4000, resulting in 20 to 100 million 100 bp long paired-end reads per sample. The sequencing failed for one macrogynous-polygynous queen. Sequences were trimmed for minimum quality and adapters using Trimmomatic version 0.38 (Bolger *et al.*, 2014) and afterwards, the quality was assessed using FastQC version 0.11.8 (Andrews *et al.*, 2010).

Transcriptome assembly & annotation

We used a draft genome of *T. rugatulus* (Jongepier *et al.*, 2022) as a reference to map the reads against using HISAT2 version 2.1.0 (Kim *et al.*, 2015) together with Samtools version 1.9 (Li *et al.*, 2009). The subsequent mapping files were used as input for StringTie version 1.3.6 (Pertea *et al.*, 2015) to create a genome-guided transcriptome assembly. Backmapping rates revealed one clear outlier that was removed from further analyses (sample 562.MicMo.Q.Br, see Supplementary Table S1-3). Final replicate numbers are given in Figure 1-1. The quality of the resulting reference transcriptome was assessed using gffread version 0.11.4 and TransRate (Smith-Unna *et al.*, 2016). Out of the 74,227 total transcripts, 41,709 had an open reading frame of at least 150 bp and were used for all following analyses. The transcripts were annotated using blastx version 2.9.0 (Altschul *et al.*, 1990) against the invertebrate protein database (downloaded: 18.03.19) with an E-value threshold of 10^{-5}. To obtain information on Gene Ontology terms (Ashburner *et al.*, 2000) and KEGG pathways (Ogata *et al.*, 1999), we ran InterProScan version 5.36-75.0 (Jones *et al.*, 2014) on the translated peptide sequences (TransDecoder version 5.5.0) of the filtered transcriptome.

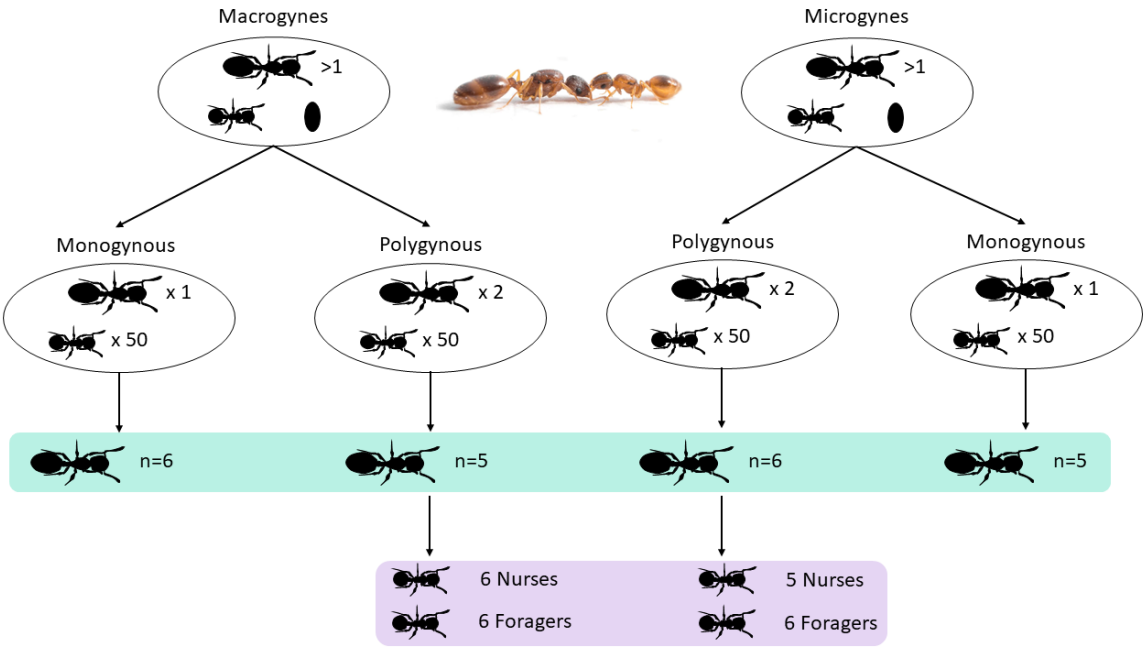

Figure 1-1 Experimental setup for RNA-Seq analysis. Colonies containing multiple queens of the same queen morph were split into a monogyne and a polygyne fraction together with 50 workers. Samples highlighted in turquoise were used for the analysis of queen brain gene expression, while those in purple were used for the analysis on workers. Photo credit: Romain Libbrecht.

Gene expression analysis

The final GTF files produced by StringTie were used to obtain transcript read counts using the *prepDE.py* script. We conducted two independent analyses on a) the queen samples and b) the worker samples. For analysis of the queen samples, we filtered for transcripts with read counts above 10 in at least four samples to obtain more reliable differentially expressed transcripts (DETs) leaving in total 28,876 transcripts for further analysis. DESeq2 version 1.24.0 (Love *et al.*, 2014) was used for gene expression analysis by performing a likelihood ratio test (LRT) comparing a full to a reduced model (see Supplementary Table S1-5 for information on models and numbers of DETs). Since we had both queen morph and social form as factors, we assessed differentially expressed transcripts: a) affected by queen morph; b) affected by social form; c) affected by the interaction of both factors. For the worker dataset, which after filtering consisted of 29,631 transcripts, we followed a similar procedure by analyzing:

a) transcripts affected by queen morph; b) transcripts affected by behavioural task; c) transcripts affected by the interaction of both factors (see Table S5 in Supplementary Information for a list of all models used). For both analyses, transcripts were considered significantly differentially expressed if FDR<0.05 and used for further analyses. We were not able to include colony ID as a factor in our DESeq2 design, as our data was not balanced across colony ID and queen morph. To see in which direction the expression of the DETs chanfes, we used DEGreport version 1.20.0 (Pantano, 2017) to assess clusters of transcripts with similar expression patterns. In the end, all differentially expressed transcripts were additionally annotated with functional information from UniProt from the organisms *Apis mellifera*, *Drosophila melanogaster* and *Caenorhabditis elegans*. Based on this additional function information we specifically searched for genes related to lifespan/longevity, fertility/fecundity and stress. All analyses were performed in R version 4.1.0 (R Core Team, 2021).

Gene Ontology Enrichment

To obtain information on the functionality of the differentially expressed transcripts, we ran a GO enrichment analysis on the lists of transcripts belonging to each cluster separately for queens and workers using topGO v2.36.0 (Alexa and Rahnenfuhrer, 2018). We used the weight01 algorithm and the Fishers exact test implemented in the package to test for overrepresentation of functional terms in our transcript lists of interest. Since different transcripts are expressed in queens and workers, we used a caste-specific set of transcripts as the universe containing only the expressed transcripts in each caste.

Queen genotyping

To gain insights into a potential genetic basis of queen caste, we used the transcriptome data to search for morph-specific variants. We followed the variant

calling workflow for RNA-Seq data proposed by the Broad Institute for pre-processing of the data, adjusting parameters only to the newer version of GATK (Van der Auwera *et al.*, 2013). The pre-processed data was afterwards used to search for fixed SNPs between macro- and microgynes in two ways:

a) Running HaplotypeCaller to generate a list of shared SNPs over all samples that was used in an additional run of HaplotypeCaller to get genotypes for only these SNPs for all samples.

b) Using the workflow for variant calling on cohorts of DNA-Seq samples provided by the Broad Institute without variant recalibration.

As the results of both workflows did not affect our conclusion, we focus in the following report on the results of workflow B, which are more contradictive to our conclusion, while the results from workflow A are found in the Supplementary Information.

The resulting SNPs were filtered to exclude indels, missing genotypes, and positions with more than two alleles. The resulting two input tables were used as input for an in-house Python script (https://github.com/mastoldt/Get_Fixed_SNPs), calculating a fixation index between 0 and 1 for each queen morph with 0 representing all samples having a genotype of 0/0, while 1 represents all samples having the genotype 1/1. Additionally, we investigated the variance explained by the two SNP sets using the R package SNPRelate (Zheng *et al.*, 2012). Additionally, differences in allele frequencies between the two morphs were tested using a Fisher's exact test implemented by Plink version 1.07. Only SNPs with a Benjamini-Hochberg-adjusted p-value below 0.05 were considered significant.

Results

Brain transcriptomes of queens

The expression analysis of queen brains was based on 28,876 transcripts that remained after filtering. Samples clustered neither according to queen morph nor social form (Figure 1-2a). The first two PCs of a Principal Component Analysis (PCA) based on all transcripts explained in total only 26% of the variance (Figure 1-2b). Overall, no clear clustering according to the factors investigated here was found. Neither did colony ID affect clustering (Supplementary Information Figure S1-1). This and the inhomogeneity of sample origin led us to not include colony ID as a factor in the differential expression analysis design.

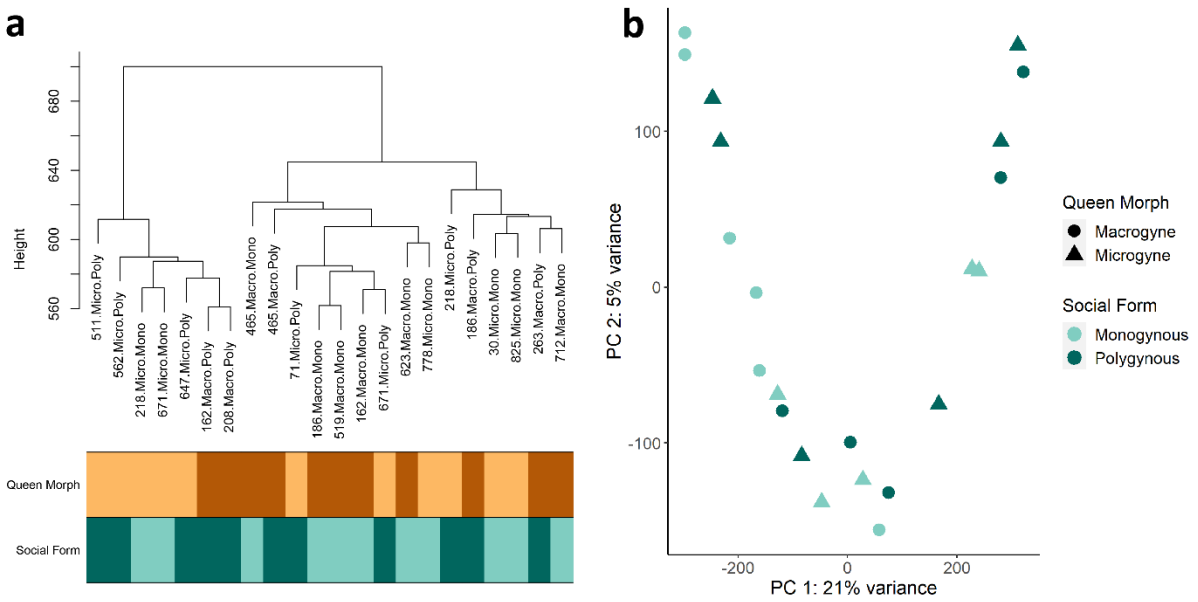

Figure 1-2 Clustering of samples according to social form and queen morph based on the filtered transcript count matrix for queens. (**a)** Sample dendrogram created using hierarchical clustering. Labels indicate sample names with the first 3-digit number corresponding to the source colony, the next part corresponding to the queen morphs with "Macro" for macrogyne queens and "Micro" for microgyne queens. The last part denotes the social form treatment with "Mono" for monogynous colonies and "Poly" for polygynous colonies. (**b)** Principal Component Analysis based on all transcripts. Samples are colored according to the social form and the shapes represent the respective queen morph.

We detected 39 transcripts affected in their expression by queen morph by using the LRT-test as implemented in DESeq2. Social structure influenced the expression of 15 transcripts. The interaction between both factors affected the highest number of

transcripts, namely 63. Clustering of the transcripts affected by queen morph resulted in two clusters, cluster Q2 containing 27 transcripts showing higher expression in macro- versus microgynes and cluster Q1 containing 12 transcripts showing the reversed pattern (Supplementary Information Figure S1-2a). Only cluster Q2 showed significant functional enrichment for "DNA integration". The transcripts affected by social organization clustered into two clusters, one containing eight transcripts higher expressed in monogyne queens (cluster Q4), while the other seven transcripts showed stronger expression in polygynous queens (cluster Q3; Supplementary Information Figure S1-2b). Both clusters showed no functional enrichment. The transcripts, whose expression was influenced by the interaction, were clustered into four clusters (Figure 1-4a). The largest cluster Q5 comprised of 31 transcripts showed a high expression for colonies with mono-macrogyne queens and a low expression for poly-macrogyne queens and a reversed pattern in colonies with microgyne queens. The cluster Q6 including 22 transcripts showed the reversed pattern. Cluster Q8 comprised of only eight transcripts showed an expression similar to cluster Q6 with expression being high for macrogynes in polygyne colonies and microgynes in monogyne ones. The only cluster showing a functional enrichment was cluster Q6, which was enriched for "isoprenoid biosynthetic process".

To identify candidates from our sets of transcripts, we used their best BLAST hits as search queries in UniProt for three model organisms: *Apis mellifera*, *D. melanogaster,* and *C. elegans.* Using a text-mining approach based on reviewed UniProt annotations, we were able to identify nine potential candidate genes involved in the regulation of lifespan (see Table 1-1), two transcripts directly related to fecundity and nine candidates involved in stress. The sets of candidates overlapped in five transcripts. Cluster Q6 of the genes influenced by the interaction of queen morph and social form

showed a high number of transcripts related to both lifespan and stress, though it was not the largest cluster, including a gene encoding a superoxide dismutase similar to a transcript found in cluster Q8 (Figure 1-5 a).

Brain transcriptomes of workers

In the analysis of worker brain gene expression depending on behavioural task and queen morph, 29,631 transcripts remained after filtering. The samples clustered neither according to queen morph, behavioural task nor colony ID in the sample dendrogram nor in the PCA including all transcripts (Figure 1-3). The results of our expression analysis on the brains of workers showed that only two transcripts were differentially expressed according to behavioural task, 14 were affected by queen morph and again the majority was influenced by the interaction, in total 57 transcripts. Transcripts differentially expressed according to queen morph clustered into two clusters: one with higher expression in macrogynes containing 11 transcripts (cluster W2) and one with higher expression in microgynes and containing three transcripts (cluster W1) (Supplementary Information Figure S1-3a). Clustering behaviour-specific transcripts resulted in two clusters consisting of one transcript each with higher expression in nurses (cluster W3 and W4). The transcripts affected by the interaction are grouped into five clusters (Figure 1-4b). The largest cluster W5 comprising 37 transcripts with low expression in foragers of microgyne colonies, was functionally enriched for "meiotic cell cycle", though this was based on a single transcript that according to our BLAST was encoding an uncharacterized protein LOC112452299 in *Temnothorax curvispinosus*. We manually blasted the nucleotide sequence of this transcript against the complete nr database using the blastx algorithm, which revealed that the next closest hit with a functional annotation was a MEIOC protein in *Acromyrmex charruanus* (Query cover: 76%, Identity: 92.89%, E-value: 0.0). MEIOC

protein is known to extend the meiotic phase in mice, allowing for completion of this reproductive event (Abby *et al.*, 2016; Soh *et al.*, 2017).

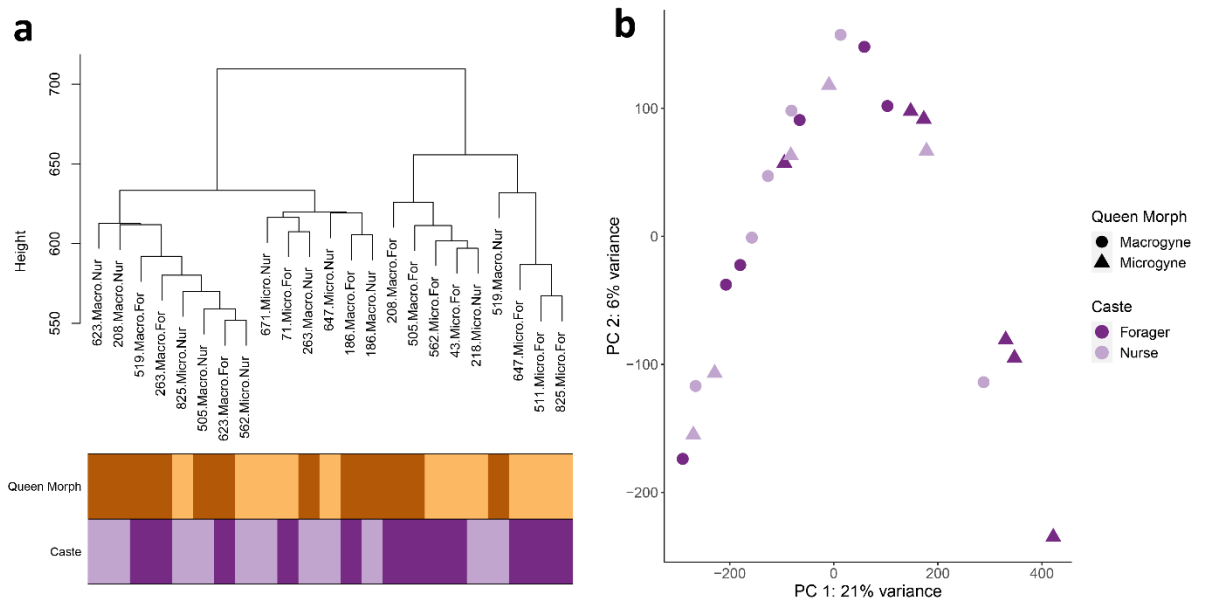

Figure 1-3 Clustering of samples according to behavioural type and queen morph based on the filtered transcript count matrix for workers. (**a)** Sample dendrogram created using hierarchical clustering. Labels again indicate the following meta-data: the first part corresponds to the source colony; the next part corresponds to the queen morph with "Macro" for the residing queens being macrogynes and "Micro" being microgyne queens. The last part represents the behavioural type of the worker samples with "For" standing for foragers and "Nur" for nurses. (**b)** Principal Component Analysis (PCA) based on all transcripts. Samples are coloured according to the behavioural type and the shapes represent the respective queen morph of the residing queens.

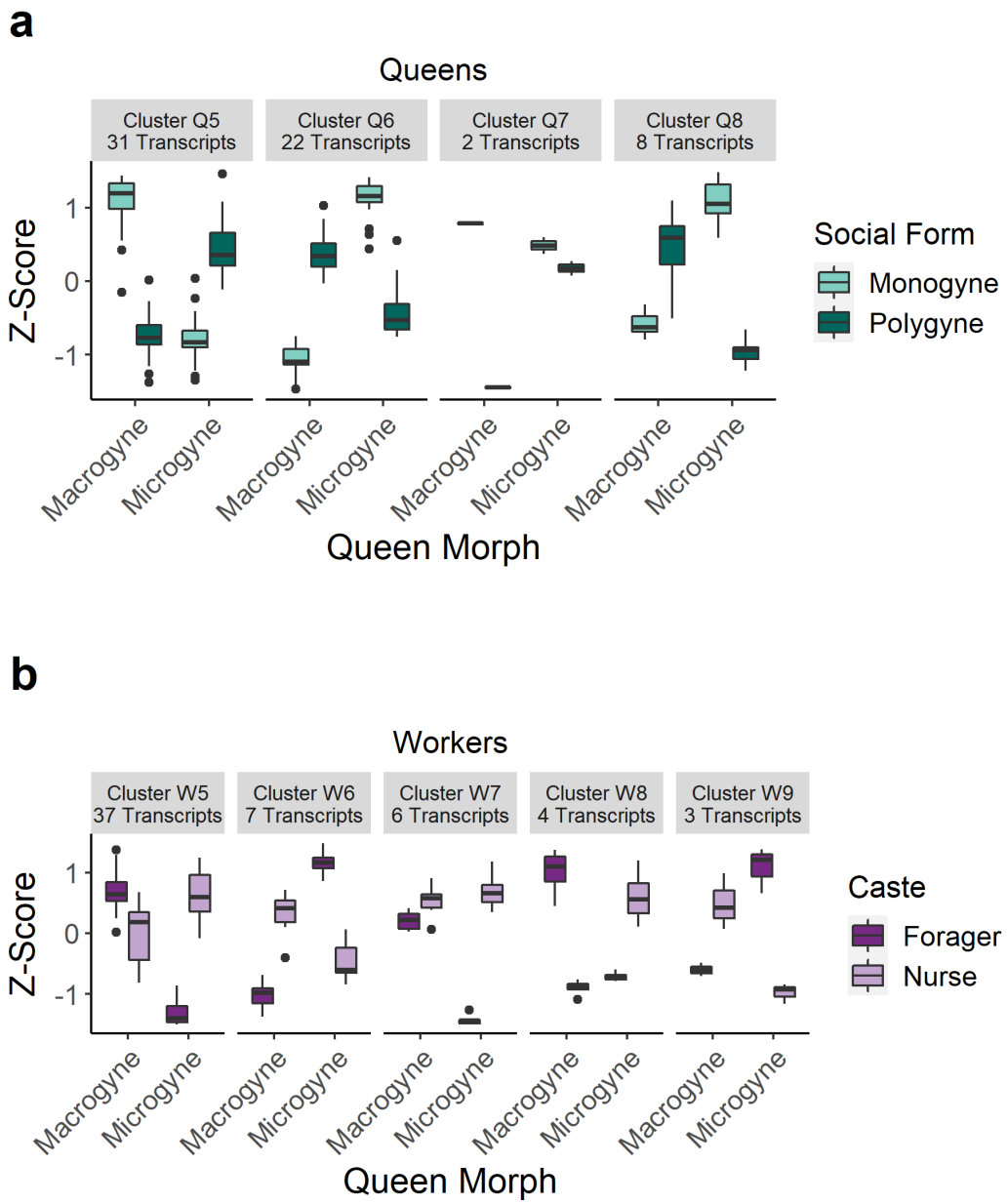

Figure 1-4 Clustering of differentially expressed transcripts using DEGreport for (**a)** DETs influenced by an interaction of queen morph and social form in queens (**b)** DETs influenced by an interaction of behavioural type of workers and queen morph in workers.

Table 1-1 List of text mining candidates among differentially expressed transcripts in queens. If available references retrieved from the functional information in UniProt are given, otherwise a literature search of the three model organisms was conducted, and otherwise, the GO ID obtained from UniProt which caused the hit in the text-mining is given.

Gene ID	Caste	Cluster	High expression in	BLAST hit in the invertebrate database	Function in *Apis*, *Drosophila* or *Caenorhabditis*	Reference
MSTRG.6350.1	Queens	Interaction 1	Macro-Mono Micro-Poly	zinc finger protein 2-like isoform X3 *Temnothorax curvispinosus*	Lifespan, Stress	Tehrani *et al.*, 2014; Singh *et al.*, 2016
MSTRG.23160.2	Queens	Interaction 1	Macro-Mono Micro-Poly	jmjC domain-containing protein 5 *Temnothorax curvispinosus*	Lifespan	Ni *et al.*, 2012
MSTRG.21619.1	Queens	Interaction 2	Macro-Poly Micro-Mono	ATP synthase subunit b mitochondrial isoform X1 *Temnothorax curvispinosus*	Lifespan, Stress	Sun *et al.,* 2014
MSTRG.11670.1	Queens	Interaction 2	Macro-Poly Micro-Mono	FMRFamide receptor-like isoform X1 *Temnothorax curvispinosus*	Stress	Iannacone *et al.*, 2017

MSTRG.6982.1	Queens	Interaction 2	Macro-Poly Micro-Mono	peptidyl-prolyl cis-trans isomerase 5 *Temnothorax curvispinosus*	Stress	GO:0070059
MSTRG.14197.1	Queens	Interaction 2	Macro-Poly Micro-Mono	fatty acid-binding protein, muscle isoform X2 *Temnothorax curvispinosus*	Lifespan	Ramachandran *et al.*, 2019
MSTRG.2959.1	Queens	Interaction 2	Macro-Poly Micro-Mono	60S ribosomal protein L9 *Megachile rotundata*	Lifespan	GO:0008340
MSTRG.10980.1	Queens	Interaction 2	Macro-Poly Micro-Mono	superoxide dismutase [Cu-Zn]-like *Temnothorax curvispinosus*	Lifespan, Stress	Ruan and Wu, 2008
MSTRG.6184.1	Queens	Interaction 2	Macro-Poly Micro-Mono	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5 mitochondrial *Temnothorax curvispinosus*	Fecundity	GO:0000003
MSTRG.7892.6	Queens	Interaction 3	Macro-Mono Micro-Mono Micro-Poly	zinc finger RNA-binding protein isoform X1 *Temnothorax curvispinosus*	Fecundity	Detwiler *et al.*, 2001

MSTRG.21766.1	Queens	Interaction 4	Macro-Poly Micro-Mono	superoxide dismutase [Cu-Zn] *Temnothorax curvispinosus*	Lifespan, Stress	Ruan and Wu, 2008
MSTRG.4840.1	Queens	Queen Morph 1	Micro	cGMP-dependent protein kinase isozyme 1 isoform X2 *Solenopsis invicta*	Lifespan	Hirose *et al.*, 2003
MSTRG.4839.1	Queens	Queen Morph 1	Micro	ATP-binding cassette sub-family C member Sur-like *Temnothorax curvispinosus*	Stress	Akasaka *et al.*, 2006
MSTRG.22936.4	Queens	Queen Morph 2	Macro	N-terminal kinase-like protein isoform X1 *Temnothorax curvispinosus*	Lifespan, Stress	Mizuno *et al.*, 2004; Yan *et al.*, 2017
MSTRG.2449.1	Queens	Queen Morph 2	Macro	peroxisome assembly factor 2 *Temnothorax curvispinosus*	Stress	Huang *et al.*, 2019
MSTRG.16927.7	Queens	Queen Morph 2	Macro	separin *Temnothorax curvispinosus*	Fecundity	Siomos *et al.*, 2001

MSTRG.12340.2	Workers	Interaction 5	Macro-Brood carer Macro-Forager Micro-Brood carer	piezo-type mechanosensitive ion channel component 2 isoform X4 *Vollenhovia emeryi*	Fecundity	Bai *et al.*, 2020
MSTRG.476.3	Workers	Interaction 5	Macro-Brood carer Macro-Forager Micro-Brood carer	kinesin-like protein unc-104 isoform X15 *Temnothorax curvispinosus*	Fecundity	GO:0000003
MSTRG.7288.12	Workers	Interaction 5	Macro-Brood carer Macro-Forager Micro-Brood carer	triosephosphate isomerase isoform X1 *Temnothorax curvispinosus*	Lifespan	Gnerer, Kreber and Ganetzky, 2006; Roland *et al.*, 2013
MSTRG.144.15	Workers	Interaction 5	Macro-Brood carer Macro-Forager Micro-Brood carer	5'AMP-activated protein kinase catalytic subunit alpha-2 isoform X4 *Pseudomyrmex gracilis*	Lifespan, Stress	Apfeld *et al.*, 2004; Lee *et al.*, 2008
MSTRG.4441.1	Workers	Interaction 8	Macro-Forager Micro-Brood carer	major royal jelly protein 1-like *Temnothorax curvispinosus*	Fecundity	Kamakura, 2011

Our text mining approach revealed three genes linked to fecundity, two to longevity, and one to stress, the last one overlapping with the ones involved in longevity (Table 1-1). The only hit we found for *A. mellifera* among all differentially expressed transcripts was one encoding major royal jelly protein 1-like, a protein known to be involved in caste determination and queen reproduction (Kamakura, 2011). This transcript was found in cluster W8 of the transcripts influenced by an interaction of queen morph and the behavioural type and was lowly expressed in foragers from colonies with a microgyne queen but high in all other groups (Figure 1-5b).

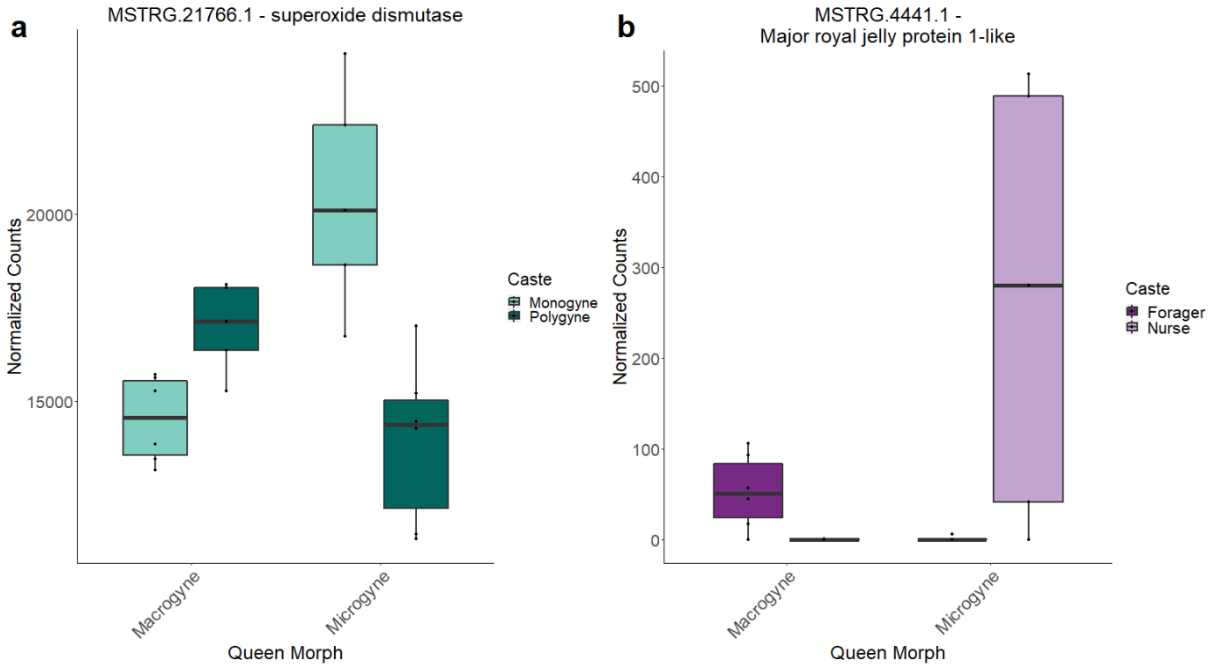

Figure 1-5 Expression of candidate transcripts in brains of queens **a** and workers **b**.

Genotyping of queen morphs

Using workflow B, we were able to detect 35,299 high confidence bi-allelic SNPs in all samples but were unable to detect any variant fixed for a single queen morph. A PCA based on all SNPs only explained a total of 13% of variance by the first two PCs (PC1:

7%, PC2: 6%), but there was a separation of the two morphs along PC1, with two of the microgyne samples still clustering closely with the macrogynes but a clear separation of the remaining microgyne samples (Figure 1-6; MANOVA: EV1: $p < 0.001$, EV2: $p = 0.622$). We identified 41 significant SNPs when testing for differences in allele frequencies between the two queen morphs, from which 40 were located inside transcript regions that we considered to be true transcripts after our filtering for a minimum open-reading frame. Three of the significantly associated SNPs were located in a transcript encoding a homeobox protein prospero-like isoform X4, a transcription factor.

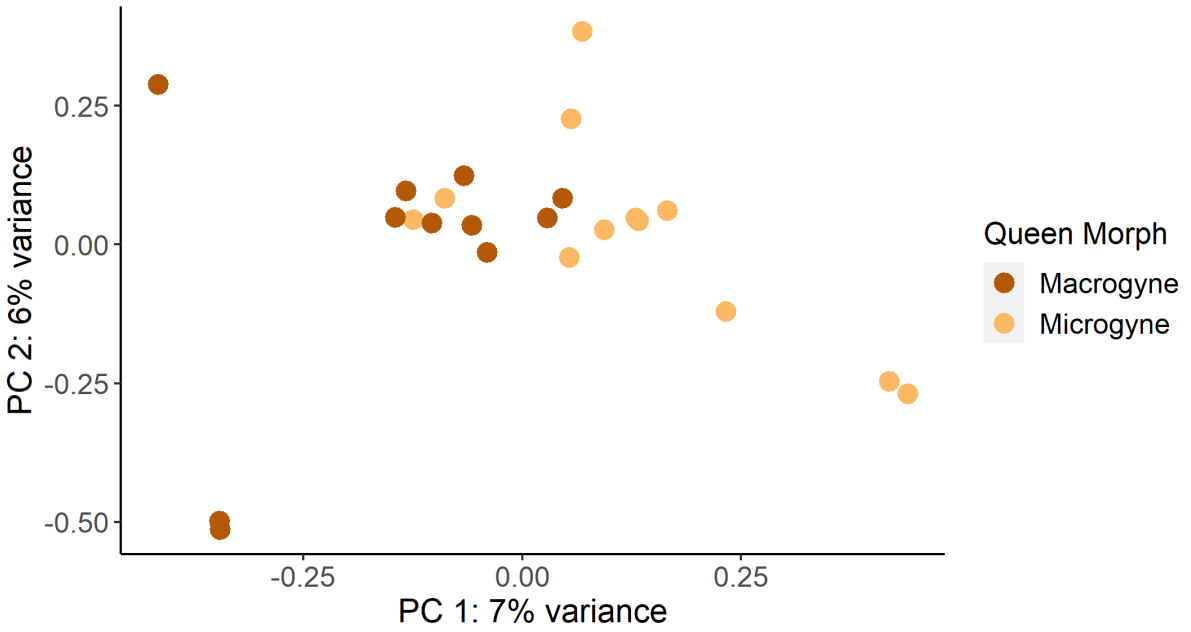

Figure 1-6 PCA of SNPs. Samples are coloured according to the respective queen morph. There is some evidence for genotypic differentiation between the queen morphs based on the variance explained by the first two PCs (MANOVA: EV1: $p < 0.001$, EV2: $p = 0.622$).

Discussion

Colonies of the ant *T. rugatulus* show a queen size polymorphism, which is associated with the social form of the colony, namely the number of residing queens. Using a

long-term experimental manipulation of ant colonies in a full-factorial design, we disentangled the effect of queen morph and social form on brain gene expression in queens of the ant *T. rugatulus*. Moreover, we analysed worker transcriptomes to investigate the effect of queen morph of the residing queen and behavioural task. Our expression analyses of queen and worker brains revealed that both depend on interactions of queen morph with either social form or behavioural task. Thus, by investigating not only the influence of queen morph but also of the other two factors we were able to unravel differences that would have been masked in a single-factor analysis. Macrogyne queens responded differently to variation in queen number compared to microgynes in the expression of a small set of genes. In a similar fashion the expression of a small set of genes in the brains of brood care or forager workers depended on whether they came from microgyne and macrogyne colonies. Furthermore, our SNP analyses did not indicate genetic variants in transcribed regions to be fixed to either queen morph but indicated 41 SNPs that varied in allele frequencies.

Queen morphs differ in their response to changes in the social environment

We experimentally disentangled queen morph from social form to study their effect but also their interaction on gene expression in queen brains. In the model system *S. invicta* where social form and queen morph are strongly associated, gene expression between the two forms differed in several hundred genes (Martinez-Ruiz *et al.*, 2020). Moreover, a study on *T. rugatulus* queens also found strong differences in fat body transcriptomes between the two morphs, especially in the expression of metabolism genes (Negroni *et al.*, 2021b). Thus, in our analyses, we would have first expected to find strong differences between either the two social forms or queen morphs. But due to our full-factorial design, which was only possible as in *T. rugatulus* the association

between queen morph and social form is not as strong, we were moreover able to analyse the interaction between these two factors. Therefore, our analyses shed light on whether the two queen morphs react to alternative social environments by expression changes in the same genes such as aggression or stress genes (Clark and Fewell, 2014). Indeed, our analysis revealed that the majority of DETs was influenced by an interaction of both factors (63 in total). We found a total of 31 transcripts to be upregulated in macrogyne queens in monogynous societies and microgyne queens in polygynous colonies. This expression pattern corresponds to the social structure both queen morphs are typically found in, in the field. Additionally, two other clusters (Q6 and Q8) showed reversed patterns with high expression in queens in their unusual environment. This indicates that the two queen morphs respond to their (un)usual environment via the expression of a similar but small set of genes. We propose social stress in an unaccustomed environment as the most likely explanation for this finding. In case the two morphs represent adaptations to their respective social form, our experimental design might have induced stress in queens when placed in a social form they are not adapted to. This form of social stress has already been partially reported in harvester ants, where queens which usually found colonies on their own, showed a higher number of aggressive events when kept with other queens (Clark and Fewell, 2014). But why would microgynes experience similar stress when kept alone? Negroni et al. previously reported that the number of eggs between mono- and polygynous colonies did not differ but that the egg-laying rate of individual queens was reduced in polygynous colonies (Negroni *et al.*, 2021b). Thus, microgyne queens which are adapted to share the task of reproduction with other queens could experience social stress when kept alone as they need to adjust their egg-laying rate accordingly. Our hypothesis is further supported by the candidate genes found among the DETs influenced by the interaction: Among those, we also detected some related to lifespan

and stress including superoxide dismutase (Beckman and Ames, 1998; Shen *et al.*, 2013).

Among the few transcripts (39) differentially expressed between the two morphs independent of social structure, we identified two candidate transcripts, encoding two kinases, of which one was higher expressed in macrogynes and the other one in microgynes. One of the transcripts identified and highly expressed in microgyne queens is a cGMP-dependent protein kinase, which is not only potentially involved in the regulation of lifespan through interfering with the insulin-pathway in *C. elegans* (Hirose *et al.*, 2003) but moreover plays a role in the adaptation to odours (Levy and Bargmann, 2020). In *S. invicta* a variant inside the *Gp-9* gene encoding for an odorant-binding protein causes differentiation between the two social forms (Krieger and Ross, 2002; Ross, 1997). Moreover, this gene was found to be differentially expressed between the two social forms showing higher expression in the SB/Sb phenotype (Lucas *et al.*, 2015). Although only based on a single transcript our finding could suggest that for microgyne queens in *T. rugatulus* this gene decreases sensitivity to odours for example from other nestmate queens as they occur in polygynous colonies. Odorant-binding proteins in insects are usually expressed in the antennae (Pikielny *et al.*, 1994), thus future studies should aim at also investigating the transcriptomic signatures in the antennae. Overall, we only found little evidence that genes related to lifespan are differentially expressed between the two morphs, fitting the observation by Negroni et al. that survival under oxidative stress does not differ between the morphs (Negroni *et al.*, 2021b).

A genetic basis for queen morph in *T. rugatulus*?

When looking at the variants in our transcripts, thus limited to the protein-coding regions, we did not find evidence for a strong genetic basis of queen size dimorphism

in the ant *T. rugatulus* as none of the variants was fixed to a single morph. But based on all variants we found a slight differentiation between the two morphs even if this was only explained by a small proportion of the total variance as well as 41 SNPs with their allele frequencies explained by morph. This could indicate an ongoing differentiation of the two morphs, which makes *T. rugatulus* an interesting model system to study the evolution of social forms, such as in *S. invicta* and *F. selysi* (Purcell *et al.*, 2014; Wang *et al.*, 2013; Brelsford *et al.*, 2020). To finally answer the question of whether there is genetic differentiation between the two queen morphs in *T. rugatulus*, we would need genome-wide resequencing data of both morphs to conduct a genome-wide screen for differentiated SNPs and inversions.

Queen morph influences worker life-history traits in *T. rugatulus*

As in *S. invicta* the social morph also influences worker traits such as size (Goodisman *et al.*, 1999), we were interested to see how queen morph affects the brain gene expression of different workers in *T. rugatulus*, i.e., those that work mostly inside the nest as nurses and those that are mainly outside as foragers. Our primary expectation was that workers would show the strongest differences in expression between caste, i.e., foragers and nurses. Next to this, we assumed that queen morph might influence worker gene expression as previous studies showed differences in colony dynamic between the morphs. For example, microgyne queens are fed more often by workers, which might trigger the expression of metabolism- and nutrition-related genes in these workers (Negroni *et al.*, 2021b). While we only found a small number of genes to be differentially expressed in all three analyses, we found that most genes are influenced by an interaction of queen morph and behavioural task (57 transcripts). Interestingly, in a cluster highly expressed in foragers of macrogyne and nurses of microgyne colonies (W8), one of the transcripts was encoding major royal jelly protein 1-like. In honeybees

nine major royal proteins exist, the one found in this study, major royal jelly protein 1, is encoded by the *mrjp1* gene (Buttstedt *et al.*, 2014). This gene was previously found to be higher expressed in the head of forager bees compared to caged workers but no different to nurses (Buttstedt *et al.*, 2013) and is suggested to be important for nutrition and social behaviour in honeybees (Schmitzová *et al.*, 1998; Drapeau *et al.*, 2006). In honeybee nurses, royal jelly is produced to feed the larvae and to destine their caste. Interestingly, vitellogenin, a protein also important for caste determination in ants (Kohlmeier *et al.*, 2018), was shown to be the source of the royal jelly produced by workers (Amdam *et al.*, 2003). The high expression in nurses of microgyne colonies might thus indeed be explained by the higher feeding activity of queens (Negroni *et al.*, 2021b). Additionally, we were able to detect some candidate transcripts related to longevity, fecundity, and stress including superoxide dismutase, but further functional studies e.g., using RNAi knockdown would be necessary to evaluate their role in shaping worker life-history in relation to the colony queen morph.

Conclusion

Our study investigating the molecular responses of queens and workers to different social environments and behavioural tasks reveals a strong interaction with queen morph. This suggests that indeed these two phenotypes independent of their genetic basis are well adapted to a different social niche and respond when it changes. Future studies should investigate in more detail the potential genetic basis of queen morph in this species; our preliminary analyses indicate no fixed genetic differences albeit allele frequencies appear to vary between the morphs, which corroborates earlier findings of some but not a strict inheritance of queen morph.

Author Contributions

The study was conceptualized by M.A.N, B.F. and S.F. Ants were collected by M.A.N, B.F. and S.F. Experimental manipulations and RNA extractions were performed by M.A.N. Establishment of bioinformatic workflow, data analysis and data visualization were performed by M.S. The original draft was written by M.S. and revised by all authors.

Supplementary Materials

Gene counts matrix, lists of DEGs, GO enrichment results and SNP data are deposited on Seafile for committee members. The following supplementary information will be published together with the manuscript:

Materials & Methods

Ant collection and maintenance

T. rugatulus colonies were collected in rock crevices and under stones in oak-pine forests of the Chiricahua Mountains, Arizona, the USA in August 2015 (coordinates: Table S1). At the lab, ants were transferred to plastered nest boxes containing artificial nest sites consisting of a Plexiglas cavity sandwiched between two microscope slides (7.5 cm x 2.5 cm x 0.5 cm) and kept at 22°C with a 12:12 light: dark cycle. Ants were fed with crickets and honey twice weekly and provided with water ad libitum.

Experimental manipulation

Queens were classified into micro- or macrogyne based on the body size index (Rüppell *et al.*, 1998), a measure which is closely correlated to their dry weight:

$$\frac{head\ width + \sqrt{thorax\ length * thorax\ width}}{2}$$

Queens with an index smaller than 0.82 were classified as microgynes, queens with an index above 0.85 as macrogynes (see also Negroni *et al.*, 2021b). A total of 91

polygynous colonies (average colony size: 217.71 ± 169.33 workers), 44 with exclusively macrogyne queens, and 47 with only microgyne queens were selected for further experiments. To experimentally vary social structure, that is queen number, we split 86 of the 91 colonies. Each experimental colony received 50 workers with a similar proportion of each behavioural type (nurses, guards, foragers), as well as 12 larvae, while all eggs were removed. Monogyne colonies received one queen of the respective queen morph, while polygynous colonies received two. Four to six weeks after splitting, experimental colonies were transferred and cooled down to artificial winter conditions (5°C) for twelve weeks. After this period, they were gradually warmed up and again kept at 22°C for 18 weeks until the end of the experiment. Overall, colonies remained in our experimental condition for over four months, so that queens and workers could adjust to the experimental conditions, i.e., colony size and queen number.

Sampling and RNA extraction

At the end of the experiment, the head of one queen from six colonies was decapitated and flash-frozen using liquid nitrogen. Neither the number of workers nor the number of queens of the source colonies did differ between the macro- and the microgyne samples (Mann-Whitney-U workers: z-value -0.15, p-value 0.88; queens: z-value 1.23, p-value 0.22), and the same was true between the mono- and polygynous samples (Mann-Whitney-U workers: z-value -0.46, p-value 0.65; queens: z-value -0.09, p-value 0.92; see Supplementary Table 1). The same was done for one nurse and one forager for the polygynous colonies. Brains were dissected in less than 5 minutes on ice in 1% PBS and immediately crushed into 50 µl of Trizol before storage at -80°C until extraction. RNA was extracted using the RNAeasy mini extraction kit (Qiagen) following the standard protocol.

Sequence Data

Samples were sent to Beijing Genomics Institute (BGI) Hongkong for sequencing on an Illumina HiSeq 4000, resulting in 20 to 100 million 100 bp long paired-end reads per sample. The sequencing failed for one sample, thus for the macrogynous-polygynous treatment, we had five instead of six replicates (see Supplementary Table 2). Sequences were quality and adapter trimmed using Trimmomatic version 0.38 (Bolger *et al.*, 2014) with non-default parameters HEADCROP:11 TRAILING:3 SLIDINGWINDOW:4:15 and afterwards, the quality was assessed using FastQC version 0.11.8 (Andrews *et al.*, 2010).

Transcriptome assembly & annotation

We used a draft genome of *T. rugatulus* (Jongepier *et al.*, 2022) as a reference to map the reads using HISAT2 version 2.1.0. BAM files were sorted and indexed with Samtools version 1.9 (Li *et al.*, 2009) and used as input for StringTie version 1.3.6 (Pertea *et al.*, 2015) to create a genome-guided transcriptome assembly. We checked backmapping rates of all samples and identified one clear outlier, which was removed accordingly (sample 562.MicMo.Q.Br, see Supplementary Table S3). Final replicate numbers are given in Figure 1-1. To assess the nucleotide sequences of the transcripts we ran gffread version 0.11.4 on the merged GTF file. We checked the transcriptome quality using TransRate (Smith-Unna *et al.*, 2016). Out of the 74,227 total transcripts, 41,709 had an open reading frame of at least 150 bp according to TransRate and were used for all following analyses. The transcripts were annotated using blastx version 2.9.0 (Altschul *et al.*, 1990) against the invertebrate protein database (downloaded: 18.03.19) with an E-value threshold of 10^{-5}. To obtain information on Gene Ontology terms (Ashburner *et al.*, 2000) and KEGG pathways (Ogata *et al.*, 1999), we ran InterProScan version 5.36-75.0 (Jones *et al.*, 2014) on the translated peptide sequences (TransDecoder version 5.5.0) of the filtered transcriptome.

Gene expression analysis

The final GTF files produced by StringTIe were used to obtain transcript read counts using the *prepDE.py* script. We conducted two independent analyses on a) the queen samples and b) the worker samples. For analysis of the queen samples, we filtered for transcripts with read counts of >= 10 in at least four samples to obtain more reliable differentially expressed transcripts (DETs) leaving in total 28,876 transcripts for further analysis. DESeq2 version 1.24.0 (Love *et al.*, 2014) was used for gene expression analysis by performing a likelihood ratio test (LRT) comparing a full to a reduced model. Since we had both queen morph and social form as factors, we assessed differentially expressed transcripts the following ways: a) transcripts affected by queen morph (comparing a full model containing queen morph and social form as fixed factors to the reduced model containing only social form); b) transcripts affected by social form (comparing a full model containing queen morph and social form as fixed factors to a reduced one containing only queen morph); c) transcripts affected by the interaction of both factors (comparing a full model containing both fixed factors as well as their interaction to a reduced one containing no interaction). For the worker dataset, which after filtering consisted of 29,631 transcripts, we followed a similar procedure by analyzing: a) transcripts affected by queen morph (comparing a full model containing queen morph and behavioural type as fixed factors to a reduced one containing only the behavioural type); b) transcripts affected by behavioural task (comparing a full model containing queen morph and worker task as fixed factors to a reduced one containing only queen morph); c) transcripts affected by the interaction of both factors (comparing a full model containing both fixed factors as well as their interaction to a reduced one containing no interaction). For both analyses, transcripts were considered significantly differentially expressed if FDR < 0.05. Only these transcripts were used for further analyses. We were not able to include colony ID as a factor in our DESeq2

design, as our data were not full-factorial regarding colony ID and queen morph. To see the direction of expression of the DETs, we used DEGreport version 1.20.0 (Pantano, 2017) to assess clusters of transcripts with similar expression patterns. We used the log2-transformed counts of the different lists of DETs as input. In the end, all differentially expressed transcripts were additionally annotated with functional information from UniProt based on their BLAST hit using an in-house script. We only retrieved reviewed entries this way from the organisms *Apis mellifera*, *Drosophila melanogaster* and *Caenorhabditis elegans*. Based on this additional function information we specifically searched for genes related to lifespan/longevity, fertility/fecundity and stress. All analyses were performed in R version 4.1.0 (R Core Team, 2021).

Gene Ontology Enrichment

To obtain information on the functionality of the differentially expressed transcripts, we ran a GO enrichment analysis on the lists of transcripts belonging to each cluster separately for queens and workers using topGO v2.36.0 (Alexa and Rahnenfuhrer, 2018). We used the weight01 algorithm and the Fishers exact test implemented in the package to test for overrepresentation of functional terms in our transcript lists of interest. Since different transcripts are expressed in queens and workers, we used a caste-specific set of transcripts as the universe containing only the expressed transcripts each.

Variant Calling based on RNA-Seq data

After generating an index file of the genome of *T. rugatulus* and mapping the samples against it using STAR v2.7.3a (Dobin *et al.*, 2013) with default parameters, we created a new genome index using all the splice junctions identified by the previous step for all samples together as input for the parameter --sjdbFileChrStartEnd and specifying additionally --sjdbOverhang 75. Afterwards, samples were again mapped against the

newly created index using STAR with default parameters. The resulting mapping files were converted to the BAM format using Samtools, read group information was added and reads were sorted by coordinates using the function AddOrReplaceReadGroups implemented in Picard v2.21.2 (Broad Institute, 2019). Duplicate entries were marked using the function MarkDuplicates with the parameter CREATE_INDEX set to true and VALIDATION_STRINGENCY set to SILENT. Using the tool SplitNCigarReads of the GATK toolkit v4.1.4.0 (Depristo *et al.*, 2011) we reassigned mapping qualities using default settings. This pre-processed data was afterwards used to search for fixated SNPs between macro- and microgynes in two ways:

a) We ran HaplotypeCaller implemented in GATK on every sample using the non-default settings --donot-use-soft-clipped-bases TRUE, --stand-call-conf 40.0 and --output-mode EMIT_VARIANTS_ONLY. Afterwards, we concatenated the SNPs found from every sample, removed duplicate entries, and created a BED file containing the SNP ID, the position-1, and the position as columns. This was used as input for the second round of HaplotypeCaller with the same settings and additionally using the BED file as input for the -L parameter, thus searching only for SNPs in the regions specified in the BED file. Thereafter SNP records of all samples were merged using bcftools and converted into an output table containing the genotype for every sample using bcftools, removing indel entries, missing entries, and multiallelic sites.

b) We used HaplotypeCaller on every sample using the non-default settings -ERC GVCF --dont-use-soft-clipped-bases TRUE, and --output-mode EMIT_VARIANTS_ONLY. The resulting GVCF files were combined using the tool CombineGVCFs implemented in GATK and using this, joint variants were called using the tool GenotypeGVCFs. Again, we created an output table containing the genotype for every sample with indel, missing and multiallelic entries removed. The resulting two input tables were used as

input for an in-house Python script, calculating a fixation index between 0 and 1 for each queen morph with 0 representing all samples having a genotype of 0/0, while 1 represents all samples having the genotype 1/1.

Results

Transcriptome assembly of *T. rugatulus*

The genome-guided assembly of *T. rugatulus* consisted of 41709 transcripts with a minimum ORF length of 150 bp. The GC content of the transcriptome was 42% and the N50 5311 bp. Of those transcripts, 99% had the best BLAST hits against sequences from Hymenopteran species suggesting a low amount of contamination. Based on this transcriptome the analyses for queen and worker samples were performed independently from each other.

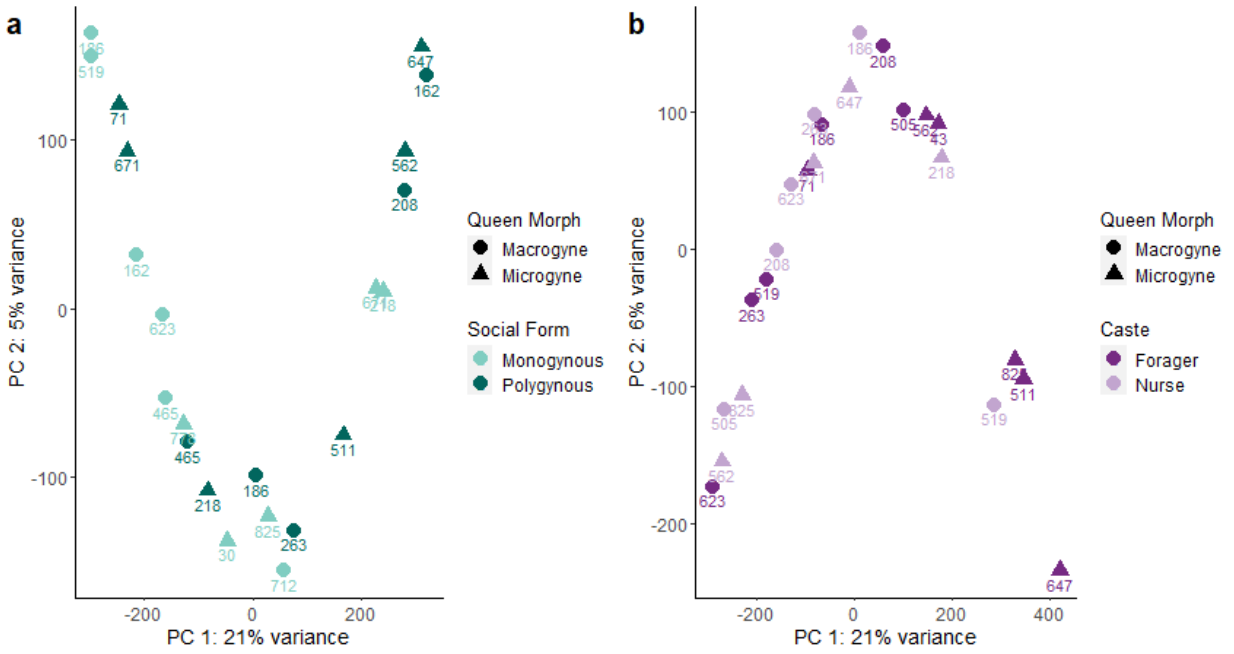

Figure S1-1 PCAs on brain transcriptome data including all expressed genes with labels corresponding to source colony ID for (**a**) queens and (**b**) for workers.

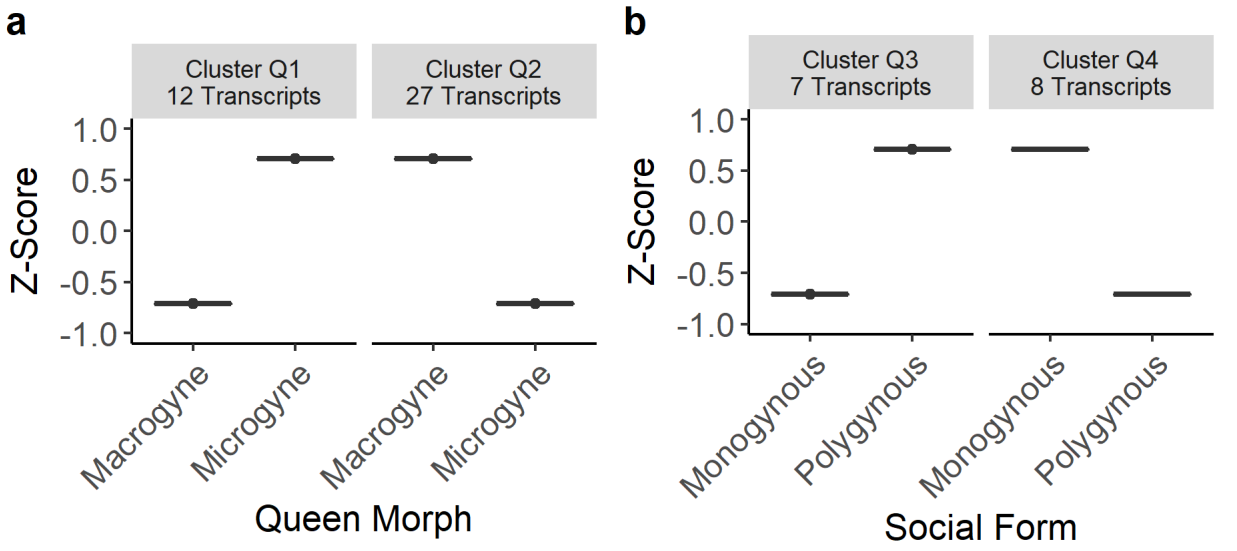

Figure S1-2 Clustering of differentially expressed transcripts in queens using DEGreport for **a.** DETs influenced by queen morph **b.** DETs influenced by social form.

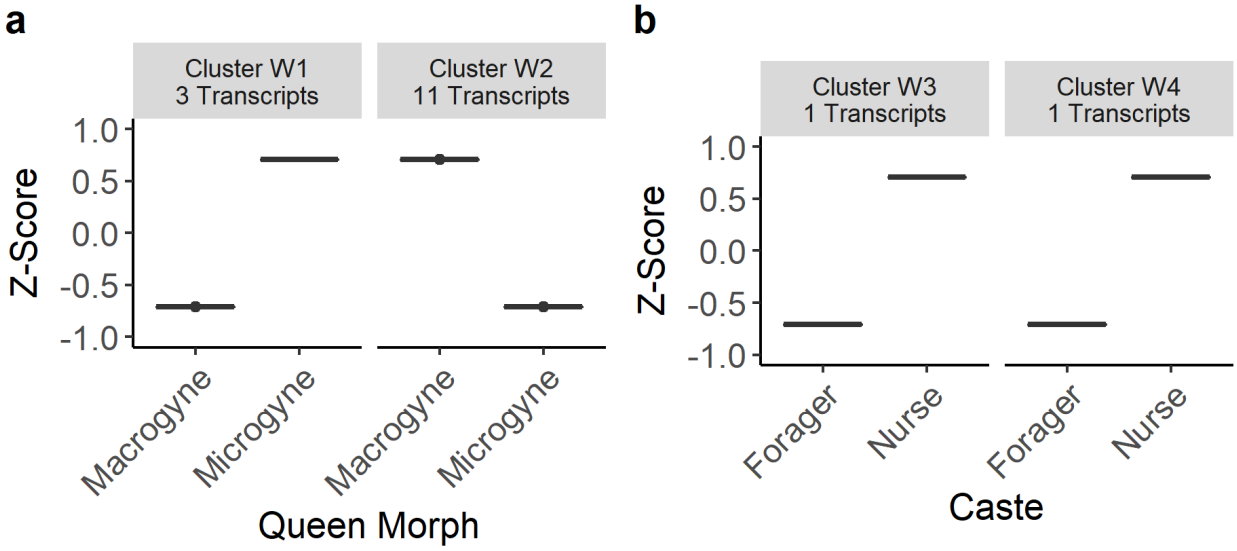

Figure S1-3 Clustering of differentially expressed transcripts in workers using DEGreport for **a**. DETs influenced by queen morph **b.** DETs influenced by behavioural type.

Table S1-1 Information about experimental colonies for RNA-Seq before splitting experiment.

Colony	Queen Morph	Workers	Queens	Coordinates
162	Macrogyne	157	7	31.909071; -109.253337
186	Macrogyne	160	7	31.909071; -109.253337
208	Macrogyne	159	8	31.909071; -109.253337
263	Macrogyne	298	8	31.909071; -109.253337
465	Macrogyne	661	5	31.909071; -109.253337
519	Macrogyne	238	9	31.850508; -109.325624
623	Macrogyne	487	10	31.850508; -109.325624
712	Macrogyne	85	1	31.850508; -109.325624
71	Microgyne	342	18	31.921641; -109.268378
218	Microgyne	771	13	31.909071; -109.253337
30	Microgyne	481	7	31.921641; -109.268378
43	Microgyne	191	5	31.921641; -109.268378
511	Microgyne	134	2	31.850508; -109.325624
562	Microgyne	174	4	31.850508; -109.325624
647	Microgyne	367	10	31.850508; -109.325624
671	Microgyne	138	8	31.850508; -109.325624
778	Microgyne	108	11	31.855962; -109.329143
825	Microgyne	110	8	31.850508; -109.325624

Table S1-2 Colony information about RNA-Seq samples.

Colony	Queen Morph	Social Form	Caste	Sample ID
30	Microgyne	Monogynous	Queen	30-MicMo-Q-Br
43	Microgyne	Polygynous	Forager	43-MicRef-Wf-Br
71	Microgyne	Polygynous	Queen	71-MicRef-Q-Br
71	Microgyne	Polygynous	Forager	71-MicRef--Wf-Br
162	Macrogyne	Monogynous	Queen	162-MacMo-Q-Br
162	Macrogyne	Polygynous	Queen	162-MicRef-Q-Br
186	Macrogyne	Monogynous	Queen	186-MacMo-Q-Br
186	Macrogyne	Polygynous	Queen	186-MacRef-Q-Br
186	Macrogyne	Polygynous	Forager	186-MacRef--Wf-Br
186	Macrogyne	Polygynous	Nurse	186-MacRef--Wi-Br

208	Macrogyne	Polygynous	Queen	208-MacRef-Q-Br
208	Macrogyne	Polygynous	Forager	208-MacRef--Wf-Br
208	Macrogyne	Polygynous	Nurse	208-MacRef--Wi-Br
218	Microgyne	Monogynous	Queen	218-MicMo-Q-Br
218	Microgyne	Polygynous	Queen	218-MicRef-Q-Br
218	Microgyne	Polygynous	Nurse	218-MicRef--Wi-Br
263	Macrogyne	Polygynous	Queen	263-MacRef-Q-Br
263	Macrogyne	Polygynous	Forager	263-MacRef--Wf-Br
263	Macrogyne	Polygynous	Nurse	263-MacRef--Wi-Br
465	Macrogyne	Monogynous	Queen	465-MacMo-Q-Br
465	Macrogyne	Polygynous	Queen	465-MacRef-Q-Br
505	Macrogyne	Polygynous	Forager	505-MacRef--Wf-Br
505	Macrogyne	Polygynous	Nurse	505-MacRef--Wi-Br
511	Microgyne	Polygynous	Queen	511-MicRef-Q-Br
511	Microgyne	Polygynous	Forager	511-MicRef--Wf-Br
519	Macrogyne	Monogynous	Queen	519-MacMo-Q-Br
519	Macrogyne	Polygynous	Forager	519-MacRef--Wf-Br
519	Macrogyne	Polygynous	Nurse	519-MacRef--Wi-Br
562	Microgyne	Monogynous	Queen	562-MicMo-Q-Br
562	Microgyne	Polygynous	Queen	562-MicRef-Q-Br
562	Microgyne	Polygynous	Forager	562-MicRef--Wf-Br
562	Microgyne	Polygynous	Nurse	562-MicRef--Wi-Br
623	Macrogyne	Monogynous	Queen	623-MacMo-Q-Br
623	Macrogyne	Polygynous	Forager	623-MacRef--Wf-Br
623	Macrogyne	Polygynous	Nurse	623-MacRef--Wi-Br
647	Microgyne	Polygynous	Queen	647-MicRef-Q-Br
647	Microgyne	Polygynous	Forager	647-MicRef--Wf-Br
647	Microgyne	Polygynous	Nurse	647-MicRef--Wi-Br
671	Microgyne	Monogynous	Queen	671-MicMo-Q-Br
671	Microgyne	Polygynous	Queen	671-MicRef-Q-Br
671	Microgyne	Polygynous	Nurse	671-MicRef--Wi-Br
712	Macrogyne	Monogynous	Queen	712-MacMo-Q-Br
778	Microgyne	Monogynous	Queen	718-MicMo-Q-Br
825	Microgyne	Monogynous	Queen	825-MicMo-Q-Br

825	Microgyne	Polygynous	Forager	825-MicRef--Wf-Br
825	Microgyne	Polygynous	Nurse	825-MicRef--Wi-Br

Table S1-3 Quality information about RNA-Seq samples. The sample given in bold was removed from the analysis due to a low backmapping rate.

Sample	Number of reads	GC Content	Backmapping Rate
30-MicMo-Q-Br	86,054,482	40.0	81.06
43-MicRef-Wf-Br	23,546,308	41.0	92.78
71-MicRef-Q-Br	47,057,380	41.0	79.07
71-MicRef--Wf-Br	90,323,144	41.0	93.62
162-MacMo-Q-Br	92,559,698	41.0	93.76
162-MacRef-Q-Br	27,078,742	40.0	91.71
186-MacMo-Q-Br	47,583,170	42.0	94.43
186-MacRef-Q-Br	72,976,442	41.0	93.38
186-MacRef--Wf-Br	61,929,020	42.0	92.99
186-MacRef--Wi-Br	19,715,300	41.0	92.66
208-MacRef-Q-Br	25,606,982	40.0	89.18
208-MacRef--Wf-Br	77,482,236	41.0	92.42
208-MacRef--Wi-Br	23,406,280	42.0	93.58
218-MicMo-Q-Br	25,604,636	40.0	91.50
218-MicRef-Q-Br	59,288,244	42.0	92.34
218-MicRef--Wi-Br	65,900,734	41.0	92.04
263-MacRef-Q-Br	69,749,118	41.0	92.79
263-MacRef--Wf-Br	100,873,464	42.0	94.38
263-MacRef--Wi-Br	24,259,890	43.0	92.46
465-MacMo-Q-Br	35,639,098	42.0	93.07
465-MacRef-Q-Br	44,338,886	42.0	93.40
505-MacRef--Wf-Br	108,981,344	41.0	92.93
505-MacRef--Wi-Br	95,731,448	42.0	93.85
511-MicRef-Q-Br	18,555,954	41.0	92.56
511-MicRef--Wf-Br	22,530,680	40.0	90.96
519-MacMo-Q-Br	47,077,740	42.0	94.03
519-MacRef--Wf-Br	91,813,170	40.0	93.40

519-MacRef--Wi-Br	40,866,478	42.0	73.34
562-MicMo-Q-Br	**76,725,504**	**43.0**	**46.64**
562-MicRef-Q-Br	91,563,336	39.0	88.17
562-MicRef--Wf-Br	32,476,962	41.0	92.46
562-MicRef--Wi-Br	69,104,880	42.0	93.85
623-MacMo-Q-Br	79,376,374	41.0	94.20
623-MacRef--Wf-Br	96,599,364	43.0	94.56
623-MacRef--Wi-Br	102,727,588	41.0	93.21
647-MicRef-Q-Br	76,273,132	41.0	87.09
647-MicRef--Wf-Br	78,336,894	40.0	77.42
647-MicRef--Wi-Br	53,133,024	42.0	92.86
671-MicMo-Q-Br	96,250,910	41.0	92.44
671-MicRef-Q-Br	83,208,536	42.0	93.71
671-MicRef--Wi-Br	109,005,260	42.0	92.80
712-MacMo-Q-Br	38,806,176	41.0	93.30
778-MicMo-Q-Br	74,437,942	42.0	86.04
825-MicMo-Q-Br	28,177,196	41.0	93.33
825-MicRef--Wf-Br	81,447,840	42.0	76.18
825-MicRef--Wi-Br	61,159,170	42.0	93.81

Table S1-4 Quality information on the assembly of *T. rugatulus* after filtering for ORF >150bp. Produced using TransRate.

Parameter	Value
Number of sequences	41709
Smallest sequence	450
Largest sequence	33483
Number of bases	166742150
Mean sequence length	3997.75
Number of sequences shorter than 200 bp	0
Number of sequences longer than 1000 bp	38381
Number of sequences longer than 10000 bp	1826
Number of sequences with ORF	41709
Mean ORF percent	37.49
N90	2131

N70	3729
N50	5311
N30	7376
N10	11250
GC content	0.42
Bases N	3

Table S1-5 Summary of the models used to analyze brain gene expression in queens and workers in respect to different factors and the total number of differentially expressed genes (adjusted p<0.05) for each factor.

Dataset	Factor of interest	Full model	Reduced model	# DEGs
Queens	Queen morph	design= ~Social Form + Queen Morph	reduced =~Social Form	39
Queens	Social form	design= ~Queen Morph + Social Form	reduced =~Queen Morph	15
Queens	Queen morph * Social Form	design= ~Queen Morph + Social Form + Queen Morph : Social Form	reduced =~Queen Morph + Social Form	63
Workers	Queen morph	design= ~Behavioral Type + Queen Morph	reduced =~Behavioral Type	14
Workers	Behavioral type	design= ~Queen Morph + Behavioral Type	reduced =~Queen Morph	2
Workers	Queen morph * Behavioral type	design= ~Queen Morph + Behavioral Type + Queen Morph : Behavioral Type	reduced =~Queen Morph + Behavioral Type	57

Chapter 2

Social isolation causes downregulation of immune and stress response genes and behavioural changes in a social insect

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Based on:

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Abstract

Humans and other social mammals experience isolation from their group as stressful, triggering behavioural and physiological anomalies that reduce fitness. While social isolation has been intensely studied in social mammals, it is less clear how social insects, which evolved sociality independently, respond to isolation. Here we examined whether the typical mammalian responses to social isolation, e.g., an impaired ability to interact socially and immune suppression are also found in social insects. We studied the consequences of social isolation on behaviour and brain gene expression in the ant *Temnothorax nylanderi*. Following isolation, workers interacted moderately less with adult nestmates, increased the duration of brood contact, and reduced the time spent self-grooming, an important sanitary behaviour. Our brain transcriptome analysis revealed that only a few behaviour-related genes had altered their expression with isolation time. Rather, many genes linked to immune system functioning and stress response had been downregulated. This probably sensitizes isolated individuals to various stressors, in particular, because isolated workers exhibit reduced sanitary behaviour. We provide evidence of the diverse consequences of social isolation in social insects, some of which resemble those found in social mammals, suggesting a general link between social well-being, stress tolerance, and immune competence in social animals.

Keywords: immune suppression, social insects, stress, *Temnothorax*, transcriptomics

Introduction

Social isolation is a topic of increasing relevance for public health, particularly regarding an ageing human population, as the negative effects of isolation are more prevalent later in life (Nicholson, 2012; Courtin and Knapp, 2017). Moreover, the effects of social isolation have gained increasing importance recently due to the quarantine measures being taken to avoid the spread of novel infectious diseases. A strong reduction in social contacts represents a mortality risk, due to its positive association with high blood pressure, high cholesterol levels, smoking, self-neglect, and cognitive decline (Cacioppo *et al.*, 2011; Pantell *et al.*, 2013; Brennan *et al.*, 2019). Social isolation has been defined in many ways, with the most general one relating to suffering from reduced contact with group members compared to the desired level (Hawkley and Capitanio, 2015). Not only do humans experience the negative consequences of social isolation, but so do other animals with a social lifestyle. It has been proposed that the strength of response to social isolation may even assist to characterize a species' level of sociality or interdependence of members of a social group (Lihoreau *et al.*, 2009; Wang *et al.*, 2016).

Owing to its relevance for humans, most research on social isolation has been conducted on small mammals, like rats and mice, as well as on mammals resembling humans in their social organization, such as prairie voles. With isolation, social mammals become more aggressive towards unfamiliar conspecifics, demonstrate less interest in social interactions, and may fail to recognize their own group members (Wise, 1974; Lukkes *et al.*, 2009; Zhao *et al.*, 2009). The absence of social interactions in social mammals can also reduce boldness and risk-taking, increase neophobia, and impede learning processes (Fone and Porkess, 2008; Grippo *et al.*, 2008; Pan *et al.*, 2009). While some experiments have demonstrated an increase in physical activity

with isolation, others have reported no change or even a decrease (Silva-Gómez *et al.*, 2003; Weiss *et al.*, 2004; Grippo *et al.*, 2008). The behavioural responses following isolation are correlated with shifts in physiology and brain gene expression. The levels of some stress-related hormones, such as cortisol, increase with isolation in mammals; while others, such as corticosterone, exhibit no clear pattern (Kanitz *et al.*, 2004; Lukkes *et al.*, 2009; Hawkley *et al.*, 2012). Social isolation is associated with the activity of the hypothalamic-pituitary-adrenocortical axis, which regulates the mammalian response to stress (Cacioppo *et al.*, 2015). The absence of social contacts also causes suppression of the immune system, which in turn can lead to a higher risk of cancer and chronic inflammation (Wu *et al.*, 2000; Hawkley *et al.*, 2012; Cruces *et al.*, 2014).

Changes in the expression of genes related to stress response and anxiety behaviours have also been linked to social isolation. For example, in prairie voles, isolation leads to the overexpression of corticotrophin-releasing factor, vasopressin, and oxytocin in the hypothalamus. The latter two are sociality-related genes that are also linked to anxiety-related behaviours (Pan *et al.*, 2009). In male mice, early social isolation leads to motor hyperactivity and behavioural disinhibition, caused by a robust downregulation of serotonin receptors in the brain (Bibancos *et al.*, 2007). Female mice with a history of social isolation, in contrast, exhibit higher levels of anxiety and upregulation of brain-derived neurotrophic factors in the cerebral cortex (Kumari *et al.*, 2016).

Sociality has repeatedly evolved not only in mammals but also in insects, with eusocial insects exhibiting the highest level of social organization. Social insects demonstrate a division of labour between queens and workers, and often also between workers of different castes, such as nurses and foragers. Social isolation has severe consequences for social insects. Isolated social insect workers die faster than grouped ones, due to

lacking those resources regularly provided by nestmates and to the general stress-induced by isolation, resulting in faster respiration, more rapid depletion of body reserves, and/or inefficient digestion (Boulay, 1999; Szczuka *et al.*, 2011; Modlmeier *et al.*, 2013; Koto *et al.*, 2015). Social isolation also modifies behaviour. Aggression may either increase or decrease with isolation, probably due to changes in the cuticular hydrocarbon profile that is important for nestmate recognition, or in the brain structure (corpora allata size) of isolated workers (Breed, 1983). For example, while aggression towards (former) nestmates may increase with the duration of isolation, the aggression against non-nestmates decreases (Boulay and Lenoir, 2001; Lenoir *et al.*, 2001). Antennation between reunited workers following short isolation decreases, whereas trophallaxis (social food exchange) increases (Boulay, 1999; Cybulska *et al.*, 2000; Korczyńska *et al.*, 2005; Wagner-Ziemka *et al.*, 2008). When social insects are isolated during development, their developmental and/or behavioural maturation is impaired, leading to slower growth of the mushroom body and/or a reduction in brood-care activity (Champalbert and Lachaud, 1990; Seid and Junge, 2016). Finally, the presence of conspecifics can moderate or buffer the stress induced by competitors or predators (Bowen *et al.*, 2013; Tian *et al.*, 2017). The mechanisms of molecular response to social isolation have rarely been studied in insects, compelling us to determine whether the phenotypic responses to isolation are regulated via similar molecular pathways in insects and mammals. Certain behaviours, such as novelty-seeking or responses to social stimuli, are controlled via similar molecular pathways in honeybees and humans, suggesting deep evolutionary conservation of some genes (Liang *et al.*, 2012; Shpigler *et al.*, 2017).

We focused on the ant *Temnothorax nylanderi*, which dwells in colonies of a few dozen workers on average and sought to examine changes in worker phenotypes

triggered by different periods of isolation. Isolated workers of *T. nylanderi* demonstrated increased mortality and lower body reserves at death compared to grouped workers (Modlmeier *et al.*, 2013). We hypothesized that social isolation would affect behaviour and gene expression and that these effects would resemble and be mechanistically similar to stress-induced responses. We predicted worker activity and their tendency to move along the arena's edges to first increase and then decrease with isolation, as workers initially search for their lost colony, but later reduce activity when failing to find it or to escape from the arena. Because social stress changes self-grooming patterns (Denmark *et al.*, 2010), and self-grooming is an important aspect of social immunity in social insects (Theis *et al.*, 2015), we predicted that this hygienic behaviour would decline with isolation. Isolated social mammals demonstrate less interest in social interactions following their return to the group (Wise, 1974; Lukkes *et al.*, 2009; Zhao *et al.*, 2009). We anticipated reduced contacts with nestmates following a lengthy period of isolation, but more frequent contacts with nestmates following a shorter period of isolation (a hump-shaped pattern). We predicted that the time allocated to tending the brood would decline, together with a general decline in social behaviours with isolation, but we did not base our expectation on concrete evidence from prior studies. In respect to brain gene expression, we predicted social isolation to modify the expression of genes controlling those behaviours that change with isolation, such as general care behaviours (towards the brood, queen, or nestmates), which are regulated via, e.g., *vitellogenin-like A* in *Temnothorax* ants (Kohlmeier *et al.*, 2018). We also predicted that the regulation of immune genes would be altered, as immune suppression is a characteristic consequence of isolation in other social animals (Hawkley *et al.*, 2012). It is difficult to foresee the direction of change. While some genes involved in immune system functioning are downregulated with social isolation in humans, other genes are upregulated (Cole *et al.*, 2007). Finally, we

predicted that those genes related to improving tolerance to stressors, such as oxidative stress, would be downregulated, as isolation makes social animals more vulnerable to stress (Zlatković *et al.*, 2014).

Material and Methods

Ant collection and maintenance

Temnothorax nylanderi establishes colonies of a few dozen workers, which inhabit cavities in acorns and sticks on the floor of European deciduous forests. Colonies contain a single queen and may occupy more than one nest (they are monogynous and polydomous; Kramer *et al.*, 2014). We collected 14 queenright colonies in December 2018 from Lennebergwald, near Mainz, Germany, and brought them to the laboratory (129 ± 24 workers per colony; mean ± 1 SD; at least 30 larvae in each colony). Each colony was kept in an artificial nest (50×10×3 mm length×width×height), placed inside a box with a plaster floor, and divided into three chambers. Water, half a cricket, and a droplet of honey were provided once a week. The colonies were kept in a climate cabinet at 21°C and a photoperiod of 12h:12h light:dark cycle.

Isolation experiment and behavioural measurements

The experiment took place in January-March 2019. Nurses, young workers (< 1-year-old) tasked with brood care, were individually isolated from 14 focal colonies for increasing durations: no isolation or 0 hours (control), 1 hour, 24 hours (1 day), 168 h (7 days), or 672 h (28 days). For each colony, the experiment started on a different day, between 21 and 45 days post collection (Supplementary Material, S1). Isolated workers were placed individually in Petri dishes (5 cm diameter), each containing two tiny plates (d = 0.5 cm) with water and honey, which were replaced twice weekly. For each isolation duration, seven workers per colony were used, resulting in 35 workers per

colony times 14 colonies, totalling 490 workers. We compared the body mass of workers isolated for different durations (one worker per colony and isolation duration) but found no changes in mass with isolation duration ($P > 0.20$). The following three behaviours were recorded from the same ant in the Petri dish of each focal worker: activity; tendency to move along the arena's edges (hereafter, edge preference); and time spent self-grooming. The activity was measured by video-recording the isolated worker for 10 min. and summing the time it was active/moving. Edge preference was measured by taking 60 screenshots, one every 10 sec., of the 10 min. video, measuring the distance of the worker from the edge, and calculating the average distance in all screenshots. Self-grooming was measured by documenting the time (for 5 min.) the worker was cleaning itself. Three additional behaviours were recorded from different ants following the introduction of either a nestmate larva, a dead nestmate worker, or a dead non-nestmate worker into the isolation arena (the Petri dish): the total time the worker spent in contact with larvae, contact duration with a nestmate and contact duration with a non-nestmate conspecific. These behaviours were quantified for 10-min., based on films of separate workers. Each worker was tested only once. We used dead workers as opponents to eliminate the sources of variation in behaviour of the tested ant workers, related to their responses to the different behaviour of live ants introduced into their isolation arena (similar to Crosland, 1990; Scharf *et al.*, 2011). The opponents were killed by freezing at -20°C when all workers were taken out of the colony for the experiment and were kept under a deep freeze. They were singly defrosted 1 min. before usage. One isolated worker per isolation period was used for two measurements of body reserves, based on photos taken under a stereomicroscope: the gaster width to head width ratio and the body mass to head width ratio. Head width is invariable in adult insects and is generally used as a measure of body size. Gaster width can vary with the amount of body reserves, the

filling of the crop, and ovary development in queens. A previous study on the studied ant (*T. nylanderi*) has demonstrated a stronger correlation between gaster-to-head width and survival time under isolation than in groups (Modlmeier *et al.*, 2013). Mass was measured using an analytical scale (accuracy of 0.01 mg). For both measurements, a larger value indicates more reserves relative to body size, similar to the Body Mass Index. Finally, two workers per colony and isolation period were pooled for brain gene expression analyses, to obtain a sufficient amount of RNA for the analysis of gene expression and to reduce the between-worker variance within each colony. All tests were conducted between 09:30-15:30, with workers from the same colony being tested at the same time of day (±1 h). None of the workers died a natural death during the experiment, although several workers (<10) drowned in the honey plate and were removed from the analysis.

Statistical analyses

We performed all the analyses with the software R (v. 4.0.2). Before analysis, the data were transformed into a long format with an explanatory variable "measurement" containing the eight measurements as levels and a response variable "value" containing the scaled values for each measurement. As the eight measurements related to behaviour and body reserves could be interrelated, we conducted a multivariate mixed-effect model to quantify the effect of isolation on those measurements. We tested for the effect of isolation duration, as a continuous variable, on the eight measurements by contrasting these two linear mixed-effect models, each built using the lmer() function of the lme4 package (Dworkin & Bolker, 2019):

- Full model: lmer(value ~ measurement + measurement:time + (measurement-1|colony)
- Reduced model: lmer(value ~ measurement + (measurement-1|colony)

We compared the models with an approximate F-test based on the Kenward-Roger approach, using the KRmodcomp() function from the pbkrtest R package. We plotted the intercept with the confidence intervals for the interaction term of each measurement with isolation duration, which revealed that some measurements had changed accordingly. We confirmed these multivariate analyses with independent models (Supplementary Material, S2).

Differential gene expression

We flash-froze two workers per colony per isolation period in liquid nitrogen and transferred them to vials at -80°C. The workers' brains were dissected and placed together in Trizol. RNA was extracted using the RNeasy Mini Extraction kit (Qiagen) and was sent to Beijing Genomics Institute (BGI) for library construction and sequencing of 100 base pair (bp) paired-end reads using BGISeq (for raw reads see NCBI, BioProject PRJNA608848). The pooled brains contained 0.0208-0.1201µg of RNA. The reads were quality- and adapter-trimmed (Trimmomatic v0.39: Bolger *et al.*, 2014) and passed a quality check (Andrews *et al.*, 2010). We used a genome-guided assembly, and the paired forward and reverse reads were mapped against the genome of *T. nylanderi* (Jongepier *et al.*, 2022; Hisat2 v2.1.0: Kim *et al.*, 2015). We assembled the RNA reads into potential transcripts (StringTie v1.3.6: Pertea *et al.*, 2015) based on the mappings obtained against the genome. We extracted the transcript sequences (gffread v0.11.4: Pertea and Pertea, 2020) and used transcripts of an open reading frame of at least 150 bp for later analyses. The assembly of the trimmed paired reads using StringTie resulted in 74,928 contigs, with a mean length of 3,093 bp. The transcriptome filtered after ORF length and minimum read count comprised 41,467 contigs. We compared the transcripts to known invertebrate gene products (protein sequences downloaded from NCBI in April 2019, a database created using

makeblastdb (Blastx v2.9.0: Altschul *et al.*, 1990)), using an E-value threshold of 10^{-5}. We annotated our translated transcripts for the Gene Ontology (GO) analysis (Transdecoder v5.5.0: Haas *et al.*, 2013; Interproscan v5.34-73.0: Jones *et al.*, 2014; Gene Ontology: Ashburner *et al.*, 2000). Later analyses were conducted in R (R Core Team, 2019). We filtered for the transcripts, having at least 10 read counts in at least six samples. We used DESeq2 (DESeq2 v1.24.0: Love *et al.*, 2014) to identify differentially expressed genes. We used likelihood-ratio tests (LRT) to compare a full model (~colony + bs(isolation time)) to a reduced model (~colony). This allowed us to identify genes affected by isolation time while controlling for colony effects. We used the bs() function (default parameters) from the R package splines (3.6.0) to also detect non-linear changes in expression over isolation time. The transcripts of interest were checked for GO enrichment using topGO (topGO v2.36.0: Alexa and Rahnenfuhrer, 2018) (Supplementary Data, S3). We used the degPatterns function (with parameter minc = 3) of the R package DEGreport (DEGreport v1.20.0: Pantano, 2017) to cluster differentially expressed genes according to their expression patterns over isolation time (Supplementary Data, S4).

Results

Changes in behaviour and body reserves

Our multivariate analysis revealed an interaction between isolation duration and the behavioural and morphological measurements ($F_{8,438.3} = 2.13$, $P = 0.03$). The slope of changes over isolation duration differed from zero for three of our measurements: self-grooming, contact with larvae, and contact with a nestmate, indicating that they had changed with social isolation (Fig. 2-1a). With increasing isolation, workers exhibited shorter durations of self-grooming and contact with adult nestmates, but longer durations of interactions with larvae (Fig. 2-1b-d). Activity, edge preference, and

contact with non-nestmates did not shift with social isolation. Body reserve measurements also did not vary with the duration of isolation. Independent analyses confirmed these findings while indicating that the effect of isolation on behaviour was moderate (Supplementary Material, S1). Colonies were kept for different periods in the laboratory (21-58 days) before the experiment commenced. Therefore, workers from the 1-month isolation treatment of the 1st experimental colonies and workers from the control/ 1h /1-day treatment of the last experimental colonies were tested around the same time (Supplementary Data, S1). While isolation is confounded with time in the laboratory for each colony, the time varies so strongly across colonies that it is unlikely that time in lab maintenance can explain our findings.

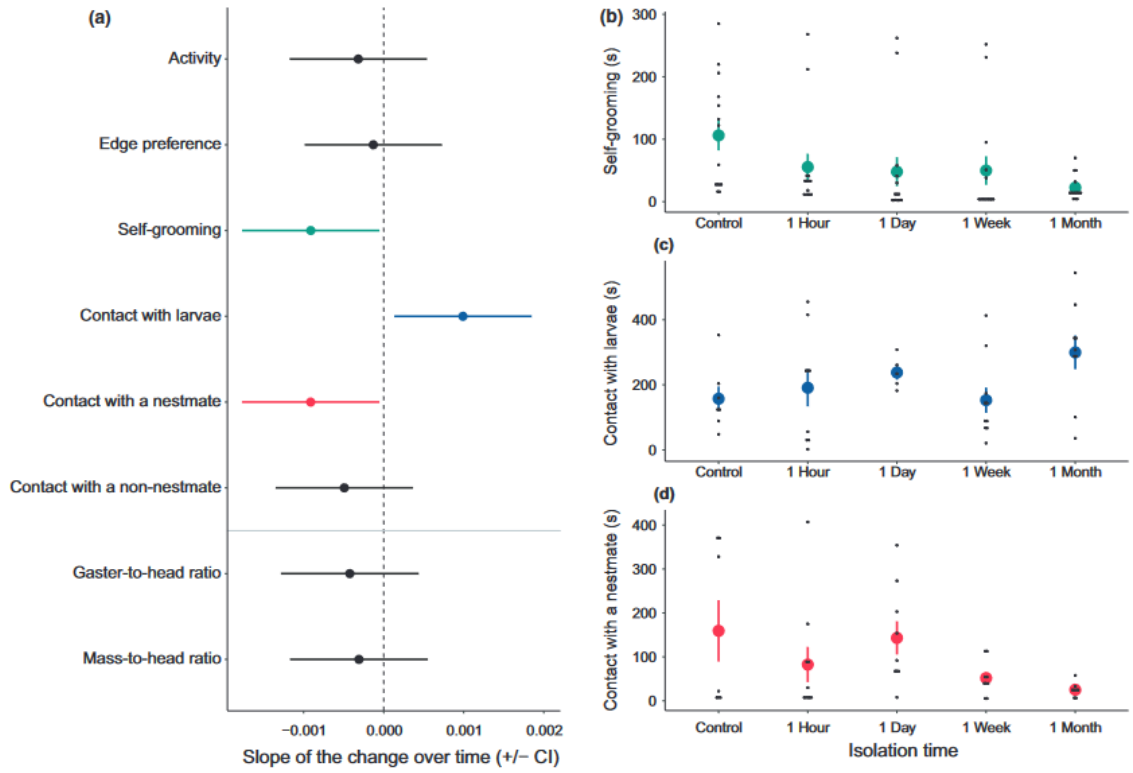

Figure 2-1 **(a)** Multivariate analysis examining whether isolation duration affects the phenotypic measurements. Isolation affected only self- grooming, duration of contact with larvae, and duration of contact with a nestmate. CI stands for the 95% confidence intervals. **(b)** The negative effect of isolation duration on self- grooming, **(c)** the positive effect of isolation duration on the duration of contact with larvae; and **(d)** the negative effect of isolation duration on the duration of contact with a nestmate. In **(b–d)**, Mean ±1 SE are presented as well as all data points (black dots).

Changes in brain gene expression

In total, 87 contigs were differentially expressed with isolation duration (False Discovery Rate < 0.05). According to the BLASTx search against the invertebrate database (August 2019), several differentially expressed genes were related to immune system functioning and stress. We summarized the functions of interesting candidate genes (Table 2-1). Adenosine deaminase 2-like decreased with isolation duration (Fig. 2-2a). This gene is involved in immune response, the differentiation of monocytes, inflammatory and injury responses, phagocytosis and melanization/encapsulation of foreign bodies (Zavialov *et al.*, 2010; Novakova and Dolezal, 2011). Aromatic-L-amino-acid decarboxylase-like plays a role in the production of dopamine and serotonin (Han *et al.*, 2010; Vavricka *et al.*, 2014). We found an increase in its production after a single day of isolation, followed by a decrease back to its original levels (Fig. 2-2b). The expression of four Cytochrome P450 genes either decreased after a single day of isolation or increased and then decreased with isolation duration (Fig. 2-2c and 2-2d). These Cytochrome P450 genes are involved in immune defence and help to remove toxic materials such as insecticides (Yocum *et al.*, 2018; Zhang *et al.*, 2016). The expression of a gene involved in thermal tolerance, and probably also in coping with other stressors (Heat shock 70 kDa protein cognate 5; Yuan *et al.*, 2017) decreased with isolation, following an increase after one day of isolation (Fig. 2-2e). The expression of a gene that catabolizes juvenile hormone (Juvenile hormone epoxide hydrolase 1-like; Toxopeus *et al.*, 2016; Allen *et al.*, 2018) decreased with the isolation period (Fig. 2-2f). This probably results in higher levels of juvenile hormones with isolation, unless juvenile hormone levels are elevated in parallel with isolation, for which we did not find any evidence. The apparent link between the possible maintenance of higher levels of juvenile hormone and longer durations of brood care by isolated workers may

also play a role in the ontogeny of ant behaviour, and in particular the transition from intranidal to extranidal activities in this and other ant species.

The transition from intranidal to extranidal activities is accompanied by an increase of juvenile hormone levels (e.g., Jaycox, 1976). Our findings – social isolation leading to an increase in contacts with brood and to a decrease in the expression of the gene catabolizing juvenile hormone (which should lead to increased levels of that hormone) – are somewhat contradictory. However, it is also known that extranidal workers respond readily to brood found outside the nest (Lenoir, 1981). The expression of a gene involved in TOR signalling (Protein charybde-like; Gotoh *et al.*, 2018) decreased with isolation (Fig. 2-2g). Interference with this pathway is associated with stress and shortened longevity (Amdam, 2011). A gene involved in controlling the inhibition of the activity of the insect visceral muscles (Myosuppressin; Egerod *et al.*, 2003; Lee *et al.*, 2015) increased with isolation (Fig. 2-2h), potentially interfering with food intake and digestion. To check whether time in the laboratory or handling could explain the transcriptomic changes we report, we did two additional comparisons. We contrasted gene expression immediately following isolation with 1-day isolation and 1-h isolation with 1-month isolation. Five out of eight candidate genes from our gene expression analysis were already differently expressed after 1-day of isolation indicating that the detected differences are triggered by isolation time. Similarly, we found five of eight candidates to be differently expressed between 1-h and 1-month isolation, revealing that handling alone i.e., transfer into an arena, which was identical in both treatments, cannot explain the described changes (Supplementary Data, S2). These additional contrasts, therefore, confirm that isolation duration is indeed responsible for the reported transcriptomic shifts.

Discussion

Social insects are surrounded by nestmates. Except for rare occasions, such as during social withdrawal when workers are injured/infected, queens found a colony, or individually foraging workers, social interactions make up most of their daily life. Isolation over longer periods should therefore lead to profound changes in behaviour and physiology. We found clear changes in brain gene expression and alterations in three important behaviours, although the behavioural shifts were not highly pronounced. As *T. nylanderi* workers live in small societies and mostly forage on their own, they are possibly more resilient and can withstand periods of solitude. We found that the self-grooming sanitary behaviour decreased with social isolation. Following isolation, ant workers also contacted their nestmates slightly less when re-encountering their worker sisters after hours or days. With increasing isolation time, workers moderately increased their contacts with larvae when given the opportunity. Other behaviours, such as activity or the response to non-nestmates and their body reserves did not change following social isolation. Our transcriptome analyses revealed strong changes in the expression of over 80 genes in the brains of workers following isolation. Isolated workers downregulated immune and stress-related genes, indicating that isolation causes immune suppression in social insects. This finding parallels the decrease in self-grooming behaviours detected with ongoing isolation, making workers that suffer from social isolation more susceptible to infection. Many downregulated genes have functionalities, such as chemical stress resistance, suggesting that social isolation may limit the ability of ants to cope with these stressors too.

Table 2-1 Candidate genes changing their expression with the isolation of ant workers. I, D, and ID stand for an increase, a decrease, or an increase followed by a decrease in expression with isolation duration over time and the FDR (False Discovery Rate, or type 1 error) value is equivalent to the P-value. Tc = *Temnothorax curvispinosus*, Wa = *Wasmannia auropunctata*, Cc = *Cyphomyrmex costatus*. The function of the up- or downregulated genes is suggested based on functional annotation and existing literature.

Blast Annotation (Species)	I/D	Log-fold ch., FDR	Function (based on previous literature)	References
Adenosine deaminase 2-like (Tc)	D	1.54, 5.8×10-8	Immunity: differentiation of macrophages inflammatory responses.	(Zavialov *et al.*, 2010; Novakova and Dolezal, 2011)
Aromatic-L-amino-acid decarboxylase-like (Tc)	ID	0.04, 1.2×10-6	Serotonin/Dopamine production, neurotransmission, insect immune response.	(Han *et al.*, 2010; Vavricka *et al.*, 2014)
Juvenile hormone epoxide hydrolase 1-like (Tc)	D	1.22, 0.0001	Catalyses juvenile hormone (JH) hydrolysis; JH is involved in caste determination, mediation of behaviour.	(Toxopeus *et al.*, 2016; Allen *et al.*, 2018)
Myosuppressin (Tc)	I	1.22, 0.0002	Decapeptide inhibiting insect visceral muscles, e.g., impedes food intake.	(Egerod *et al.*, 2003; Lee *et al.*, 2015)
Cytochrome P450 6a2-like (Tc)	ID	3.80, 0.0005	Insecticide breakdown, insect hormone metabolism, possibly involved in the ant immune response.	(Brun *et al.*, 1996; Ratzka *et al.*, 2011)
Cytochrome P450 4C1-like isoform X1 (Tc)	D	4.31, 0.0006	Insect hormone metabolism, insecticide breakdown, also in ants.	(Zhang *et al.*, 2016)
Cytochrome P450 6a14 (Tc)	ID	0.34, 0.003	Insect hormone metabolism, insecticide breakdown, seasonal expression in bees.	(Yocum *et al.*, 2018)
Cytochrome P450 4C1-like (Tc)	D	1.66, 0.004	Insect hormone metabolism, insecticide breakdown, also in ants.	(Yang *et al.*, 2013b; Zhang *et al.*, 2016)
predicted Heat shock 70 kDa protein cognate 5 (Wa)	ID	0.35, 0.005	Response to stress, heat stress, upregulated under viral stress.	(Yuan *et al.*, 2017)
Something about silencing protein 10 (Tc)	ID	0.16, 0.027	Roles in: gene silencing, the structure of silenced chromatin, gene regulation during development, RNA binding.	(Peters *et al.*, 2003)
Predicted Protein charybde-like (Cc)	D	-0.24, 0.034	Apoptotic process, negative regulation of signal transduction, regulation of TOR signalling, expressed in the ant accessory glands.	(Gotoh *et al.*, 2018)

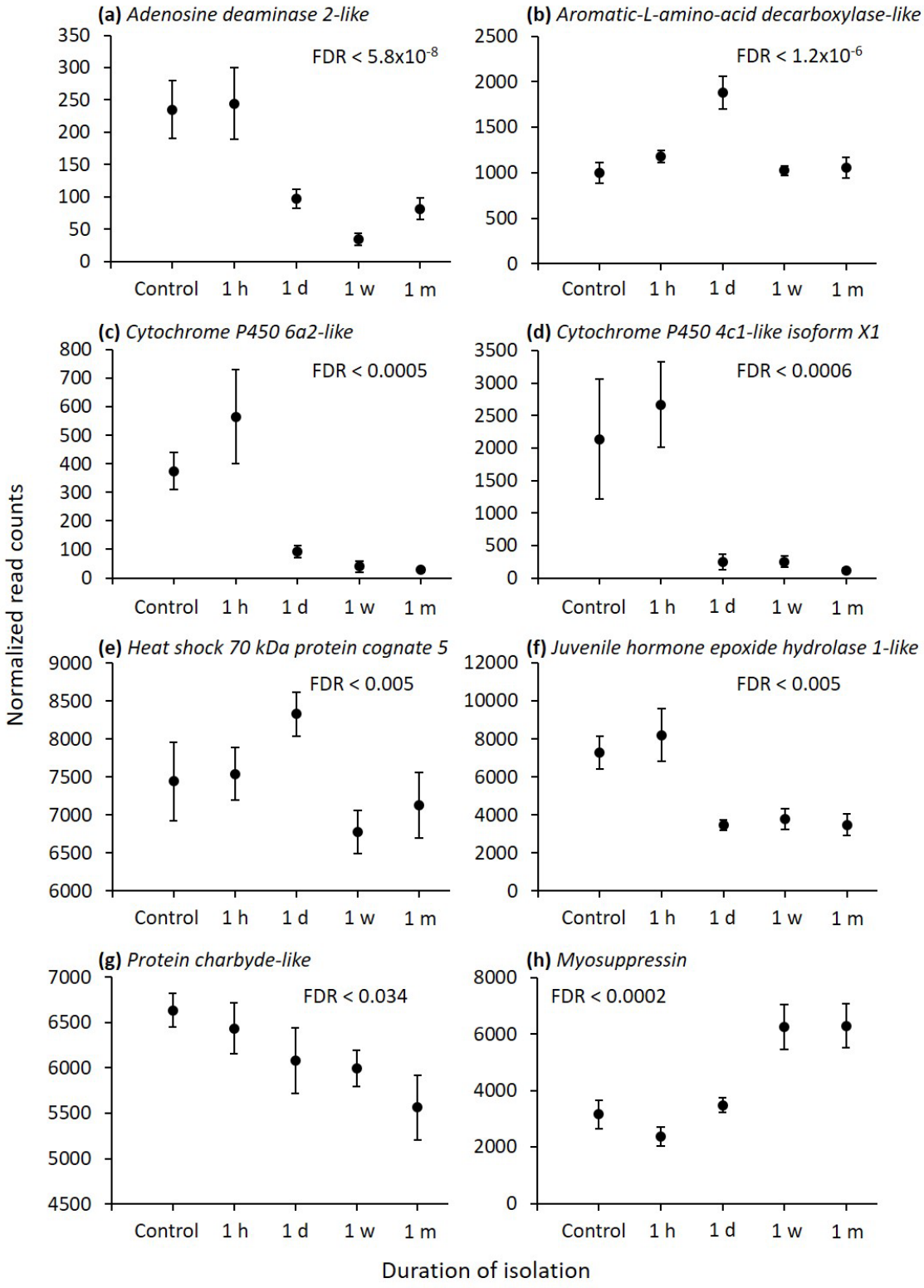

Figure 2-2 Candidate gene expression changes as a function of the isolation period. **(a)** Adenosine deaminase 2- like, **(b)** aromatic- L- amino- acid decarboxylase-like, **(c)** cytochrome P450 6a2-like, **(d)** cytochrome P450 4C1-like isoform X1, **(e)** heat shock 70 kDa protein cognate 5, **(f)** juvenile hormone epoxide hydrolase 1- like, **(g)** protein charybde-like and **(h)** myosuppressin. False discovery rate (FDR) values are provided. Mean ± 1 SE are presented as well as all data points (black dots).

Behavioural and body reserve changes with isolation

With increasing isolation duration, workers displayed moderately less interest in contacting nestmates, when a nestmate worker was re-introduced into their arena. Similar findings of lower antennation rates following social isolation were reported in the ant *Camponotus acvapimensis* (Cybulska *et al.*, 2000). The reduced interest in nestmates following isolation resembles that observed in small mammals, which often exhibit a reduction in social interactions, linked to an increase in anxiety-like behaviours following social isolation (e.g., Lukkes *et al.*, 2009). A possible, social insect-specific cause of the social contact reduction could be that of a gradual divergence in the cuticular hydrocarbon profile of workers isolated from their nestmates. Such a shift has been shown in the ant *Aphaenogaster senilis*, where it led to increased aggression between nestmates following isolation (Lenoir *et al.*, 2001; Ichinose and Lenoir, 2009). Our focal ants' responses towards non-nestmates did not shift with isolation and were generally weak. We never observed overt aggression probably due to the generally low aggression levels in our focal species (Beros *et al.*, 2017), or because encounters happened outside of the nest, which is generally defended (Scharf *et al.*, 2012b). Studies on insects have demonstrated mixed patterns regarding changes in aggression with isolation (Breed, 1983; Boulay and Lenoir, 2001; Korczyńska *et al.*, 2005; Liu *et al.*, 2011; Stevenson and Rillich, 2013).

Self-grooming is an effective sanitary behaviour used by ants to clean their cuticle from fungal or bacterial pathogens (Tragust *et al.*, 2013). It is also involved in other functions, such as homogenization of the colony odour by spreading over the body the contents of post-pharyngeal glands exchanged between ant workers via trophallaxis and allogrooming (Soroker *et al.*, 1995). Exposure to a pathogenic fungus was shown to cause an increase in self-grooming but a decrease in allogrooming in the ant *Lasius*

neglectus (Theis *et al.*, 2015). Here, we found that workers exposed to social isolation showed a reduction in self-grooming, which, in addition to the downregulation of immune genes, is expected to make isolated workers more susceptible to pathogens. Contact with re-introduced larvae moderately increased following prolonged isolation, which could be partially explained by the finding from earlier work on ants, of a negative association between the intensity of larval care and self-grooming (Westhus *et al.*, 2014). The evidence from social mammals is to the contrary: isolation and the deprivation of maternal care in juvenile rats lead to their later reduction in care of their own offspring (Lovic *et al.*, 2001).

Social isolation can affect the activity patterns of animals. In the ant *Camponotus fellah,* isolated workers become more active, but only on the first day of isolation (Koto *et al.*, 2015). Rodents exhibit diverse activity changes with social isolation (Grippo *et al.*, 2008; Silva-Gómez *et al.*, 2003; Weiss *et al.*, 2004). *Temnothorax* workers become typically more active with age (Kohlmeier *et al.*, 2017), but our experiments found no change in activity with social isolation over time. Caste too can have a strong influence on the response to social isolation, with nurses being more ready to engage in social contact, while foragers are more interested in their immediate abiotic environment (Wagner-Ziemka *et al.*, 2008; Mazurkiewicz *et al.*, 2016). Ant nurses and foragers may also differ in their mortality in conditions of social isolation (Boulay, 1999). The tendency to move along the test arena's edges reflects either the risk that the tested animal perceives or its attempt to escape a closed area. Although this tendency has been shown to change with isolation duration in rats and zebrafish (Djordjevic *et al.*, 2012; Shams *et al.*, 2015), we did not observe any such change here.

Body reserves did not change with isolation. This deviates from previous findings for the same species, which demonstrated lower body reserves in isolated than in

grouped workers (Modlmeier *et al.*, 2013). The difference between the two studies lies in the fact that our workers were allowed to feed, whereas in the study of Modlmeier *et al.* (2013) the workers were starved. Our finding also contrasts those of other studies on isolated ants that have reported digestion problems (Howard and Tschinkel, 1981; Koto *et al.*, 2015), which should lead to reduced body reserves. It is possible that other nutritional deficits exist but are not detectable using this rough estimator, and more accurate biochemical methods are required to detect the effect of social isolation.

Three confounding factors that we were unable to separate from isolation duration might have affected our results. First, the period that each colony was maintained in the laboratory is confounded with isolation duration. During this period, other behavioural changes could have taken place, such as a transition from nursing to foraging in some workers. Foragers could be more interested in returning brood to the nest (Lenoir, 1981), which fits the observed behavioural change of increased interactions with brood following isolation. Furthermore, the transition from intranidal to extranidal activities might have been accelerated in isolated ants by the possibly higher levels of juvenile hormone in the brain of isolated workers (e.g., Jaycox, 1976). However, given the lifespan of *Temnothorax* workers of several years and the once per annum production of new workers, behavioural progression is unlikely to explain the observed behavioural changes given the timeframe of our study. Second, isolation is confounded with changes in nest structure (to a Petri dish), which may also affect behaviour. However, this should have only affected the ants in the control, as thereafter the nest structure remained unmodified. Third, isolation was confounded with protein-free nutrition and the absence of brood. In social Hymenopterans, larvae are often responsible for breaking down the protein into amino acids and provisioning the workers (Schultner *et al.*, 2017). Thus, some behavioural changes, such as higher

attendance to larvae upon reunification could result from a reduction in the proteins available to the isolated workers.

Brain gene expression changes with isolation

We can demonstrate that the expression of stress or immune system-associated genes was linked to isolation duration. The downregulation of genes involved in the immune response should impede the ability of ant workers to confront a challenge to the immune system. Our findings thus fit the results on other group-living insects and mammals, indicating that the deterioration of the immune system following isolation is widespread across social animals: earwigs and termites isolated after fungal infection died faster than those also infected but kept in groups (Rosengaus *et al.*, 1998; Hughes *et al.*, 2002), and isolated mammals have been shown to have a higher risk of developing cancer (Wu *et al.*, 2000). The mechanisms behind a suppressed immune system include lower levels of hemocyte cells in the hemolymph of isolated moths (Pavlushin *et al.*, 2019); and lower activity of natural killer cells and lymphocytes in lonely/isolated humans and mice than in non-lonely/grouped ones (Kiecolt-Glaser *et al.*, 1984; Cruces *et al.*, 2014).

Also, genes related to the neutralization of toxins are downregulated with isolation in our ants. These genes are involved in insecticide resistance in fruit flies, whiteflies, and fire ants (Brun *et al.*, 1996; Yang *et al.*, 2013a; Zhang *et al.*, 2016). Consequently, isolation should make insects more vulnerable to insecticides and toxins. Isolation in small mammals leads to elevated oxidative stress in the brain, because more reactive substances are produced and the activity of antioxidant enzymes is lower (Huong *et al.*, 2005; Shao *et al.*, 2015), suggesting that isolated small mammals cope less well with toxic materials. Similarly, social isolation increases the expression of heat-shock

proteins in mammals, and such upregulation may help to protect the brain against reactive oxygen species (Zlatković *et al.*, 2014).

Some genes were initially expressed more intensely with short isolation duration and then expressed less when isolation was prolonged. We found such a pattern for the gene aromatic-L-amino-acid decarboxylase-like, involved in the production of dopamine and serotonin (Han *et al.*, 2010; Vavricka *et al.*, 2014). These neurotransmitters are linked to anxiety-related behaviours in mammals (Brandão *et al.*, 2015; Marcinkiewcz *et al.*, 2016), and an increase in anxiety is a typical response of mammals to social isolation (Bibancos *et al.*, 2007; Pan *et al.*, 2009). We are not suggesting that animals lacking social contact are more anxious. Rather, we are reporting on a change following isolation in the expression of a gene involved in molecular pathways associated with anxiety in mammals. In insects, serotonin is involved in the mediation of behaviour and physiology, such as feeding, sleeping, and learning (Vleugels *et al.*, 2015). Serotonin and dopamine also play a role in the mediation of aggressive behaviour and other social activities in ants (Kostowski and Tarchalska, 1972; Szczuka *et al.*, 2013). In contrast to the observed change in this gene's expression, which reached its peak after a single day of isolation, we did not detect any behavioural changes. Koto et al. (2015) observed an increase in the activity of isolated workers, but only on the first day of isolation, which appears to correspond well to our finding: insect activity is often positively correlated with serotonin levels (Kamyshev *et al.*, 1983; Kloppenburg *et al.*, 1999).

The decrease in expression of a gene responsible for catabolizing juvenile hormone with isolation duration could lead to higher circulating levels of juvenile hormone. In agreement, honeybees and locusts raised/maintained in isolation demonstrated higher levels of juvenile hormone (Injeyan and Tobe, 1981; Breed, 1983; Huang and Robinson,

1992). Myosuppressin, a gene involved in controlling the inhibition of the activity of the insect visceral muscles, and which could interfere with food intake and digestion, is upregulated with isolation. The upregulation of myosuppressin may indicate impaired digestion in isolated workers. Isolated workers were shown to have retained food in their foregut (Koto *et al.*, 2015), and myosuppressin can inhibit foregut activity (Aguilar *et al.*, 2004). This upregulation may explain why the body reserve measurements did not differ between isolated and control ants: food was retained in the gut but was not digested, leading to no observed change in morphology.

Conclusion

We have demonstrated that pronounced changes in gene expression, correlated with social isolation, take place in *T. nylanderi* workers. These changes may have adverse effects on the ants as they indicate suppression of the immune system, lower resistance to other stressors, interference with food intake and digestion, and a possible increase in circulating juvenile hormone. Such changes might contribute to a shortened lifespan in isolated workers, as detected for this and other ants. We found moderate changes in social interactions following isolation: ant workers were less inclined to contact their adult nestmates, while increasing the duration of contacts with brood. The reduction in self-grooming with ongoing isolation should reduce the removal of pathogens from the cuticle and, in combination with downregulation of immune genes, might make isolated workers more prone to infection. Future studies should examine how behavioural and physiological immunity variables interact and analyse the behavioural responses to isolation in more depth. Such studies should also include physiological and neurobiological factors. Only such multifaceted examinations can help us to reach a firm conclusion regarding the comprehensive effects of isolation in social species.

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Author Contributions

I.S., S.F., and R.L. designed the study and interpreted its results. S.F. and I.S. collected the ant colonies. I.S., A.L.H., and M.K. conducted the experiment. R.L., E.J. and I.S. analysed the behavioural data. M.S., R.L. and I.S. analysed the transcriptomics data. E.J. contributed genomic data of the studied species. I.S. and S.F. wrote the manuscript. The study was conducted in the laboratory of S.F. All authors contributed to writing and approved the final manuscript.

Supplementary Materials

The following supplementary materials are available online at https://onlinelibrary.wiley.com/doi/10.1111/mec.15902:

S1: Behavioural dataset and schedule, S2: Independent analyses of behavioural, body reserve measurements, and pairwise comparisons of gene expression between shorter and longer isolation durations, S3: GO enrichment of genes in clusters, S4: Differentially expressed genes according to isolation duration.

Raw reads can be accessed from SRA under BioProject ID PRJNA608848.

Chapter 3

Molecular (co)evolution of hymenopteran social parasites and their hosts

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Abstract

Social parasitism describes a fascinating way of life in which species exploit the altruistic behaviour of closely related, social species. Social parasites have repeatedly evolved in the social Hymenoptera, including ants, bees, and wasps. The common ancestry and shared (social) environment with their hosts facilitates the study of molecular adaptations to the parasitic lifestyle. Moreover, when social parasites are widespread and virulent, they exert strong selection pressure on their hosts, leading to the evolution of defence mechanisms and triggering a coevolutionary arms race. Recent advances in sequencing technology now make it possible to study the molecular basis of this coevolutionary process. In addition to describing the latest developments, we highlight open research questions that could be tackled with genomic, transcriptomic, or epigenetic data.

Highlights

- Social parasites are characterized by a loss of odorant receptor genes
- Different genes underlie the strategies used by different social parasites
- Wasps and bees adapt to a parasitic lifestyle by re-shaping existing gene-regulatory networks
- Novel molecular approaches could shed light on social parasite-host coevolution

Introduction

Social parasites exploit the altruistic behaviour of social species to raise their offspring (Davies and Brooke, 1989; Brandt *et al.*, 2005a; Thorogood *et al.*, 2019), similar to avian brood parasites. This form of parasitism evolved several times independently, in particular in the social Hymenoptera (Hölldobler and Wilson, 1990; Hines and Cameron, 2010; Borowiec *et al.*, 2021) and there in all three clades: Social bees, wasps, and ants. These host-parasite relationships typically evolve from common ancestors, with social parasites and their hosts being close relatives, a phylogenetic pattern previously described by Emery (Emery, 1909). Evidence from parasitic bees suggests that parasites commonly exploit their sister species (strict definition Emery's rule) at emergence while targeting increasingly more distant and diverse hosts over time (loose definition) (Sless *et al.*, 2022). The lifestyles of social parasites vary in their degree of host dependency. Facultative social parasites can live on their own, but often use hosts to establish new colonies. Queens of the Cape honeybee *Apis mellifera capensis* can found their own colony and live independently of their host, yet, their parasitic workers disperse to *A. m. scutellata* colonies to exploit their social behaviour and to reproduce via thelytokous parthenogenesis (Neumann and Hepburn, 2002). Obligate social parasites are dependent on their host for their entire life, or at least some stages of it. In the ant *Lasius umbratus*, freshly mated parasitic queens temporarily depend on their host during the founding phase to raise their worker offspring, which later completely replaces the host workers (Buschinger, 1986). A recent phylogenetic study of *Formica* ants has shown that such temporary parasitic lifestyles can evolve into permanent social parasitism, including dulosis (Borowiec *et al.*, 2021). Parasitic inquiline species found in wasps, bumblebees and ants, have convergently lost the worker caste and instead, these queens use the host's workforce to raise their sexual offspring (Cervo, 2006; Buschinger, 2009; Dronnet *et al.*, 2005). A

less extreme, but still partial loss of worker function is seen in dulotic ants (sometimes referred to as "slave-making"), where workers have lost the ability to feed themselves and to perform nursing or foraging tasks. Consequently, they permanently depend on host workers for their survival (Cervo, 2006; Foitzik *et al.*, 2001; Jongepier *et al.*, 2014), but also acquired novel behavioural traits such as the ability to conduct raids or to reproduce in the queen's presence.

Most social parasites are rare. Yet, dulotic ants and many socially parasitic wasps can be so widespread and virulent that they trigger a perpetual antagonistic cycle of host adaptation and parasite counter-adaptation (Ortolani and Cervo, 2009, 2010; Brandt *et al.*, 2005b; Foitzik *et al.*, 2001), called coevolution. The escalation dynamics driven by strong reciprocal selection are known as co-evolutionary arms races (Thompson, 1989, 2005). Differences in parasite prevalence among communities and limited gene flow between populations may trigger a geographic mosaic with coevolutionary hot and cold spots (Jongepier *et al.*, 2014; Thompson, 2005; Bauer *et al.*, 2009; Kleeberg *et al.*, 2015; Kaur *et al.*, 2019). Given the close phylogenetic relationship and shared environment of social parasites and their hosts, they are ideal to study coevolutionary dynamics with fewer confounding factors than seen in other systems, which is important for identifying the expression and regulation of genes associated with social parasitism and host defences (Emery, 1909). In this review, we first summarize which changes in gene expression, genes and genomes have contributed to the evolution of socially parasitic lifestyles. We then discuss studies that investigate the molecular basis of social parasite-host coevolution. Finally, we propose how recent advances in omics technologies can be used to uncover not only molecular adaptations to social parasitism but also to illuminate the coevolutionary dynamics between social parasites and their hosts. In this regard, approaches used in human-pathogen systems could be

applied to social insect systems to open new avenues in the study of coevolution between social parasites and their hosts.

Genomic changes with social parasitism

Relaxed selection on traits that lose importance in a parasitic lifestyle (Benoit *et al.*, 2016; Chang *et al.*, 2015; Tsai *et al.*, 2013; Kirkness *et al.*, 2010) causes obligate parasites of all types to often show signs of gene loss to extreme genome reduction (Slyusarev *et al.*, 2020). Thus, genomes of highly specialized social parasites may be expected to contain fewer genes when host exploitation renders some social traits, such as foraging and brood care, obsolete (Cervo, 2006; Buschinger, 2009). A recent study on *Acromyrmex* inquiline ants provided evidence for genome erosion and relaxed selection in these workerless social parasites and suggested that gene loss was triggered by genomic rearrangements and fusion (Schrader *et al.*, 2021). In contrast, a comparative study on the parasitic bumblebees of the subgenus *Psithyrus* observed increased chromosome numbers and genome sizes comparable to that of the common non-parasitic ancestor, while loss of eleven odorant receptor genes was also detected (Sun *et al.*, 2021).

In dulotic ants, Jongepier et al. likewise reported the convergent loss of odorant receptor genes and a severe reduction of gustatory genes in their genomes across multiple independent origins of dulosis, however, without reduction in genome size (Jongepier *et al.*, 2022). In social insects, these olfactory receptors are important for nestmate recognition and communication, but especially for the detection of volatiles such as sex pheromones or food cues (Yan *et al.*, 2020a). These gene families were found to have expanded massively during the evolutionary transition to insect eusociality (Zhou *et al.*, 2012, 2015; Legan *et al.*, 2021; Robertson and Wanner, 2006; Sadd *et al.*, 2015). Notably, many of these olfactory receptors belong to the 9-exon

subfamily, which plays an important role in chemical communication through the detection of cuticular hydrocarbons (Zhou *et al.*, 2015; McKenzie and Kronauer, 2018; Engsontia *et al.*, 2015). A loss of these genes suggests that selection on genes important for olfaction in social parasites is relaxed, probably caused by a reduction in social tasks that require close communication. In contrast, no evidence of gene loss in *Pogonomyrmex* and *Vollenhovia* inquilines was found and regulatory changes within multifunctional genomes were proposed to be associated with the transition towards social parasitism in these ants (Smith *et al.*, 2015).

Genes associated with social parasitism

A first step in elucidating how social parasitism evolved is to identify genes or allelic variants associated with or causing parasitic behaviour which are expected to be under strong selection (Wallberg *et al.*, 2016; Feldmeyer *et al.*, 2017; Fouks and Lattorff, 2016). Rather than identifying "genes responsible for social parasitism", empirical studies have discovered genes associated with specific parasitic traits. For example, in dulotic ants of the genus *Temnothorax*, which are active only during summer raids, genes related to circadian rhythms are under positive selection, possibly explaining the divergent activity patterns of these parasites (Feldmeyer *et al.*, 2017). Accordingly, changes in circadian rhythms have also been found in parasitic paper wasps (Ortolani and Cervo, 2009). Furthermore, genes involved in the synthesis of cuticular hydrocarbons are also under selection in dulotic ants (Feldmeyer *et al.*, 2017). Again, this could be related to their lifestyle, as many social parasites such as dulotic ants avoid detection by carrying fewer cuticular hydrocarbons used in host recognition (Kaur *et al.*, 2019; Jongepier and Foitzik, 2016a; Kleeberg *et al.*, 2017). Interestingly, only a single gene from a dataset of raiding dulotic ants was found to be under selection in all three species, likely explained by the divergent raiding strategies

employed by these dulotic ants (Feldmeyer *et al.*, 2017). Several studies on the socially parasitic Cape honeybee *Apis mellifera capensis* have identified genomic regions with signatures of selection (Wallberg *et al.*, 2016; Aumer *et al.*, 2019), which were suggested to be associated with thelytoky, a form of asexual reproduction used by parasitic workers. A recent genome-wide association study (GWAS) failed to confirm that the loci identified by these studies are indeed associated with thelytoky, except for a sole gene, for which specific allelic variants were exclusively found in thelytokous *A. m. capensis* (Yagound *et al.*, 2020). These studies and their diverse outcomes suggest that more work is needed to confirm genomic regions or genes selected for thelytoky and social parasitism in the Cape honey bee (Wallberg *et al.*, 2016; Aumer *et al.*, 2019; Yagound *et al.*, 2020; Christmas *et al.*, 2019).

Transcriptomic changes with social parasitism

Differences in phenotype, especially in closely related species, are not always due to novel alleles, genes, or genomic rearrangements, but may also be caused by phenotypic plasticity and underlying changes in gene-regulatory networks (West-Eberhard, 1989). Since host and parasite pairs share a common non-parasitic ancestor, the evolution of social parasitism may have been facilitated by altering the expression of pre-existing genes in a new parasitic context, a less costly strategy compared to the evolution of completely new sets of genes. For example, social insects have evolved two distinct female phenotypes, queens and workers, which mostly arise via differential gene expression (Smith *et al.*, 2008; Sumner, 2006). Thus, in the transition to a socially parasitic lifestyle, plastic pathways could be used to adapt, facilitating initial steps that are later hardwired through genetic assimilation (Price *et al.*, 2003). In systems where social parasites are highly reproductive in host colonies, this high fecundity is achieved either by upregulation of queen-specific genes such as transferrin

or vitellogenin as in the Cape honeybee (Korb *et al.*, 2021; Aumer *et al.*, 2018) or by downregulation of worker-biased genes as seen in the paper wasp *Polistes sulcifer* (Cini *et al.*, 2015).

In another host-parasite system, both the host ant *Temnothorax longispinosus* and its social parasite *T. americanus* use tandem-running behaviour, a recruitment strategy, during nest relocation and raiding, respectively. RNA-Seq experiments revealed that different genes are associated with these homologous tandem-running behaviours as the expression of orthologous genes was not similarly explained by behaviour (Alleman *et al.*, 2019). This suggests that the parasite has recruited novel genes that result in a phenotype similar to that of the host. However, caution should be exercised when comparing gene expression across species, as transcriptomic differences could be explained in part by differences in tissue composition of the same organs, such as in the brains of parasitic paper wasps and their hosts (Rozanski *et al.*, 2022).

A host perspective

Most of the molecular studies discussed to date have focused on the transcriptomic and genomic changes associated with the transition to a socially parasitic lifestyle (see Fig. 3-1), while the molecular traces of the escalating arms race on hosts have received less attention. Recent evidence from wasp social parasite systems suggests that hosts may also use pre-existing phenotypic plasticity to defend themselves. Cini et al. showed that parasitized workers upregulate a gene with fertility function that is normally overexpressed in queens (Cini *et al.*, 2015, 2020, 2014). Since worker reproduction is usually costly but increases host fitness when in a parasite nest, one could argue that the possibility of neutral evolution of these regulatory changes is unlikely. In fact, there is additional evidence that this host response is triggered by the presence of the parasite: *P. dominula* workers do not begin reproducing when a

conspecific unrelated queen replaces their mother (Monnin *et al.*, 2009). Further studies should unravel whether this response represents a form of rebellion or even benefits the parasite in some way.

Another interesting avenue for research is to investigate how varying selection pressures may drive the expression of genes underlying offensive and defensive traits in parasites and hosts. Parasite prevalence in the host population has been linked to the expression of phenotypic traits in ant and wasp hosts (Ortolani and Cervo, 2009; Jongepier *et al.*, 2014; Kleeberg *et al.*, 2015; Ortolani and Cervo, 2010; Jongepier and Foitzik, 2016a). These traits include increased aggressiveness, altered defence strategies, colony demography and cuticular hydrocarbon diversity (strategies reviewed in Grüter *et al.*, 2018). A first study by Kaur et al. compared the transcriptomic response of *T. longispinosus* hosts when facing their parasite *T. americanus* as a function of parasite prevalence in the community (Kaur *et al.*, 2019). This revealed that gene expression in the host brain depends on the origin of the parasite they encounter, as dulotic workers from high prevalence populations gained undetected access to the host colony (and thus did not trigger aggressive defences), indicating that the parasite leads the coevolutionary arms race. Table 3-1 provides an overview of known host defence traits in combination with hypotheses on their underlying molecular bases. These testable predictions could be considered by social insect researchers to investigate the molecular coevolution of social parasite-host systems.

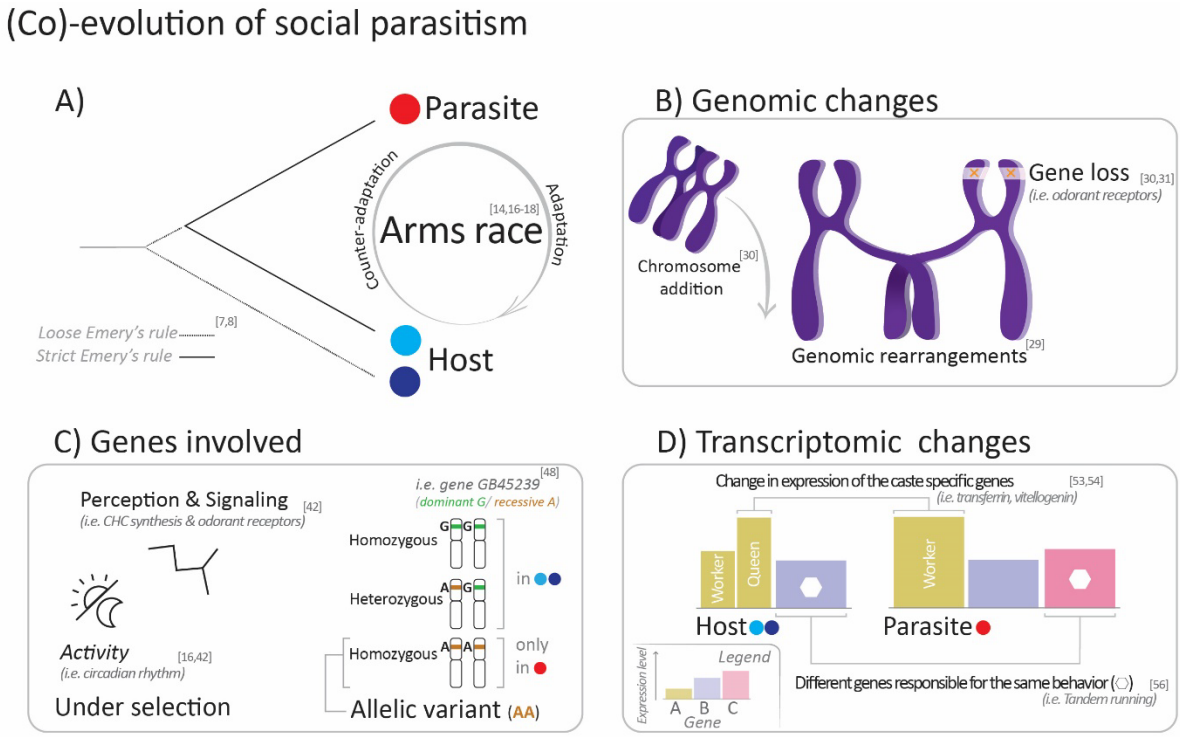

Figure 3-1 Molecular evolution of social parasites. Schematic overview of the molecular (co)evolution of social parasitism including an illustration of some examples. A) Social parasites emerge from a common ancestor with their hosts from which they are more or less closely related (Strict/Loose Emery's rule (Emery, 1909; Sless *et al*, 2022)). When parasite prevalence and virulence are high, interactions can escalate into a coevolutionary arms race of parasite adaptations and host counter-adaptations (Foitzik, Christopher J. DeHeer, *et al.*, 2001; Brandt et al. 2005; Ortolani and Cervo, 2009, 2010). Evidence of molecular changes have been found on three levels: B) Parasite adapt to their host by the addition of new chromosomes (Sun *et al*, 2021), by the loss of gene families (Jongepier *et al*, 2022; Sun *et al*, 2021) or by genomic rearrangement (e.g., crossing-over) (Schrader *et al*, 2021). C) Genes related to the specific lifestyle/infection strategy are under selection in parasites e.g., genes involved in circadian rhythm or signaling (Ortolani and Cervo, 2009; Feldmeyer *et al*, 2017). The allelic variant identified to be causal for social parasitism in the Cape honeybee is recessive so parasites are always homozygous for this allele (Yagound *et al*, 2020). D) Genes can lose their plastic expression in social parasites and be expressed across social environments as seen in parasitic paper wasps or honeybees (Korb *et al*, 2021; Aumer *et al*, 2018) or new genes can cause homologous phenotypes to the ones observed in hosts (Alleman *et al*, 2019).

Table 3-1 Molecular basis and mode of selection on host-defence traits. If selection pressure exerted by social parasites on host populations is high (as seen especially in some dulotic ants and paper wasp social parasites), hosts will develop behavioural, physiological or demographic defences (for a summary of host defences see (Grüter *et al.*, 2018). The molecular basis and mode of selection on these coevolutionary adaptations is not well understood and here we provide some predictions to guide future research.

Parasite trait	Host defence trait	Examples	Suggested molecular mechanism Mode of selection
Tracking host recognition cues via mimicry or camouflage	Increased diversity of recognition patterns to undermine host-matching	Positive correlation between parasite pressure and intercolonial CHC diversity (Martin *et al.*, 2011; Jongepier and Foitzik, 2016a)	Molecular changes (e.g. balancing/relaxed selection, increase in mutation rate, introgression, or de-canalization) on CHC synthesis genes leading to higher variation in chemical cue diversity
Display of overt aggression by the parasite to invade/raid host colonies during specific times of the year	Increase in aggression and/or optimization of fighting strategies in response to attacks	Increased aggression in hosts from highly parasitized populations, especially during seasons with frequent parasite attacks (Kleeberg *et al.*, 2014; Brandt *et al.*, 2005b)	Gain of and selection on genes linked to aggression and recognition abilities, e.g., odorant receptors Phenotypic plasticity in aggression/recognition traits via differential gene expression in response to seasonal variation in attack risks (Alleman *et al.*, 2018)
	Plasticity in behavioural defences induced by parasite presence	Plasticity in defence strategies of hosts in the presence of parasites (Jongepier *et al.*, 2014)	Behavioural plasticity linked to parasite-induced changes in gene expression
	Increase in body size and/or colony size to improve nest defence	Increase of hosts body sizes in parasites presence (Ortolani and Cervo, 2010)	Genetic or transcriptional changes in genes related to individual and colony development
Parasitic queens invading host colonies to seek adoption	Strict monogyny (in ants) facilitating the rejection of additional queens, including parasitic ones; Generally, improve in queen recognition	Link between monogyny and high parasite pressure (Herbers and Foitzik, 2002)	Selection on and expression changes in genes related to social structure and recognition
Manipulations induced by parasites to facilitate host invasion or cohabitation	Unresponsiveness to parasitic manipulation	Positive correlation between parasite prevalence and the ability of the parasite to manipulate the host (Foitzik *et al.*, 2003; Jongepier *et al.*, 2015)	Resistance to manipulation due to alteration of odorant perception/ processing

Novel molecular avenues

We propose additional strategies to decipher the molecular basis of social parasite evolution. On the genomic level, several studies have identified gene loss in parasites. However, as social parasites also exhibit novel morphological and behavioural traits, such as sabre-shaped mandibles or raids, repurposing of existing genes or gene gains should also play a role. Gains have been previously described in parasitic nematodes, where gene families involved in detoxification expanded, likely to facilitate parasite survival in the presence of defensive chemicals released by the host (Zhang *et al.*, 2020a). Future genomic studies should therefore focus more on gene gains and innovations associated with the socially parasitic lifestyle. So far, we have considered genes associated with social parasitism as separate entities, but they could also be linked and inherited as super-genes. Indeed, alternative reproductive strategies were found to be regulated via large non-recombining elements in several ant species (Lagunas-Robles *et al.*, 2021; Wang *et al.*, 2013; Purcell *et al.*, 2014; Brelsford *et al.*, 2020; Yan *et al.*, 2020b) and it is similarly possible that the switch to a novel social parasitic strategy might be linked to a parasitic supergene (Linksvayer *et al.*, 2013). In addition to studying the genomes and transcriptomes, epigenomes should be sequenced to investigate signs of epigenetic regulation that may promote rapid adaptation to interaction partners (Jablonka, 2017). In the greater wax moth *Galleria mellonella* and the red flour beetle *Tribolium castaneum*, epigenetic factors including DNA methylation, histone acetylation and the activity of miRNAs were found to contribute to rapid adaptation in response to bacterial infections (Mukherjee *et al.*, 2019; Vilcinskas, 2016). Epigenetic studies have already been performed on social insects and should be easily transferable to social parasite systems (Morandin and Brendel, 2021; Cardoso-Júnior *et al.*, 2021; Sieber *et al.*, 2021; Collins *et al.*, 2017;

Simola *et al.*, 2016). While RNA-Seq has been used extensively in hymenopteran social parasites, proteomic approaches are less frequently performed but are necessary to investigate possible post-transcriptional changes that may play a role during social parasite evolution (Hegde *et al.*, 2003). Whether similar mechanisms contribute to social parasite evolution remains to be investigated.

In addition to increasing our knowledge of the molecular mechanisms underlying the social parasite and host phenotype, future studies should aim to determine how parasite and host populations shape each other at the molecular level. This can be done by studying genes and allelic variants that influence the outcome of the coevolutionary arms race. It is likely that the expression of such genes changes in response to the prevalence of the parasite and that alleles alter their frequency accordingly. Furthermore, by using methods such as GWAS, it is possible to relate genomic positions to varying parasite load, and in the case of social parasites, to the prevalence of social parasites within a population, which are likely to drive adaptations to the interacting partner (Hirschhorn and Daly, 2005). Thus, we are in need of more population genomic studies to detect variation in genotypes linked to parasite presence. In addition, human pathogens have been studied using natural co-GWAS, in which host and infecting parasite are sampled from natural populations, to investigate whether the genomes of both interacting partners determine the infection outcome (Lees *et al.*, 2019; Ansari *et al.*, 2017). We believe that a similar approach could be used to study hymenopteran social parasites and their hosts. By sampling hosts and parasites from the same parasite colony, it is possible to determine whether the phenotype and fitness of a parasitic wasp, bee, or ant might be influenced not only by her own genome but also by the genome of the host colony in which she grew up or which she exploits as an adult. Such genotype-to-genotype interaction has already

been observed in social insects outside the context of social parasitism: In bumblebees, the expression of immune genes depends on both the genotype of the bumblebee and the genotype of the trypanosome parasite with which it is infected (Barribeau *et al.*, 2014).

Outlook

Based on the above suggestions, we propose here that ideal model systems for studying the molecular coevolution of hymenopteran social parasites and their hosts should meet the following requirements: 1) Their genomes need to be sequenced with a corresponding annotation of good quality for detecting any genomic rearrangements, but also to study the epigenome using methods such as chromatin immunoprecipitation sequencing (ChIP-seq), bisulfite sequencing or ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing). 2) Ideally, the species should belong to a clade with multiple convergent origins of parasitic behaviour to study gains and losses associated with a parasitic lifestyle. 3) If parasite prevalence varies across communities, the coevolutionary trajectory leading to different adaptations could be studied in more detail. Due to the effort of the Global Ant Genomics Alliance and others, more and more high-quality ant genomes are being sequenced, revealing multiple independent origins of social parasitism (Jongepier *et al.*, 2022; Boomsma *et al.*, 2017). Systems with coevolutionary arms races such as those described before (Ortolani and Cervo, 2010; Foitzik *et al.*, 2003) offer excellent possibilities to advance our knowledge of the molecular mechanisms driving host and parasite adaptations. In the long run, parallels to other host-parasite systems, such as avian brood parasites, will allow us to determine whether similar coevolutionary dynamics shape the evolutionary outcomes not only on a behavioural level but also at the molecular level (Kilner and Langmore, 2011).

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Author Contributions

First draft was created by M.S. Figure was created by E.C. Introduction was mainly written by M.N.M., E.C. and S.F. and manuscript was finalized by M.S. and S.F. All authors revised the manuscript constantly.

Chapter 4

Living under a social parasite: Transcriptomic changes in parasitized ant workers shed light on parasite-host coevolution

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In preparation

Abstract

The widespread lifestyle of social or brood parasites is characterised by the exploitation of the social behaviour of the hosts. Studies on how these parasites manage to infiltrate host nests or elicit host defences have yielded fascinating insights into parasite-host coevolution. Less studied, however, is why parasitized hosts direct their social behaviour toward the parasites. We investigated this question in the ant *Leptothorax acervorum*, host of the dulotic, obligate social parasite *Harpagoxenus sublaevis*. We shed light on molecular mechanisms underlying worker exploitation by contrasting tissue-specific gene expression in young host workers that we experimentally (re-)introduced as pupae into parasitic colonies, their mother, or another conspecific colony. Brain and antennal transcriptomes revealed that ant workers were affected by their adult social environment. In addition, gene expression in the antennae, but not in the brain was strongly linked to worker behaviour in the hour before sampling. Antennal gene expression shifted similarly in host workers living in parasitic colonies as in alien conspecific colonies, pointing to the altered chemical environment as the cause for those changes. Yet, transcriptomic alterations in the brain were more pronounced in workers residing in parasitic nests, indicating that social parasites influence host brain activity and in turn their behaviour. Our study revealed that the exploitation of social behaviours by brood parasites is linked to transcriptomic alterations in the central and peripheral nervous system. Future work shall investigate whether and how social parasites manipulate host behaviour as our experiments cannot distinguish between active manipulation or by-products of the altered social environment.

Keywords: Social parasitism, parasite manipulation, gene expression, slavemaking ants.

Introduction

In the animal kingdom, a great variety of fascinating social behaviours evolved from mouth-breeding in cichlids, over blood-sharing in vampire bats to honeybees that signal the location of food sources to their nestmates using a symbolic dance language (Frisch, 1967; Balshine and Abate, 2021; Wilkinson, 1990). As early as 160 years ago, Darwin was fascinated by the exploitation of these social behaviours by cuckoos or socially parasitic ants, which he described and whose evolution he discussed (Darwin, 1859). Over the past decade, with advances in omics technology, the focus of research has shifted from describing novel behaviours to determining the molecular basis of known behaviours. Not only do these studies allow the identification of genes associated with specific behaviours, but the function of these genes can provide insight into the proximate mechanisms regulating these behaviours. Most of these studies examine the molecular changes in the central nervous system, i.e., the brain, which not only processes information from the sensory organs but also directly controls behaviour and thus reflects an individuals' behavioural phenotype (Robinson *et al.*, 2008). In honeybees, brain gene expression is characteristic for the behavioural caste of workers and the function of these genes can shed lights on other life aspects such as nutrition. Foragers, for example, overexpress two genes related to trehalose metabolism, the main carbohydrate storage for insects (Whitfield *et al.*, 2003). Behaviours such as learning and teaching have been extensively studied in respect to the underlying (epi)genetic basis in social insects, in particular in the honeybee (Alleman *et al.*, 2019; Eisenhardt, 2014; Biergans *et al.*, 2012), while the molecular regulation of other behaviours remains to be explored. In addition, it is known that behaviours are often influenced by the social environment in which individuals live, their diet and the presence of parasites (Cini *et al.*, 2020; Scharf *et al.*, 2021; Lihoreau *et*

al., 2018). Controlled experimental manipulations are needed to study how exactly these factors influence behaviour and its molecular underpinnings.

Social insects are ideal models for studying behaviour because they exhibit a wide range of behaviours including teaching, farming and raiding (Franks and Richardson, 2006; Chomicki and Renner, 2016; Chadab and Rettenmeyer, 1975). Furthermore, they are characterised by a reproductive division of labour in which some individuals reproduce while others selflessly take care of them and their brood. The molecular changes associated with these social behaviours, especially the division of labour, have been studied in detail in social insects, from termites to honeybees to ants (Korb, 2016; Vikram *et al.*, 2018; Ashby *et al.*, 2016). According to Hamilton's rule, this altruistic behaviour of workers should be preferentially directed towards related conspecifics, suggesting that individuals can both signal and perceive information indicating relatedness (Hamilton, 1963).

An interesting exception to this rule are the exploited host workers of insect social parasites: These workers take care of the brood, queen and adult workers of another species (Thorogood *et al.*, 2019). This exploitation of altruistic care behaviours comes with fitness costs to the hosts, and therefore they are under selection to develop defensive traits, which include better nestmate recognition and behaviours directed against invading or raiding parasites (Grüter *et al.*, 2018). If this has been ineffective and a host worker finds herself in a parasite nest, destroying parasite brood or attempting to reproduce might be strategies to rescue fitness (Foitzik *et al.*, 2001; Davies and Brooke, 1991; Achenbach and Foitzik, 2009; Achenbach *et al.*, 2010; Czechowski and Godzińska, 2015; Cini *et al.*, 2014). Social parasites, on the other hand, are selected to suppress these rebellious instincts of their host workers by preventing hosts from sensing the parasitic environment. This raises the question of how

perception in hosts within parasitic nests is altered compared to their maternal colonies and what the subsequent consequences are for their behaviour. A cross-fostering experiment in the dulotic ant *Temnothorax americanus* showed that *T. longispinosus* host workers did not differ in their aggressiveness when together with their mother queen or a socially parasitic one (Keiser *et al.*, 2015). This suggests that parasitic queens manage to go undetected. Fewer recognition substances on the cuticle might aid in acceptance of parasitic queens, workers and brood (Kleeberg *et al.*, 2017; Jongepier and Foitzik, 2016a; Kaur *et al.*, 2019). Another method to reduce host aggression could be to match the cuticular hydrocarbon (CHC) profile of the host, either by chemical mimicry, where the parasite itself synthesises host CHCs or by chemical camouflage, where CHCs are obtained from host workers during cohabitation (Tsuneoka and Akino, 2012; Kleeberg and Foitzik, 2016; Johnson *et al.*, 2001). On the other hand, *T. longispinosus* host workers were reported to exhibit novel behaviours in parasitic nests, including the killing of parasitic pupae, a behaviour described as "rebellion" (Achenbach and Foitzik, 2009; Alloway and Pesado, 1983).

In the following, we study the ant *Leptothorax acervorum*, which is a host to the obligate European dulotic ant *Harpagoxenus sublaevis.* This social parasite also regularly exploits a smaller second host species, *L. muscorum*. Even though parasitic colonies are functional, aggressive interactions such as antennal boxing and biting frequently occur in *H. sublaevis* colonies, in particular, parasite workers show dominant interactions towards their sisters and exploited host workers (Bourke, 1988). Moreover, host workers aggress hosts of different species and their social parasites indicating that they recognize to a certain extent that their social environment is different (Heinze *et al.*, 1994). One reason might be that the two host species have disparate CHC profiles impeding chemical mimicry as a strategy as it is impossible for

the parasite to match the chemical profile of both host species at once (Bauer *et al.*, 2010). Still, *H. sublaevis* parasites have been shown to carry small amounts of host-specific compounds, mostly from the smaller host species *L. muscorum* (Bauer *et al.*, 2010). Using this study system, we wanted to investigate how the social environment of a parasitic colony influences the molecular underpinnings of social behaviour of exploited workers, as well as their recognition system. If the latter would be impeded, this might aid in the functioning of parasitic colonies despite chemical dissimilarities between parasites and hosts.

We, therefore, studied the transcriptomic response of *L. acervorum* host workers that emerged inside nests of the social parasite *H. sublaevis* to shed light on the mechanisms underlying the social behaviour of host workers in parasitic nests. We investigated changes in brain gene expression, which serves as a proxy for behavioural alterations as well as antennal gene expression, which is linked to recognition abilities in ants. By transferring workers as pupae to parasitic nests – as would happen in nature during raids (Buschinger, 1983) – we rule out any transcriptional changes during larval development in a different environment and focus on changes during the first weeks of adult life as workers. We then analysed which genes change their activity to conclude the molecular regulation of the observed behaviour and the sensory system in the context of social parasitism. If host workers can indeed perceive the social environment as parasitic and distinguish it from a conspecific environment, we would expect gene expression in the antennae and brain to change, and with it behaviour in a parasite nest. If, on the other hand, the ability of the exploited hosts to recognize nestmates is somehow impaired by the parasite, we would expect antennal gene expression to not change when living in a parasitic nest. Additionally, by recording the behaviour and spatial position of the ants shortly before

sampling, we would like to gain insights into whether and how gene expression in the brain or antenna is linked to behaviour. The importance of chemical communication in social insects may explain why a recent study of honeybees found that transcriptional activity in antennae can better explain worker behaviour than gene expression in the brain (Kennedy *et al.*, 2021). Insights into what determines social insect behaviour will ultimately help in designing future experiments to unravel the molecular basis of social behaviour.

Material and Methods

Ant collection, maintenance and experimental manipulation

Colonies of the hosts *Leptothorax acervorum* and *L. muscorum* and the dulotic ant *Harpagoxenus sublaevis* were collected in July 2020 close to Nuremberg, Germany. At the University of Mainz, ants were transferred to plastered nest boxes containing artificial nest sites consisting of a Plexiglas cavity sandwiched between two microscope slides (7.5 cm x 2.5 cm x 0.5 cm) covered with a red foil and kept at 18°C with a 12:12 light: dark cycle. Ants were fed with crickets and honey 1½ times per week and provided with water ad libitum.

For our experiment, we selected ten colonies of *L. acervorum* and five colonies each of *L. muscorum* and *H. sublaevis.* Each colony was assigned to one of five replicates. We prepared experimental colonies by standardizing colony size to one queen, 15 small larvae and 60 adult workers (30 inside + 30 outside workers). Workers were marked with a thin metal wire between thorax and abdomen (0.02 mm diameter, Elektrisola, red) to allow distinguishing them from newly emerged workers. We removed 24 pupae from each *L. acervorum* colony, of which six were returned to the mother colony (referred to as "original" treatment), and six each were transferred into another *L. acervorum* colony ("conspecific" treatment), into an *L. muscorum* colony

("heterospecific" treatment) and a colony of *H. sublaevis* ("parasitic" treatment). For the *H. sublaevis* treatment, we standardized the number of host workers to 60 as described above, but additionally added all *H. sublaevis* ants (18.4 ± 13.56 individuals). In these obligate social parasites, workers do not take over worker chores such as brood care and foraging, which are outsourced to host workers. Unfortunately, worker pupae from two of our replicates did not develop into adult workers in sufficient numbers, so we focused our transcriptomic analyses on workers from the remaining three replicates. Moreover, we observed that all *L. acervorum* pupae transferred into *L. muscorum* colonies ("heterospecific" treatment) were either killed or expelled from the colony or did not emerge into workers which is why we were unable to include this treatment in our analysis. Nevertheless, the number of transferred individuals still alive at the end of the experiment did not differ between treatments (Kruskal-Wallis: $p = 0.34$), but survival and rejection of pupae were different between the heterospecific and all other treatments (**Survival**: Kruskal-Wallis $p = 0.05$; **Rejection**: Kruskal-Wallis $p < 0.001$, Wilcoxon Heterospecific-Parasitic $p = 0.016$, Wilcoxon Heterospecific-Conspecific $p = 0.012$, Wilcoxon Heterospecific-Original $p = 0.004$). Rejection was calculated as the number of pupae outside the nest over the first three days. A summary of the experimental set-up and the final replicate number is given in Figure 4-1 (more details in Supplementary Material).

Behavioural observations and sampling

About 10 weeks (63-69 days) after the emergence of the first worker, colonies were transferred to 22°C and the red foil was removed to allow workers to adapt to light. A day thereafter, the slide nest was transferred to a fluon-treated arena (3 cm x 7.5 cm) and each colony was filmed for 100 min using a SONY FDR-AX33 camera and a Leica KL1500 LED light. Ant behaviour was analyzed per scan sampling (1 scan every 2min,

30 scans) for the last 60 min of video recording (see Supplementary Material) using QuickTime Player 7.6.6. The recorded behaviours and the location were then analysed using Principal Component Analyses (PCA) separately (see Supplementary Material) using the packages FactoMineR, factoextra, missMDA from R version 4.1.2. Individual PCs, which explain at least ten percent of the variance, were extracted to be later used to correlate behaviour and location to the transcriptomic data. Directly after filming, all unmarked ants were removed with forceps, frozen in liquid nitrogen, and stored at -80°C until dissection. Dissections were performed in November 2020.

The antennae and the brain were dissected of each individual. Both tissues were separately placed into 75µl of Trizol and stored at -80°C. mRNA was extracted using the Qiagen MiniKit following instructions and samples were sent to Novogene (Cambridge, UK) for sequencing of 150 bp long paired-end reads on a NovaSeq6000.

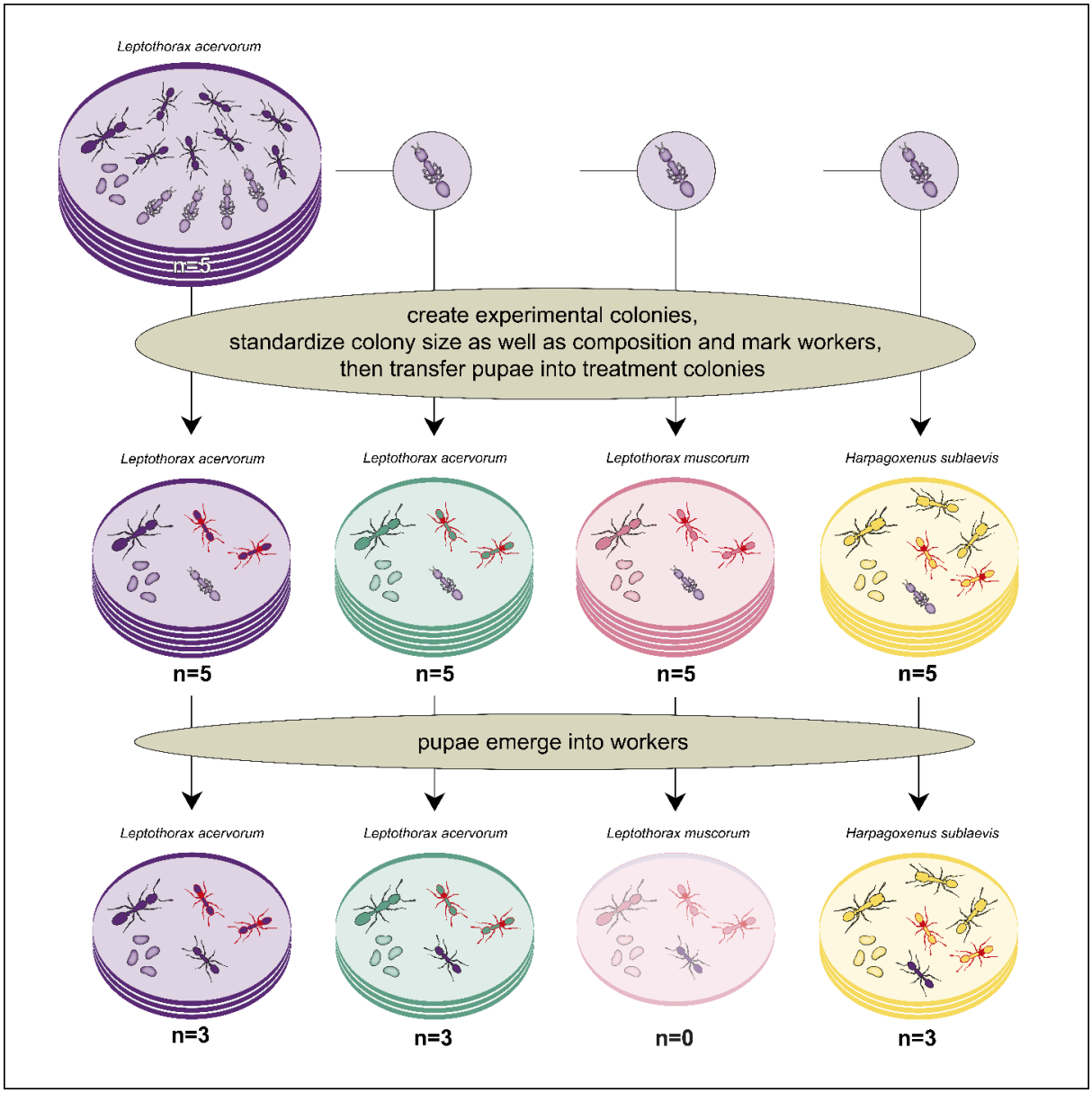

Figure 4-1 Schematic representation of the experimental design. Pupae from one *L. acervorum* colony were placed into four different experimental colonies, back into the original nest (purple), into the nest of another *L. acervorum* colony (green), into a nest of *L. muscorum* (pink) or a nest of *H. sublaevis*, the parasite (yellow). 10 weeks after the emergence of the first worker, unmarked workers were sampled from three colonies. Credit: Jenny Fuchs.

Gene expression analyses

Based on mRNA quality and quantity analyses, we decided to sequence 22 brain samples and 19 antennae samples. RNA-Seq resulted in at least 30 Mio. reads per sample for both the brain and the antennal samples. Bioinformatic analyses started by removing adapters and contaminations using FastQScreen v0.14.0 (Wingett and Andrews, 2018). Afterwards, reads were adapter- and quality-trimmed using Trimmomatic v0.39 (Bolger *et al.*, 2014) leaving at least 26 Mio. reads per sample after filtering and trimming (mean: 30.47 ± 3.05 Mio.) for the brain data and at least 27 Mio. (mean: 28.70 ± 0.65 Mio.) for the antennal data. The quality of filtered and trimmed reads was assessed using FastQC v0.11.8 (Andrews *et al.*, 2010). Reads were mapped against the genome assembly of *L. acervorum* (Jongepier *et al.*, 2022) using HISAT2 v2.1.0 (Kim *et al.*, 2015). Backmapping of the filtered and trimmed reads against the genome assembly of *L. acervorum* was above 90% for all samples (**brain**: mean: 93.29 ± 4.94 %; **antennae**: mean: 95.09 ± 0.36 %; details Table S4-1). After mapping StringTie v1.3.6 was used to create a genome-guided assembly which for the brain data consisted of 85,301 and for the antennal data of 86,415 transcripts (Pertea *et al.*, 2015). These assemblies were then filtered to only contain contigs which exceeded an open-reading frame (ORF) of 149 bp as identified by TransRate v1.0.3 (Smith-Unna *et al.*, 2016). Thus, the final genome-guided assemblies consisted of 50,616 after filtering for the brain transcriptome and 50,648 for the antennal transcriptome. Transcripts were translated to their most likely protein sequence using TransDecoder v5.5.0 (Haas *et al.*, 2013) and Gene Ontology terms were identified using InterProScan v5.51-85.0 (Jones *et al.*, 2014; Ashburner *et al.*, 2000). Thereafter, we quantified transcript and gene counts using the script prepDE.py retrieved from https://ccb.jhu.edu/software/stringtie/dl/prepDE.py (Pertea *et al.*, 2016). The following analyses were based on a gene count matrix from StringTie so that "gene" refers to

the gene model implemented in StringTie. We removed genes with less than 10 reads in at least five samples, irrespective of treatment, as they likely represent noise. The gene count matrix based on the brain data comprised 17,820 genes and 13,454 after filtering for minimum read count while the antennal gene count matrix contained 21,961 genes and 15,218 after filtering.

We used ComBat-Seq to adjust our gene count matrix for source colony ID as batch effect and treatment as the biological group (Zhang *et al.*, 2020b). We decided on this alternative approach instead of using colony ID as batch effect in DESeq2 as source colony ID was not evenly spread across all treatments. As this unbalanced design made further analyses difficult, we used whenever possible the adjusted p-values as a quality score for the reliability of differentially expressed genes (DEGs). All further analyses were performed on this adjusted count matrix.

A Principle Component Analysis (PCA) based on all genes was performed using the function plotPCA() implemented in the DESeq2 package (Love *et al.*, 2014). We used DESeq2 to model gene expression using treatment as fixed factor. Using the LRT-test, we assessed genes whose expression was more explained by the model incorporating our fixed effect. We considered all genes with an FDR-adjusted p-value below 0.05 as differentially expressed. To detect patterns across those differentially expressed genes, we used the function degPatterns from the R package DEGreport on the filtered rlog-transformed gene count matrix (Pantano, 2017). These differentially expressed transcripts were functionally annotated by using blastx v2.9.0 against the non-redundant invertebrate database (downloaded 18th March 2019) to retrieve similar proteins with an E-value below 10^{-5} (Altschul *et al.*, 1990). We used the package *topGO* to test for enrichment of GO terms in the lists of differentially expressed genes within the same cluster (Alexa and Rahnenfuhrer, 2018). Therefore, we performed a

Kolmogorov-Smirnov test using the p-value of genes as the gene score and made use of the weight01 algorithm implemented within topGO. Moreover, differentially expressed genes were annotated with their respective best BLAST hit (in respect to bit score) from their longest isoform. All of these analyses were performed in R v4.1.1 (R Core Team, 2021).

To investigate expression patterns across all genes, we performed a Weighted Gene Coexpression Network Analysis (WGCNA) using the package WGCNA (Langfelder and Horvath, 2008). For this, we used the filtered, adjusted, and variance-transformed gene count matrix to construct a scale-free network. To determine an appropriate soft-threshold power, we plotted R^2 and mean connectivity for different values and chose the one that reached an R^2 of 0.9 while having high mean connectivity (brain: 4, antennae: 7) (Zhang and Horvath, 2005). Using this soft-threshold power, we constructed a signed adjacency matrix and based on this a topological overlap matrix (TOM). Hierarchical clustering was performed using the dissimilarity defined as 1-TOM as the distance metric. We used the function cuttreeDynamic to employ a dynamic tree-cutting algorithm to identify modules with a minimum module size of 100 for the brain and 200 for the antennae. To validate our approach and choice of parameters we first visually inspected a heatmap of the TOM to check whether indeed genes that are highly correlated end up in the same module (Zhang and Horvath, 2005). We then calculated the module eigengene for each module that represents the first principal component of the expression matrix of the respective module. Thus, they can be used to summarize the expression pattern of a module. Next, we tested whether the module eigengenes correlated with the principal components explaining behaviours and locations recorded during sampling using Pearson correlation.

To be able to compare the expression of candidate genes in the respective other tissue, we ran OrthoFinder v2.5.4 to identify orthogroups between the transcriptomes in peptide sequence as identified by TransDecoder before (Emms and Kelly, 2015).

Results

Brain gene expression

RNA-Seq samples clustered in the PCA neither clearly by treatment nor by source colony ID (Fig. S4-1a). We identified 209 differently genes between treatments, which clustered into four distinct clusters (Fig. 4-2a, see Fig. 4-3 for expression patterns of candidate genes). Cluster 1 comprised 112 genes that showed a lower expression in host workers living in the parasitic environment compared to the two other treatments. Among the top 20% genes (according to adj. p-values), we identified several candidate genes with functions related to the regulation of gene expression including one encoding an RNA/RNP complex-1-interacting phosphatase, but also a gene involved in antennal development, encoding protein bric-a-brac 2, and one involved in the determination of adult lifespan in *C. elegans*, encoding F-box/SPRY domain-containing protein 1 (Hung *et al.*, 2013). Cluster 2, comprising 75 genes showed the reverse pattern with higher expression in the parasitic treatment. Again, a gene was identified as differentially expressed which encodes a protein involved in the determination of lifespan in *C. elegans*, ubiquitin modifier-activating enzyme 5 (Hertel *et al.*, 2013). The nine genes in cluster 3 showed the highest expression in the conspecific treatment, while the 13 genes in cluster 4 were highly expressed in the original treatment. Interestingly, the top DEG in cluster 3 is encoding the CLOCK protein, which is an important regulator of circadian rhythms (Matsumura *et al.*, 2014). None of the clusters of DEGs showed any significant GO enrichment with a p-value below 0.05. The

WGCNA consisted of six modules, none of which were significantly associated with treatment (Figure 4-4c) or with behaviour or spatial location the hour before sampling.

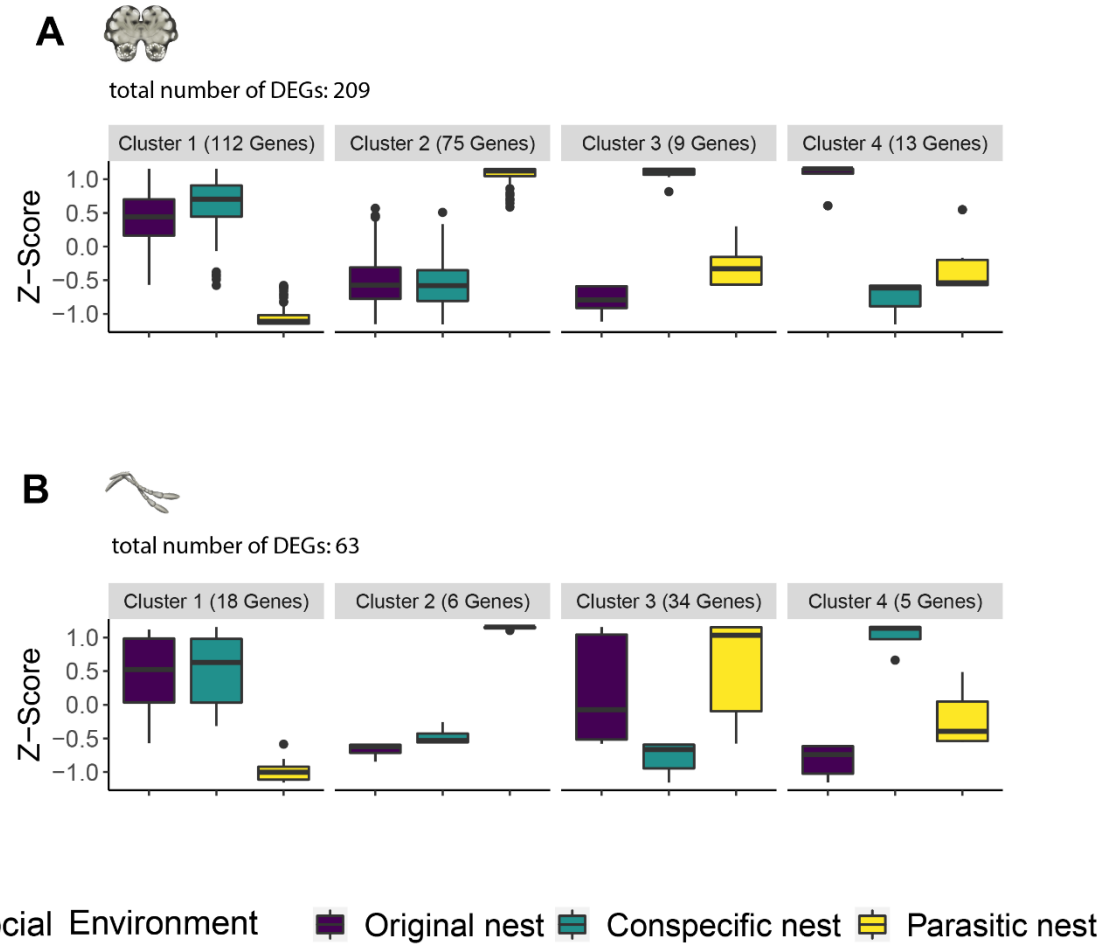

Figure 4-2 Clustering of differentially expressed genes in (**a**) the brain and (**b**) the antennae. A minimum cluster size of 5 was chosen.

Antennal gene expression

The PCA of antennae RNA-Seq samples showed neither a clear clustering by treatment nor by source colony ID (Fig. S4-1b). We identified 63 genes as differentially expressed between treatments, which could be grouped into four distinct clusters (Figure 4-2b). The largest cluster (3) comprised 34 genes and showed low expression in the conspecific treatment compared to the others. Among the five genes with the lowest adjusted p-value, we found a gene encoding C2 domain-containing protein 3-like, which is involved in membrane trafficking (Littleton, 2000). A reversed pattern of

expression was observed in cluster 4, which only comprised five genes that were lowly expressed in the conspecific treatment, one of them encoding the ejaculatory bulb-specific protein 3, an odorant-binding protein involved in broadcasting chemical signals (McKenna *et al.*, 1994). Cluster 1 and 2 comprising a total of 24 genes showed distinct high and low expression in the parasitic treatment compared to the other two, respectively. Interestingly, we again found a gene encoding a protein involved in the determination of lifespan to be expressed lowly in the parasitic treatment, lipase-3 (Fig. 4-4, Banerjee *et al.*, 2012) as well as one encoding trypsin-3 a protein involved in digestion. Again, none of these clusters showed any GO enrichment.

The coexpression network based on the antennal data consisted of 23 modules of which four were significantly linked to the first two PCs explaining behaviour or location (Figure 4-4d). Of special interest was the yellow module since its expression positively correlated with PC2 for both behaviour and location and negatively with PC1 for behaviour. This suggests that these genes are highly expressed in individuals, which more often perform walking and trophallaxis and antennate other workers while being away from the brood pile (Figure 4-4a and b). In general, the association of the expression (as defined by the module eigengene) correlated strongly with the module membership of genes in this module, meaning that genes important for the respective trait (PC here) are more central and connected to other genes within the module (Suppl. Figure S4-2). Moreover, this module comprised in total six DEGs, which showed higher mean connectivity compared to the other genes within the module (Wilcoxon $p < 0.001$). Only two of them had a BLAST hit in the invertebrate database, one, encoding the sperm flagellar protein 1-like, which was previously shown to be uniquely expressed in heads of foragers and not in nurses of *Apis cerana cerana* (Chen *et al.*, 2021). This module is strongly enriched for “transmembrane transport” and

(amongst others) “transforming growth factor beta receptor signalling pathway” (Suppl. Figure S4-2).

The other interesting module, tan, showed the exact reverse pattern, being negatively correlated with PC2 for both behaviours and location and positively with PC1 for behaviour. Therefore, high expression of these genes is found in individuals that groom or rest and are rather close to the brood. This module is enriched for terms involved in carbohydrate processing including “galactose catabolic process via UDP-galactose”, “methylglyoxal catabolic process to D-lactate via S-lactoyl-glutathione” and “pentose-phosphate shunt, non-oxidative branch”. Again, we found a strong correlation between gene significance for the respective PCs and the module membership of the genes (Suppl. Figure S4-3). This module comprised a total of eleven DEGs, which is more than expected by chance (Fisher’s Exact Test $p < 0.001$), which again were more connected compared to the other genes in this module (Wilcoxon $p < 0.001$). Amongst the differentially expressed genes in this module were two, which were already mentioned above as being differentially expressed with the lowest expression in the parasitic treatment: one encoding a lipase-3 and another encoding trypsin-3.

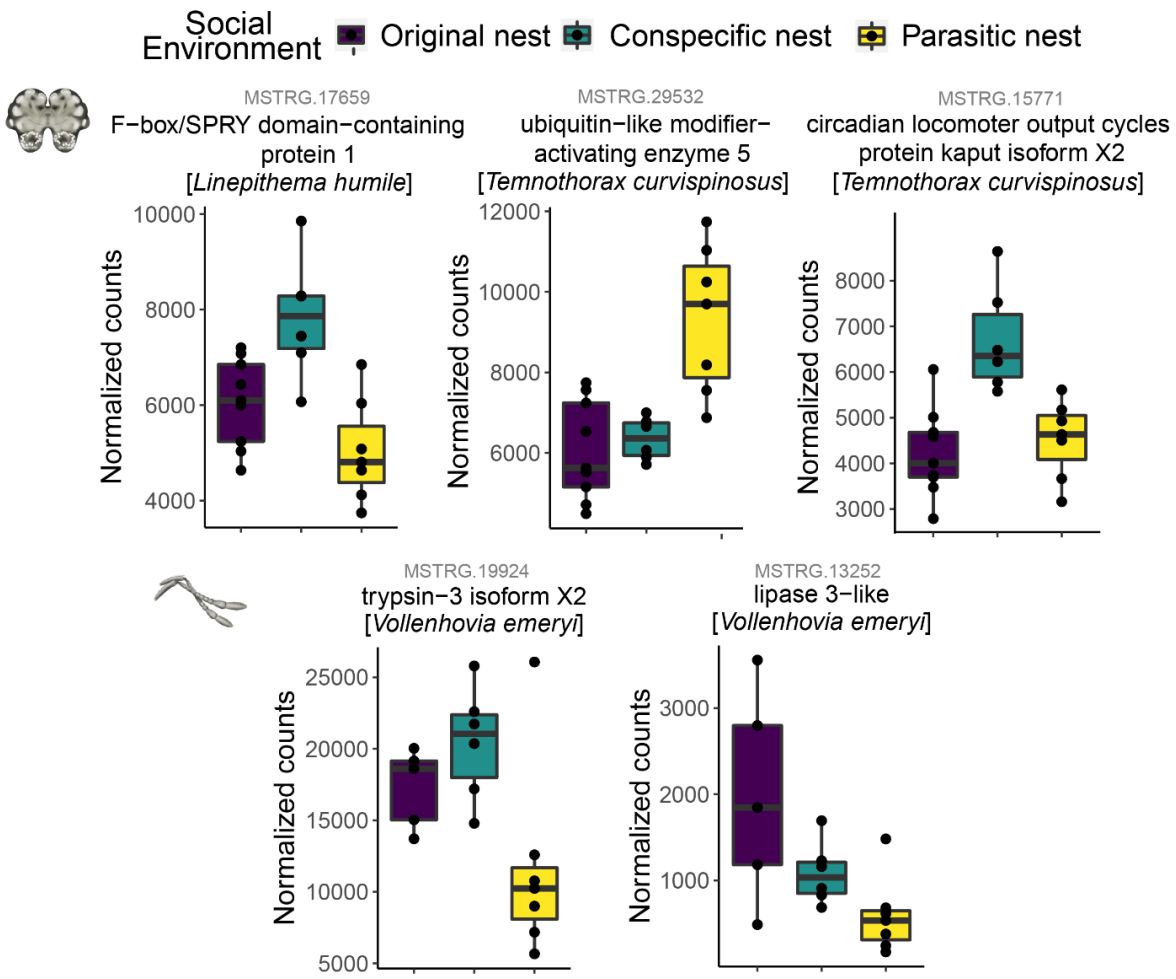

Figure 4-3 Expression of candidate genes in the brain (first row) and the antennae (second row).

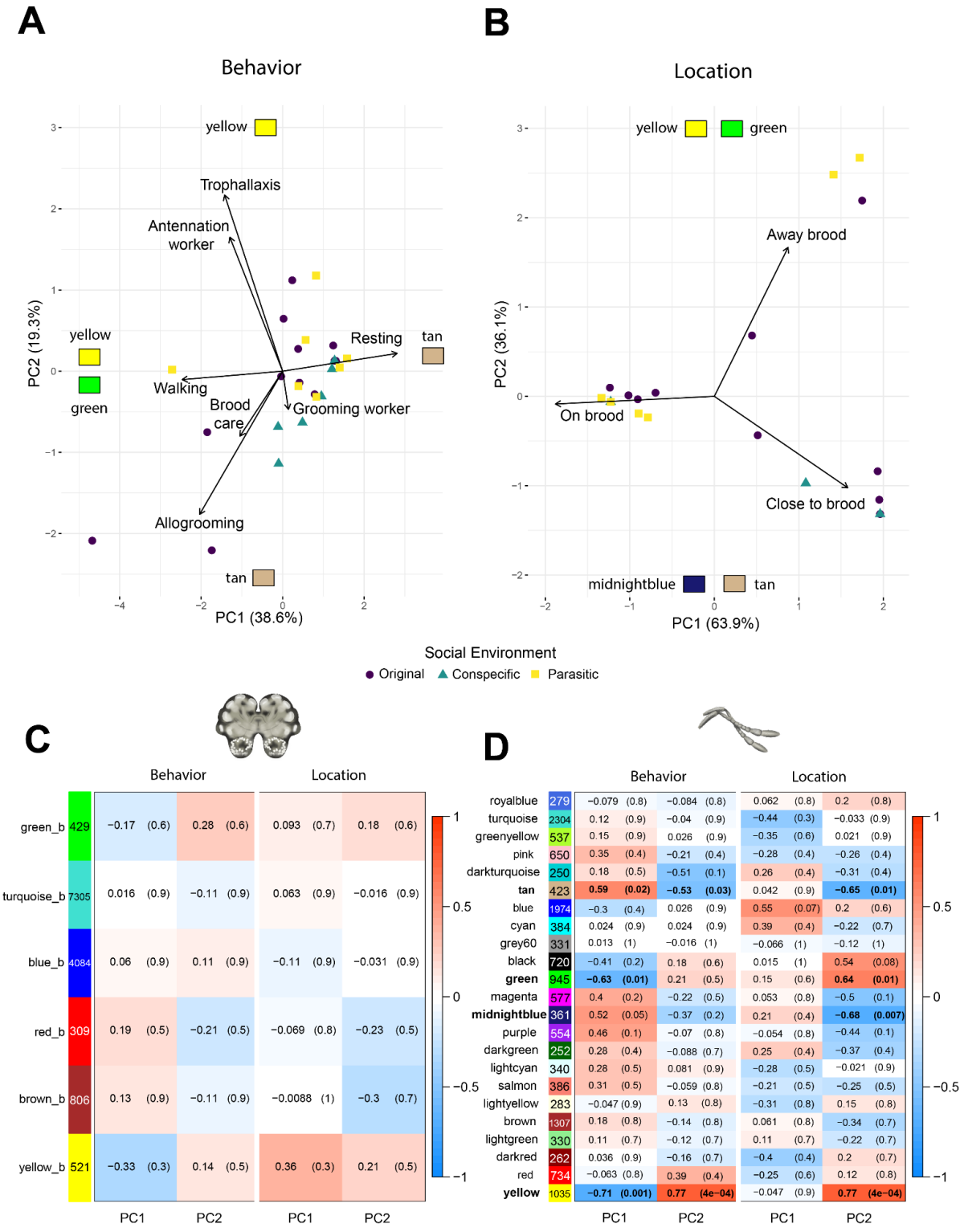

Figure 4-4 Correlation of recorded behaviours and gene expression modules. **A-B**: Principal Component Analysis of behaviour (**A**) and location (**B**) recorded during the 60 minutes before workers were sampled. Arrows represent correlations between variables and principal components. **C-D**: Correlation heatmap depicting correlation and p-values of Pearson correlation between PC values and module eigengenes in the brain (**C**) and the antennae (**D**). Modules with a p-value below 0.05 are printed in bold and the number of genes in each module is given within the coloured box next to the module name. Significant modules are also depicted in the respective PCA plot to illustrate the direction of the correlation between principal component and module eigengene.

Discussion

We combined transcriptome data from brains and antennae of host workers living in different social environments, including parasitic nests, with behavioural data to elucidate the molecular mechanisms that facilitate the maintenance of parasitic colonies and the gene networks responsible for the behaviours performed. We transferred *L. acervorum* workers as pupae to their parent nest, a conspecific nest or a parasite nest, as would be the case in nature during raiding. We then analysed the gene expression of the adult workers 10 weeks after their emergence. Thus, all the changes observed in our experiment indicate plastic responses in adult workers. By controlling for factors such as colony size and age of the workers, our study was able to uncover transcriptomic shifts related to the social environment in which the workers live. Indeed, we identified genes, whose expression changes when adult workers live with social parasites, three times as many genes in the brain than in the antennae, where gene expression deviates more in ants living in a foreign colony of the same species. This suggests that recognition of non-relatives by the host within the parasite nest is somehow impaired. Furthermore, we have identified modules of genes in the antennae that change their expression depending on the behaviours exhibited by the workers an hour earlier. Thereby we can additionally identify gene networks whose expression changes in workers exhibiting different behaviours regardless of treatment.

Brain and antennal transcriptomes shift in parasitic nests

Comparing gene expression between the treatments revealed that gene activity differed in the brains of workers living in a parasitic environment or a conspecific one. Thus, living with social parasites is different not only from living in the mother colony but also from life in an unrelated colony of the same species in the expression of a set of genes in both brain and antennae. Similarly, changes in brain gene expression have

been found in the parasitic wasp-host system of *Polistes sulcifer* and *Polistes dominula*, where transcriptomes of workers living in parasitized colonies differed from those in unparasitized wasp nests (Cini *et al.*, 2020). We found the brain transcriptomes to be more affected by the parasitic treatment (as evidenced by the number of differentially expressed genes) than the antennal ones. This might be explained by differential investment into brain regions in ants living in parasitized nests as it was previously shown for socially isolated ants in *Camponotus floridanus* (Seid and Junge, 2016). Indeed, the highest number of differentially expressed genes in the brain was downregulated in the parasitic treatment compared to the other two, supporting this hypothesis. Still, more detailed neuroanatomical experiments are needed to confirm that brain gene activity is lower in ants exploited by a social parasite. Another possible explanation for this finding might be that nestmate recognition is suppressed in parasitic nests, resulting in an antenna gene expression more similar to the control ("original") treatment. As antennae and antennal lobes are the main organs involved in nestmate recognition (Ozaki *et al.*, 2005; Stroeymeyt *et al.*, 2010), this might lead to lower discrimination abilities of hosts and thus allow for the successful maintenance of parasitic nests. Generally, in host-parasite systems, the hosts usually react strongly aggressive to social parasites, while inside the parasitic nest aggression is lower (Pamminger *et al.*, 2011). As the cooperation of host workers is essential for the functioning of the parasitic colony, parasites are under strong selection to evolve mechanisms suppressing host aggression. A previous cross-fostering experiment with a different social parasite-host system has already revealed that aggression of host workers transferred as pupae did not differ between those put back to their maternal queen or together with a parasitic one but was elevated in the presence of a conspecific queen (Keiser *et al.*, 2015). Suppression of antennal gene expression might

be one mechanism allowing for this, but how exactly this is achieved by the parasite remains unclear.

Candidate genes associated with living in a parasitic nest

We identified several genes in both tissues whose expression was divergent in the parasitic treatment compared to both the original and the conspecific treatment. Among the differentially expressed genes, we found multiple ones related to longevity both in the antennal and the brain transcriptomes (see Supplementary Material). For example, one gene encoding a ubiquitin-like modifier-activating enzyme 5 (Hertel *et al.*, 2013) was found to be highly expressed in the brains of *L. acervorum* workers inside parasitic nests while another one, encoding the F-box/SPRY domain-containing protein 1 (Hung *et al.*, 2013), showed low expression in the same treatment. Similarly, we found a gene encoding a lipase-3, also known to function in lifespan regulation (Banerjee *et al.*, 2012), to be expressed lowly in antennal transcriptomes of the parasitic treatment. Together these results suggest that longevity might be altered when *L. acervorum* workers live inside the parasitic nests of *H. sublaevis*. Lifespan genes were shown to be influenced in the brain by changes in the social environment for example in *Solenopsis* queens depending on whether they were singly-founding or group-founding (Manfredini *et al.*, 2021). This highlights the interplay between social environment and individual life-history traits such as longevity. We propose different reasons for a differential expression of longevity genes in parasitized workers: 1) The differential regulation of genes related to lifespan in parasitized individuals might be due to stress they experience in the parasitic nest. This could be induced either by the presence of the parasites themselves or via other stressors such as nutritional availability. 2) Moreover, the situation inside the parasitic nest could reflect a situation of queen loss for the host as no conspecific queen is available inside the nest. In other

systems, it has been shown that queen loss induces worker fertility and increases their lifespan (Majoe *et al.*, 2021; Giehr *et al.*, 2020; Monnin and Peeters, 1999). 3) Similarly, in the system of *Polistes sulcifer – Polistes dominula*, hosts are known to become fertile when parasitized but not when living with a conspecific but unrelated queen (Monnin *et al.*, 2009). The tight connection between fertility and longevity thus could explain our findings, even though the direction of change is unclear since in *Bombus terrestris* more fertile individuals were longer-lived while experimental induction of fertility actually shortened lifespan (Blacher *et al.*, 2017). Whether indeed hosts inside parasitic nests show a different lifespan compared to hosts living inside their source colony should be investigated in a long-term study with higher sample size.

Interestingly, a gene we found to be highly expressed in the brains of the conspecific treatment was encoding the circadian locomotor output cycles protein kaput (CLOCK), a protein known to be a key player in regulating circadian rhythm (Bell-Pedersen *et al.*, 2005). Previously it was also found to be under positive selection in social parasites of the genus *Temnothorax* compared to their hosts (Feldmeyer *et al.*, 2017). Another gene involved in the circadian rhythm, *period*, is strongly affected by the task performed in both honeybees and harvester ants (Bloch *et al.*, 2001; Ingram *et al.*, 2009). As the expression of CLOCK was similar between the original and the parasitic treatment, this suggests that the activity patterns in parasitized workers are not altered although their parasitic nestmates stay mostly inside the nest outside of raiding season.

Antennal gene expression is a strong predictor of behaviour

Behavioural observations allowed us to connect principal components of behaviour and spatial location to the brain and antennal gene expression of our focal workers. While brain gene expression showed no correlation to both behaviour and location PCs, we identified strong links to gene modules in the antennae, however, the antennal

network consisted of more modules in general. One of the antennal modules, whose expression was correlated to several PCs was also found to contain a high number of DEGs, all except one from cluster 1 with low expression in the parasitic treatment bridging the results of the differential gene expression and the network analysis. Interestingly, the enriched functions of this module include those involved in carbohydrate metabolism. Moreover, several terms suggest that the contained genes must be important for the maintenance of genomic integrity, including base-excision repair, chromatin assembly or disassembly, and the ubiquitin-dependent ERAD pathway which is required for protein degradation. As the genes of the tan module are positively correlated with being active, they might represent genes upregulated in inactive workers. These workers have previously been described as walking slowly, being located close to the centre of the nest, and being more corpulent in *Temnothorax rugatulus* (Charbonneau *et al.*, 2017). As the DEGs in the tan module showed low expression in the parasitic treatment this would suggest that host workers inside the parasitic nest spend more time being active and away from the brood than workers inside *L. acervorum* colonies. Triggers that usually signal the workers which tasks to perform might not be present in the parasitic nest in an intensity that is sufficient to induce the respective behaviour, including taking care of the parasitic brood. In the cuckoo, chicks have been shown to use a hypersignal to be fed by their host by mimicking a whole brood of host chicks to ensure feeding by the host (Davies *et al.*, 1998). Selection usually acts to direct the altruistic behaviour of hosts towards their siblings/nestmates and hypersignalling represents one possible strategy of parasites to cope with this. How exactly social parasites signal their hosts their needs and whether this signalling fails under certain conditions, remains to be investigated.

Outlook

Our results suggest that the recognition and processing of adult ants inside parasitic nests are altered probably in a way that facilitates the coexistence of two or more species inside the same nest. Using our experimental design, we were not able to disentangle whether these changes are induced via possible manipulation by the parasite or whether they are the result of different dynamics within the parasitic nest compared to conspecific nests. Further experiments are needed to test whether indeed the parasites use chemical substances to manipulate their hosts, including proteomic analyses of substances present on host heads. As another follow-up experiment, we propose to perform a time-series experiment to further investigate the establishment of parasitic colonies when new hosts are added. Together with more behavioural observations and a larger sample size, this will allow us to deeply investigate the changes in the behavioural network as well as changes in the reproductive potential of host workers (Cini *et al.*, 2020).

Acknowledgements

[removed for privacy purposes]

Author Contributions

M.S., E.C. and S.F. designed the experimental set-up and collected the ant colonies. M.S. and E.C. set up the experimental colonies and sampled workers. E.C. and M.N.M. performed dissections and E.C. performed RNA extractions. E.C. analysed behavioural

data. M.S. with the assistance of E.C. analysed the gene expression data. M.S. wrote a first draft of the manuscript and all authors revised it.

Supplementary Materials

The gene counts matrices, lists of DEGs, GO enrichment results, WGCNA and PCA results are available on Seafile for committee members. The following supplementary information will be published together with the manuscript:

Material and Methods

Ant collection, maintenance and experimental manipulation

Colonies of the hosts *Leptothorax acervorum* and *L. muscorum* and the dulotic ant *Harpagoxenus sublaevis* were collected in July 2020 close to Nuremberg, Germany. At the University of Mainz, ants were transferred to plastered nest boxes containing artificial nest sites consisting of a Plexiglas cavity sandwiched between two microscope slides (7.5 cm x 2.5 cm x 0.5 cm) covered with a red foil and kept at 18°C with a 12:12 light: dark cycle. Ants were fed with crickets and honey 1½ times per week and provided with water ad libitum.

Experimental manipulation of colonies

The experiment started on the 5th of Aug 2020 by preparing experimental colonies. For this purpose, we selected in total ten colonies of *L. acervorum*, five colonies of *L. muscorum* as well as five colonies of *H. sublaevis,* and each colony was assigned to one of five replicates. Subsequently, each replicate was processed by starting with one of the *L. acervorum* colonies and marking 30 adult workers with red metal wire (0.02 mm diameter, Elektrisola, red) without anaesthesia which were then placed into a new nest box containing a fresh slide nest together with the queen. This helped to distinguish old from newly emerged workers after the experimental period. To keep colony composition as natural as possible we first marked and transferred 15 ants that

were outside of the nest or which moved outside after little disturbance and afterwards transferred the same number of ants to the top of the brood pile. Moreover, we added 15 small brood items to the new experimental colony. Additionally, from this first colony per replicate, 24 pupae were removed and depleted with large larvae in case, not enough pupae were present in the nest. Six of those pupae were transferred into the new experimental colony which originated from the same colony (from now on referred to as "original" treatment). We repeated the procedure described above for the other *L. acervorum* colony of the same replicate, but instead transferred six pupae from the "original" colony into this new experimental colony (from now on referred to as "conspecific" treatment) as well as for the *L. muscorum* colony (from now on referred to as "heterospecific" treatment). For the *H. sublaevis* colony, we standardized the number of host workers as described above and additionally added all ants of *H. sublaevis* (18.4 ± 13.56 individuals), as these do not contribute to the usual colony chores and thus do not represent "real" workers (from now on referred to as "parasitic"). Afterwards, the remaining replicates were treated the same way. All instruments were cleaned with ethanol between colonies to avoid any transfer of cues from one colony to another which might influence the acceptance of workers and brood items in the new experimental colonies. During the first two days after marking, dead marked ants were removed from the colony as those probably died of complications of the marking itself and new ants were marked and added to the respective colony. Moreover, we checked the acceptance of the pupae after one day and if pupae were still outside the slide nest, we placed them into the nest entrance. On the next day, if pupae were still not accepted, we removed them from the nest. Colonies were observed every day and the number of marked and unmarked individuals was noted down as well as the number of dead ants (which were removed afterwards).

To make sure that pupae which were transferred into the experimental colonies would be of similar age at the end of the experimental period, we removed all non-hatched pupae from the nests two weeks after the set-up of the experimental colonies. For replicate 2 and 4, none of the brood items had emerged until then, which is why in the remaining manuscript we analysed replicates 1, 3, and 5. Moreover, we observed that all *L. acervorum* pupae transferred into *L. muscorum* colonies ("heterospecific" treatment) were either killed or expelled from the colony or did not emerge into workers which is why we were not able to include this treatment in our analysis. The number of transferred individuals still alive at the end of the experimental period did not differ between the treatments (Kruskal-Wallis: $p = 0.34$), but acceptance of pupae was significantly different between the heterospecific and all other treatments (**Survival**: Kruskal-Wallis $p = 0.05$; **Acceptance**: Kruskal-Wallis $p < 0.001$, Wilcoxon Heterospecific-Parasitic $p = 0.016$, Wilcoxon Heterospecific-Conspecific $p = 0.012$, Wilcoxon Heterospecific-Original $p = 0.004$). It must be noted that acceptance here was calculated as the number of pupae outside the nest over the first three days.

Behavioural observations and sampling

About 10 weeks (69 days) after the emergence of the first worker, colonies were transferred to 22°C and the red foil was removed to allow workers to adapt to light. A day thereafter, the slide nest was transferred to a fluon-treated arena (3 cm x 7.5 cm) and each colony was filmed for 100 min using a SONY FDR-AX33 camera and a Leica KL1500 LED light. Directly after filming, all non-marked ants were removed with forceps, frozen in liquid nitrogen, and stored at -80°C until dissection. Ant behaviour was analyzed per scan sampling (1 scan every 2min, 30 scans) for the last 60 min of the video recording (see Supplementary Material) using QuickTime Player 7.6.6. Dissections were performed in November 2020. The antennae and the brain were dissected of each individual. Both tissues were separately placed into 75µl of Trizol and

stored at -80°C. mRNA was extracted using the Qiagen MiniKit following instructions and samples were sent to Novogene (Cambridge, UK) for sequencing of 150 bp long paired-end reads on a NovaSeq6000.

Gene expression analyses

Based on mRNA quality and quantity analyses, we decided to sequence 22 brain samples and 19 antennae samples. RNA-Seq resulted in at least 30 Mio. reads per sample for both the brain and the antennal samples. Bioinformatic analyses started by removing adapters using FastQScreen v0.14.0 (Wingett and Andrews, 2018) and raw reads mapping against *Homo sapiens*, *Escherichia coli* or vectors (retrieved from http://www.ncbi.nlm.nih.gov/VecScreen/UniVec_Core.html). Afterwards, reads were adapter- and quality-trimmed using Trimmomatic v0.39 (Bolger *et al.*, 2014) leaving at least 26 Mio. reads per sample after filtering and trimming (mean: 30.47 ± 3.05 Mio) for the brain data and at least 27 Mio. (mean: 28.70 ± 0.65 Mio) for the antennal data. The quality of filtered and trimmed reads was assessed using FastQC v0.11.8 (Andrews *et al.*, 2010). Reads were mapped against the genome assembly of *L. acervorum* (Jongepier *et al.*, 2022) using HISAT2 v2.1.0 specifying --dta as a non-default parameter (backmapping rates: Table S1) (Kim *et al.*, 2015). Backmapping of the filtered and trimmed reads against the genome assembly of *L. acervorum* was above 90% for all samples (**brain**: mean: 93.29 ± 4.94 %; **antennae**: mean: 95.09 ± 0.36 %). The resulting SAM files were converted into BAM format using Samtools v1.9, sorted after coordinates (Li *et al.*, 2009) and used as input for StringTie v1.3.6 to create a GTF file for each sample (Pertea *et al.*, 2015). These were subsequently merged and the corresponding transcripts were extracted from the genome assembly using gffread v0.11.4 resulting in 85,301 transcripts (brain) and 86,415 (antennae) respectively (Pertea and Pertea, 2020). We ran TransRate v1.0.3 to identify those transcripts for which the open-reading frame (ORF) exceeded 149 bp (Smith-Unna *et al.*, 2016).

Transcripts were translated to their most likely protein sequence using TransDecoder v5.5.0 (Haas *et al.*, 2013) and used as input for InterProScan v5.51-85.0 (Jones *et al.*, 2014) to retrieve functional annotations including Gene Ontology (GO) terms (Ashburner *et al.*, 2000). To remove any newly identified transcripts, we removed GTF files with an ORF of less than 150 bp length. The final genome-guided assembly consisted of 50,616 after filtering for the brain transcriptome and 50,648 for the antennal transcriptome. Thereafter, we quantified transcript and gene counts using the script prepDE.py retrieved from https://ccb.jhu.edu/software/stringtie/dl/prepDE.py (Pertea *et al.*, 2016). The following analyses were based on a gene count matrix from StringTie so that "gene" refers to the gene model implemented in StringTie. We removed genes with less than 10 reads in at least five samples, irrespective of treatment, as they likely represent noise. The gene count matrix based on the brain data comprised 17,820 genes and 13,454 after filtering for minimum read count while the antennal gene count matrix contained 21,961 genes and 15,218 after filtering.

We used ComBat-Seq to adjust our gene count matrix for source colony ID as batch effect and treatment as the biological group (Zhang *et al.*, 2020b). We decided on this alternative approach instead of using colony ID as batch effect in DESeq2 as source colony ID was not evenly spread across all treatments. As this unbalanced design made further analyses difficult, we used whenever possible the adjusted p-values as a quality score for the reliability of the differentially expressed gene (DEG). All further analyses were performed on this adjusted count matrix.

A Principal Component Analysis (PCA) based on all genes was performed using the function plotPCA() implemented in the DESeq2 package (Love *et al.*, 2014). We used DESeq2 to model gene expression using treatment as fixed factor. Using the LRT-test, we assessed genes whose expression was more explained by the model incorporating

our fixed effect. We considered all genes with an FDR-adjusted p-value below 0.05 as differentially expressed. To detect patterns across those differentially expressed genes, we used the function degPatterns from the R package DEGreport on the filtered rlog-transformed gene count matrix (Pantano, 2017). These differentially expressed transcripts were functionally annotated by using blastx v2.9.0 against the non-redundant invertebrate database (downloaded 18th March 2019) to retrieve similar proteins with an E-value below 10^{-5} (Altschul *et al.*, 1990). We used the package topGO to test for enrichment of GO terms in the lists of differentially expressed genes within the same cluster (Alexa and Rahnenfuhrer, 2018). Therefore, we performed a Kolmogorov-Smirnov test using the p-value of genes as the gene score and made use of the weight01 algorithm implemented within topGO. Moreover, genes were annotated with their respective best BLAST hit (in respect to bit score) from their longest isoform. All of these analyses were performed in R v4.1.1 (R Core Team, 2021).

To investigate expression patterns across all genes, we performed a Weighted Gene Coexpression Network Analysis (WGCNA) using the package WGCNA (Langfelder and Horvath, 2008). For this, we used the filtered, adjusted, and variance-transformed gene count matrix to construct a scale-free network. To determine an appropriate soft-threshold power, we plotted R^2 and mean connectivity for different values and chose the one that reached an R^2 of 0.9 while having high mean connectivity (=4) (Zhang and Horvath, 2005). Using this soft-threshold power, we constructed a signed adjacency matrix and based on this a topological overlap matrix (TOM). Hierarchical clustering was performed using the dissimilarity defined as 1-TOM as the distance metric. We used the function cuttreeDynamic to employ a dynamic tree-cutting algorithm to identify modules with a minimum module size of 100. To validate our approach and choice of parameters we first visually inspected a heatmap of the TOM to check

whether indeed genes that are highly correlated end up in the same module (Zhang and Horvath, 2005). Moreover, we performed a GO enrichment analysis on the modules using Fisher's exact test to determine whether they indeed represent biologically relevant clusters of genes. We then calculated the module eigengene for each module that represents the first principal component of the expression matrix of the respective module. Thus, they can be used to summarize the expression pattern of a module. Next, we tested whether the module eigengenes can be explained by our treatments using a Kruskal-Wallis test followed by a Wilcoxon test or by behaviours and locations recorded during sampling using Pearson correlation. We tested whether any of our identified modules contained more of the differentially expressed genes than expected by chance using Fisher's exact test.

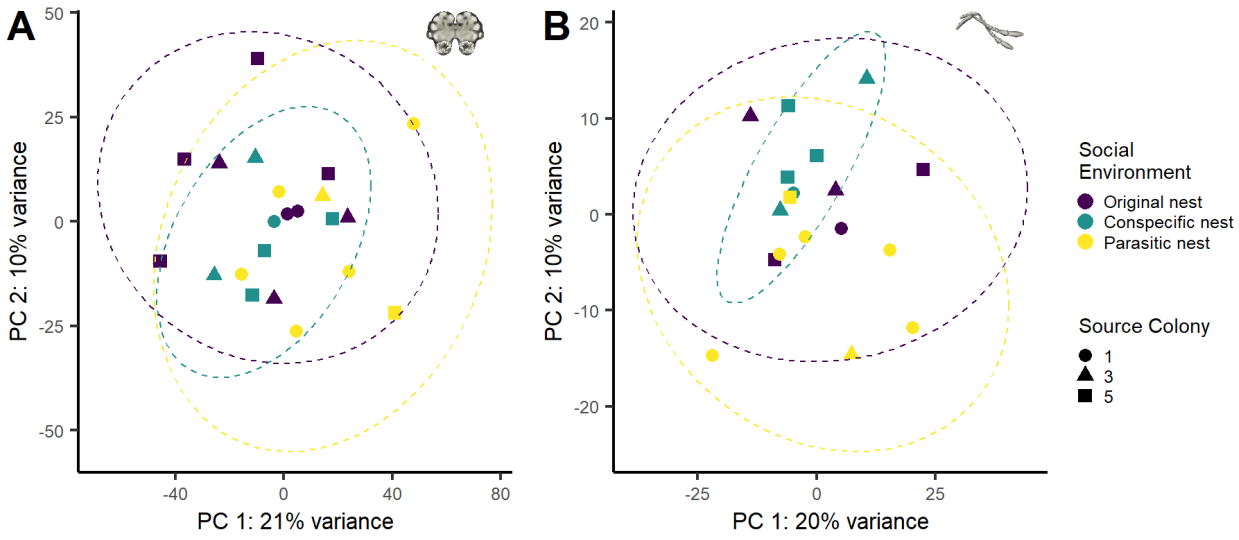

Figure S4-1 Principal Component Analysis of (**A**) brain transcriptomes and (**B**) antennal transcriptomes.

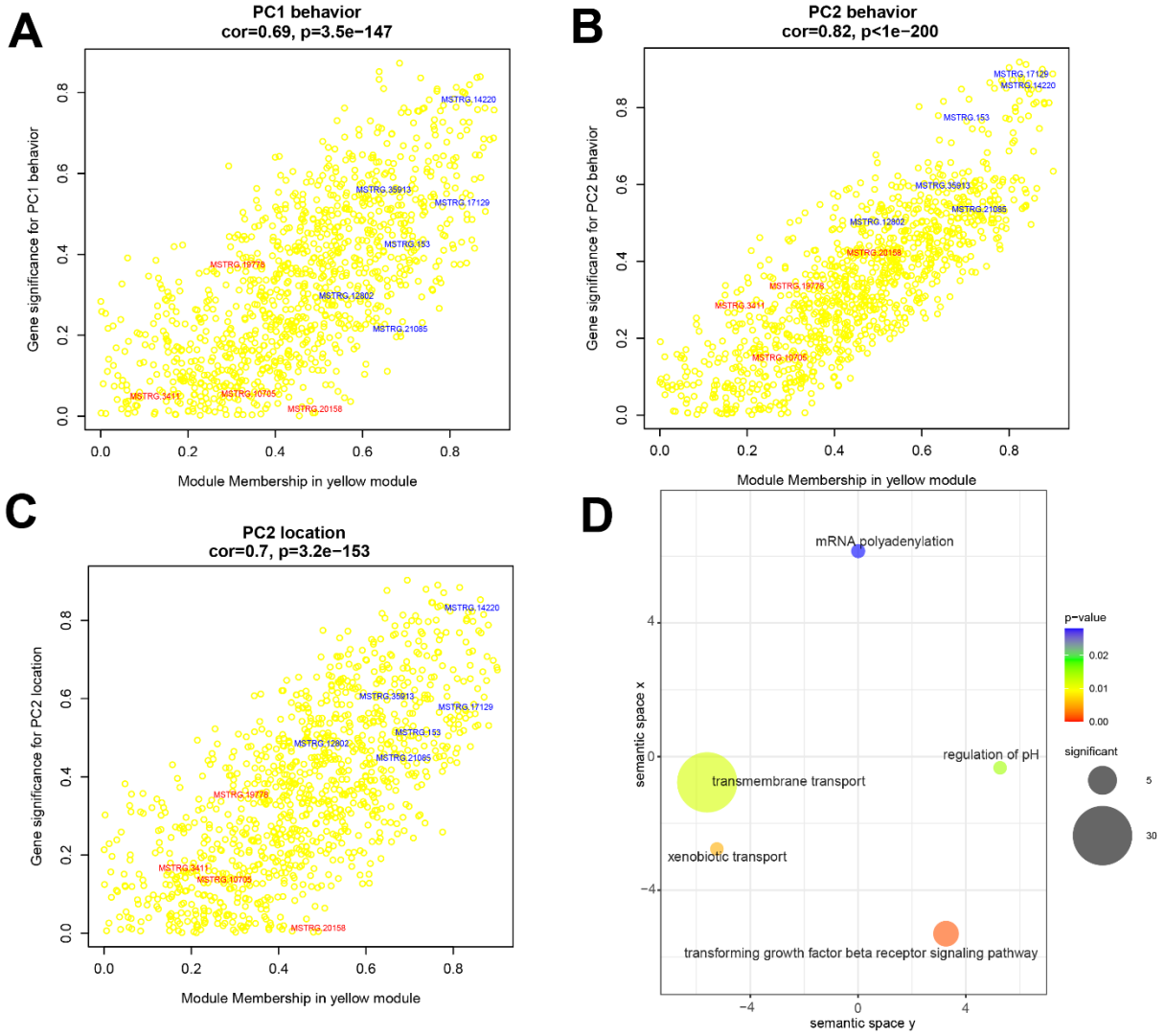

Figure S4-2 Correlations in the yellow module between the module membership and the gene significance between the module eigengene and (**A**) PC1 of the behavioural data, (**B**) PC2 of the behavioural data and (**C**) PC2 of the location data. Differentially expressed genes within the module are labelled using blue font while odorant receptors are labelled in red. (**D**) Graphical representation of the GO terms enriched within the yellow module with $p < 0.05$ within the two-dimensional semantic space. Colours of circles correspond to the p-value while size corresponds to the number of genes with the respective GO annotation.

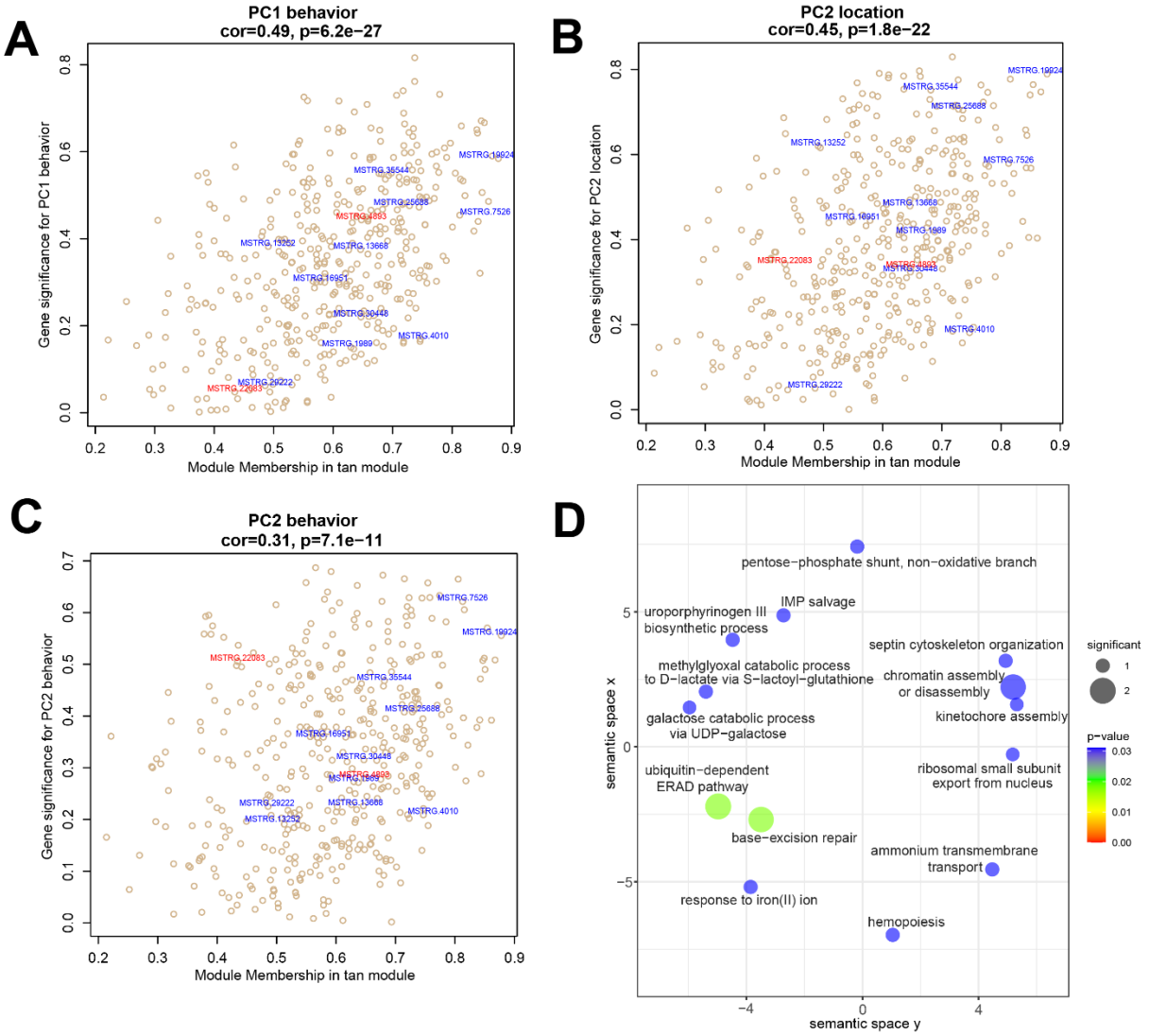

Figure S4-3 Correlations in the tan module between the module membership and the gene significance between the module eigengene and (**A**) PC1 of the behavioural data, (**B**) PC2 of the behavioural data and (**C**) PC2 of the location data. Differentially expressed genes within the module are labelled using blue font while odorant receptors are labelled in red. (**D**) Graphical representation of the GO terms enriched within the tan module with $p < 0.05$ within the two-dimensional semantic space. Colours of circles correspond to the p-value while size corresponds to the number of genes with the respective GO annotation.

Table S4-2 Number of reads in antennal and brain samples after each quality control step within the bioinformatic pipeline.

Sample	Tissue	Read orientation	Raw reads	Filtered reads	Filtered and trimmed reads	Percent reads remaining
C11b	Brain	forward	35488522	34264961	32796431	92.4141924
C11b	Brain	reverse	35488522	34262287	32796431	92.4141924
C31b	Brain	forward	31752497	31261309	29750171	93.69395736
C31b	Brain	reverse	31752497	31259134	29750171	93.69395736
C33b	Brain	forward	33010634	32161384	30720619	93.06279607
C33b	Brain	reverse	33010634	32174621	30720619	93.06279607
C51b	Brain	forward	47556188	42672684	39607708	83.28612882
C51b	Brain	reverse	47556188	42793001	39607708	83.28612882
C52b	Brain	forward	31342468	28849830	26251349	83.75648338
C52b	Brain	reverse	31342468	28942352	26251349	83.75648338
C55b	Brain	forward	30875312	30239801	29022194	93.99805903
C55b	Brain	reverse	30875312	30241930	29022194	93.99805903
O14b	Brain	forward	33499354	32546949	31010856	92.57150451
O14b	Brain	reverse	33499354	32550954	31010856	92.57150451
O15b	Brain	forward	31781295	31300548	29730881	93.54836233
O15b	Brain	reverse	31781295	31295017	29730881	93.54836233
O31b	Brain	forward	31964573	31562420	29979068	93.78842007
O31b	Brain	reverse	31964573	31558549	29979068	93.78842007
O32b	Brain	forward	33216325	32691117	30990795	93.29989094
O32b	Brain	reverse	33216325	32694656	30990795	93.29989094
O35b	Brain	forward	31833180	30703431	29328348	92.1313799
O35b	Brain	reverse	31833180	30718339	29328348	92.1313799

O51b	Brain	forward	32963707	31859964	30264495	91.81156415
O51b	Brain	reverse	32963707	31882026	30264495	91.81156415
O52b	Brain	forward	30486729	29909926	27223109	89.29494863
O52b	Brain	reverse	30486729	29934856	27223109	89.29494863
O54b	Brain	forward	30753323	29627340	27088304	88.08252689
O54b	Brain	reverse	30753323	29656301	27088304	88.08252689
O55b	Brain	forward	31108289	30736616	29224418	93.94415103
O55b	Brain	reverse	31108289	30729784	29224418	93.94415103
S12b	Brain	forward	32213918	31534248	30019540	93.18810584
S12b	Brain	reverse	32213918	31541883	30019540	93.18810584
S13b	Brain	forward	30643025	30194243	28580846	93.27031519
S13b	Brain	reverse	30643025	30203326	28580846	93.27031519
S14b	Brain	forward	33353635	32455529	31177864	93.47666004
S14b	Brain	reverse	33353635	32473579	31177864	93.47666004
S15b	Brain	forward	32601376	32170555	30494816	93.53843224
S15b	Brain	reverse	32601376	32175770	30494816	93.53843224
S16b	Brain	forward	32805218	31520717	30161534	91.94126983
S16b	Brain	reverse	32805218	31538399	30161534	91.94126983
S32b	Brain	forward	31142119	30702450	28928381	92.89149849
S32b	Brain	reverse	31142119	30702467	28928381	92.89149849
S51b	Brain	forward	44182917	41020024	38097724	86.22727196
S51b	Brain	reverse	44182917	41080477	38097724	86.22727196
C11A	Antennae	forward	29819813	29409634	27758145	93.08624772
C11A	Antennae	reverse	29819813	29418419	27758145	93.08624772
C31A	Antennae	forward	31611249	31227507	29763013	94.15323324
C31A	Antennae	reverse	31611249	31227721	29763013	94.15323324
C33A	Antennae	forward	30312100	29721513	28050053	92.53747843

C33A	Antennae	reverse	30312100	29718499	28050053	92.53747843
C51A	Antennae	forward	30241713	29585067	28062809	92.79503777
C51A	Antennae	reverse	30241713	29577446	28062809	92.79503777
C52A	Antennae	forward	29688359	29272185	27836010	93.76068916
C52A	Antennae	reverse	29688359	29280756	27836010	93.76068916
C55A	Antennae	forward	30743186	30240124	28679733	93.28809643
C55A	Antennae	reverse	30743186	30235252	28679733	93.28809643
O14A	Antennae	forward	30875658	30205465	28835854	93.39348816
O14A	Antennae	reverse	30875658	30219652	28835854	93.39348816
O15A	Antennae	forward	30881312	30532305	29014385	93.9545088
O15A	Antennae	reverse	30881312	30518916	29014385	93.9545088
O32A	Antennae	forward	31047593	30640241	28983014	93.35027678
O32A	Antennae	reverse	31047593	30642733	28983014	93.35027678
O35A	Antennae	forward	31369279	30829443	29211609	93.12170994
O35A	Antennae	reverse	31369279	30825702	29211609	93.12170994
O54A	Antennae	forward	30489834	30003538	28456121	93.32986529
O54A	Antennae	reverse	30489834	30008838	28456121	93.32986529
O55A	Antennae	forward	30987482	30599583	29017098	93.64135492
O55A	Antennae	reverse	30987482	30591331	29017098	93.64135492
S12A	Antennae	forward	30574870	30109860	28579208	93.4728684
S12A	Antennae	reverse	30574870	30111767	28579208	93.4728684
S13A	Antennae	forward	29825739	29458050	27850944	93.37888996
S13A	Antennae	reverse	29825739	29448115	27850944	93.37888996
S14A	Antennae	forward	31099108	30723989	29026963	93.33696323
S14A	Antennae	reverse	31099108	30732119	29026963	93.33696323
S15A	Antennae	forward	30673696	29823148	28248624	92.09396872
S15A	Antennae	reverse	30673696	29830585	28248624	92.09396872

S16A	Antennae	forward	31220273	30827450	29298362	93.84402885
S16A	Antennae	reverse	31220273	30834334	29298362	93.84402885
S32A	Antennae	forward	30328817	29957871	28450573	93.80706475
S32A	Antennae	reverse	30328817	29956134	28450573	93.80706475
S51A	Antennae	forward	32437587	32032408	30183150	93.04992384
S51A	Antennae	reverse	32437587	32028656	30183150	93.04992384

Table S4-2 Ethogram used in the behavioural analyses.

Behaviour	Description
Antennation	Antennal contact between ants by tickling
Active	Any conspicuous ant movement at normal video speed
Auto-grooming	Ant grooming its bodyparts
Allogrooming	ant grooming or being groomed by another ant
“Spiderman”	Individual sticking to the upper glass of the nest upside down
On brood	Ant having at least one of its legs touching the brood
Queen_proximity	An ant body size distance from the focal ant
Walk	Ant having a direction with its 6 legs moving

Chapter 5

Ant behaviour and brain gene expression of defending hosts depend on the ecological success of the intruding social parasite

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Abstract

The geographic mosaic theory of coevolution predicts that species interactions vary between locales. Depending on who leads the coevolutionary arms race, the effectivity of parasite attack or host defence strategies will explain parasite prevalence. Here, we compare behaviour and brain transcriptomes of *Temnothorax longispinosus* ant workers when defending their nest against an invading social parasite, the slavemaking ant *T. americanus*. A full-factorial design allowed us to test whether behaviour and gene expression are linked to parasite pressure on host populations or the ecological success of parasite populations. Albeit host defences were shown before to co-vary with local parasite pressure, we found parasite success to be much more important. Our chemical and behavioural analyses revealed that parasites from high prevalence sites carry fewer recognition substances and are less often attacked by hosts and this link was further supported by gene expression analysis. Our study reveals that host-parasite interactions are strongly influenced by social parasite strategies, so that variation in parasite prevalence is determined by parasite traits rather than the efficacy of host defence. Gene functions associated with parasite success indicated strong neuronal responses in hosts, including long-term changes in gene regulation indicating an enduring impact of parasites on host behaviour.

Keywords: Coevolution, parasite prevalence, transcriptomics, social parasites, slavemaking ants, *Temnothorax longispinosus*

Introduction

The evolution of traits and species is largely driven by interactions with other species (Thompson, 1999; Betts *et al.*, 2018). In particular, antagonistic host-parasite associations can escalate in Red Queen dynamics (van Valen, 1973) or coevolutionary arms-races (Dawkins and Krebs, 1979; Tellier *et al.*, 2014; Nash *et al.*, 2008), with parasites perfecting host exploitation and hosts mounting ever better defences. Depending on gene flow and differences in selection pressures between sites (Thompson, 2005), hosts or parasites might locally adapt to the opponent (Gurney *et al.*, 2017) or develop more general resistance or offensive traits (Koskella and Parr, 2015). Escalation of the Red Queen dynamics can lead to population extinction and replacement, as recently shown for the pathogenic poplar rust fungus in France (Persoons *et al.*, 2017). Often the species that is better adapted to its opponent is considered to be ahead in the evolutionary arms race. However, recent theoretical models indicate that this is not necessarily the case, as the magnitudes of local and global adaptation are often independent, and the association between local adaptation and coevolutionary advantage is less strong (Nuismer, 2017). Antagonistic coevolution between parasites and hosts has been studied intensely from a theoretical perspective, in laboratory experimental evolution set-ups and comparative field studies (Lion and Gandon, 2015; Kerstes and Martin, 2014; Feeney *et al.*, 2014). We start to gain insights into how hosts alter their gene expression in response to parasite contact (Dennis *et al.*, 2017; Feldmeyer *et al.*, 2016; Martinson *et al.*, 2014; Barribeau *et al.*, 2014; Libersat *et al.*, 2018), and into the transcriptomic and genomic basis of parasite evolution and host-parasite coevolution (Alleman *et al.*, 2018; Feldmeyer *et al.*, 2017; Feis *et al.*, 2018; Cini *et al.*, 2015; Smith *et al.*, 2015). Yet, genes and pathways underlying traits important for coevolutionary interactions are often unknown and this is particularly

true for some of the most fascinating coevolutionary field models, the brood or social parasites, such as the cuckoos or slavemaking ants.

Brood parasites do not exploit the body of their hosts, but their social or care behaviours (Davies *et al.*, 1989). Well studied are the avian brood parasites, such as cuckoos or cowbirds that take advantage of the caring behaviour of other birds to circumvent the costs of parental care (Brooke and Davies, 1988; Davies *et al.*, 1996; Stoddard and Hauber, 2017). The social parasites of the ants and wasps parasitize entire animal societies (Hölldobler and Wilson, 1990; Buschinger, 2009). As in other host-parasite systems, social parasites and their hosts engage in coevolutionary arms races (Soler and Møller, 1990; Davies, 2000; Jongepier *et al.*, 2014; Brandt *et al.*, 2005a). According to Emery's rule, social parasites are often closely related to their hosts (Emery, 1909; Smith *et al.*, 2013) (but see (Carpenter and Perera, 2006; Sumner *et al.*, 2004; Huang and Dornhaus, 2008)), so that the population size, generation time and evolutionary potential of both opponents are largely similar (Pennings *et al.*, 2011) making the study of reciprocal adaptation particularly interesting.

Here, we focus on the coevolutionary interactions between the acorn ant *T. longispinosus* and its social parasite *Temnothorax americanus* (Ward *et al.*, 2015), a closely related slavemaking ant (Beibl *et al.*, 2005; Feldmeyer *et al.*, 2017), hereafter referred to as "the parasite". Via recurrent and destructive slave raids, this obligate social parasite exerts severe selection on its main host (Brandt and Foitzik, 2004). Local parasite pressure causes a reduction in host density and colony size and induces changes in the social structure, intra-colonial relatedness and allocation strategies of its host *T. longispinosus* (Foitzik and Herbers, 2001b; Foitzik *et al.*, 2009). Indeed, a geographic mosaic of coevolution (Thompson, 1999) is evident in population differences in host defence portfolios including behavioural, chemical and life-history

traits of hosts that can be chiefly explained by geographic variation in parasite pressure (Jongepier *et al.*, 2015; Jongepier and Foitzik, 2016a; Jongepier *et al.*, 2014). In low parasite pressure populations, *Temnothorax* hosts respond to the parasite with coordinated fights, while they move from a fight to a flight strategy in more highly parasitized locales. *Temnothorax* hosts also exhibit an inducible increase in aggression after parasite contact (Pamminger *et al.*, 2011; Scharf *et al.*, 2011), suggesting that the permanent expression of high aggression in the absence of the enemy can be costly. Yet, host colonies responding highly aggressive towards non-nestmate conspecifics fare better during parasite attacks, possibly due to interactions with *T. longispinosus* slaves that accompany slavemakers on raids (Pamminger *et al.*, 2012; Kleeberg *et al.*, 2014) and aggression increases with parasite pressure over the range of two *Temnothorax* host species (Kleeberg *et al.*, 2015) pointing to convergent coadaptation.

To direct aggression towards invading social parasites, these enemies have to be recognized. In order to circumvent host counter-attacks, *Temnothorax*, *Polyergus* or *Harpagoxenus* slavemakers either mimic the chemical recognition profiles of their hosts (D'Ettorre *et al.*, 2002; Brandt *et al.*, 2005b; Bauer *et al.*, 2009), carry fewer recognition substances (Kleeberg *et al.*, 2017), or trace the chemical signature of their local host population (Achenbach *et al.*, 2010). Hosts can respond to these chemical adaptations by intercolonial diversification in cuticular hydrocarbon profiles, which has been shown both in *Temnothorax* and *Formica* ant hosts (Martin *et al.*, 2011; Jongepier and Foitzik, 2016a). Social parasites also use the secretion of the Dufour's gland as a chemical weapon (Allies *et al.*, 1986; Jongepier *et al.*, 2015) to elicit fights among host defenders to deter host attacks. Parasites benefit from manipulating host aggression because the likelihood that intruding parasites survive host encounters and the

parasite prevalence in the field is linked to their ability to elicit skirmishes among hosts (Achenbach *et al.*, 2010).

Many of our behavioural studies investigated geographic variation in host defences (Pamminger *et al.*, 2013; Jongepier and Foitzik, 2016b; Jongepier *et al.*, 2014, 2015; Kleeberg *et al.*, 2015) while controlling for parasite population. The studies that did compare parasite populations (Foitzik *et al.*, 2003; Brandt and Foitzik, 2004; Foitzik *et al.*, 2009), detected variation in behavioural or chemical strategies of parasites and at times even local adaptation. Investigating variation between parasite populations is more difficult as social parasites are less common than hosts and most *T. americanus* colonies are small, containing on average less than five slavemakers. Behavioural differences between host or parasite populations could be due to genetic differences in protein-coding sequences or due to variation in gene expression e.g. caused by changes in gene regulatory sequences. The latter is more likely, as rapid adaptation is often based on shifts in gene expression (Campbell-Staton *et al.*, 2017; Ghalambor *et al.*, 2015). Indeed, if parasites manage to evade detection, they might not elicit any host attacks and this would leave a clear footprint in the transcriptomes of host defenders. A first gene expression study on the two focal species from a single locale identified interesting candidate genes, whose expression is associated with the raiding state in *T. americanus* and host nest defence in *T. longispinosus* (Alleman *et al.*, 2018).

Here, we combine behavioural experiments, chemical analyses of cuticular hydrocarbon profiles and transcriptomics aided by the newly sequenced genome of the ant *T. longispinosus* (which we publish alongside this study), to test whether host defences and brain gene expression of defending host workers are linked to parasite pressure on host populations or the ecological success of parasite populations. We used a full-factorial design for our parasite intrusion experiment that allowed us to

disentangle the influence of slavemaker origin from host origin on parasite-host interactions. As host populations differ in defence trait expression depending on local parasite pressure with colonies under low parasite pressure fighting rather than fleeing (Brandt and Foitzik, 2004; Jongepier *et al.*, 2014, 2015; Kleeberg *et al.*, 2015; Jongepier and Foitzik, 2016a; Pamminger *et al.*, 2013), we predicted that variation in host behaviour and gene expression should depend on parasite pressure, e.g. hosts from less parasitized sites, that attack intruding slavemakers, should upregulate the expression of aggression genes in their brains.

However, given the evidence of variation between parasite populations (Brandt and Foitzik, 2004; Foitzik *et al.*, 2003, 2009), it is also possible that parasite prevalence is more strongly linked to the efficacy of behavioural or chemical offensive strategies of parasite populations. If so, we predict that slavemakers from sites where they are common should manage to evade host detection and will be less frequently attacked. As hosts detect parasites by their chemical profile and recognition cues are known in *T. longispinosus* (Jongepier and Foitzik, 2016a), we expected that a high parasite prevalence is linked to a lower expression of these recognition cues on the cuticle of slavemakers. Whatever will explain the behavioural responses of host colonies to slavemaker intruders, host or parasite origin, variation in host behaviour, especially attack *vs.* no attack, should be reflected in the expression of behavioural defence genes in the brain.

Materials & Methods

Collection sites and parasite pressure

Temnothorax longispinosus is a primary host of the obligate social parasite and slavemaking ant *T. americanus* and occurs in Eastern North America. Colonies of *T. longispinosus* and its social parasite for the behaviour and transcriptome project were

collected in May-June 2017 at eight sites across their range (Supplementary material-I, Table T-1). The social parasite *T. americanus* was found at five sites with varying prevalence, which was estimated by the number of social parasite colonies per colony of the primary host in the community. The generation time of *Temnothorax* ants is about 10 years and population sizes are large, so parasite prevalence should be relatively stable over time. However, as social parasites are patchily distributed (Herbers and Foitzik, 2002), only intensive sampling will result in reliable estimates of parasite prevalence. We, therefore, combined our 2017 collection data with published long-term data (Brandt and Foitzik, 2004; Jongepier *et al.*, 2014; Foitzik *et al.*, 2009; Herbers and Foitzik, 2002), obtained over the last years at these locales. Some parasite colonies exploit slaves from two *Temnothorax* host species. In order to not overestimate parasite pressure on our focal host, we only included half of these mixed colonies when estimating parasite prevalence.

We used a full-factorial design so that host colonies from each population were subjected to the intrusion by a slavemaker of each parasite population. This allowed us to disentangle the impact of host and parasite origin on the outcome of the behavioural interaction and associated gene expression. Therefore, we used information on parasite prevalence in the local ant community for both the host and the parasite population. We used the term parasite prevalence$_{HOST}$ to indicate parasite prevalence of the host colony's source population and parasite prevalence$_{PARASITE}$ to specify parasite prevalence in the population of the slavemaker intruder. Whereas parasite prevalence in the host population provides information on the strength of parasite pressure on the host, we discuss below that parasite prevalence could also be regarded as a measure of the ecological success of the social parasite population.

Ant collection and maintenance

Ant colonies were detected in acorns, sticks and cracks of rocks. Each colony was separately transferred with some leaf litter into a Ziploc bag. Colonies were fed with cookie crumbs and moisture was maintained with water-soaked cotton. They were kept at 8°C until the transfer from the field to the laboratory, which was done within 16 days of collection. In the laboratory, each colony was given an artificial nest site to relocate, made out of a 4mm Plexiglas, with a 50 x 10mm cavity, sandwiched between two glass slides. Nests were kept in plastered three-chambered nest boxes to avoid desiccation. Ad libitum supply of water and honey was provided and ants were fed twice a week with crickets. Host colonies were kept at 18°C to 22°C with 16L: 8D cycle to slow down brood development. *T. americanus* colonies were kept at 25°C with a14L: 10D cycle. A week before the intrusion test, host colonies were transferred to the 25°C climate chamber for acclimation.

Intrusion tests and sampling regime

Intrusion tests included a careful introduction of a live slavemaking worker (the parasite) into a host nest. We blocked the nest entrance for 1h to ensure intense contact between parasites and hosts (Jongepier *et al.*, 2014). The intrusion test included 96 queenright *T. longispinosus* colonies from eight sites each with at least 15 workers. We selected the experimental ant colonies so that there were no population differences in colony size (Kruskal-Wallis test: $\chi^2 = 2.21$, df = 7, p = 0.947). We used 96 *T. americanus* workers from 31 colonies from four sites (on average workers from 7.8 colonies per site) for the intrusion tests (see Supplementary Files, Summary_tables_BEH_CHC_GC $_{\text{Avg n SD}}$).

In the field, hosts encounter social parasites only during the raiding season from July to September. Parasite encounter alters host responses at least for a fortnight

(Kleeberg *et al.*, 2014). To ensure a similar non-induced state in host colonies, we a) collected colonies a month before the onset of the raiding season, b) kept host and parasite colonies in separate boxes to avoid accidental encounters and c) used each host colony only once. Host colonies had therefore no encounters with its slavemaker parasites for at least one year. We performed the intrusion tests during the raiding season over four consecutive days (18th-21st July 2017). We controlled for light, temperature (25°C), humidity and time of the day by performing the experiments in a climate-controlled environmental chamber between 14:00-16:00. Each experiment was set up by the same experimenter and recorded on video. After 45min, we recorded the number of workers involved in aggressive responses towards the intruder. An aggressive response included behaviours such as mandible opening (a threat behaviour), holding, biting and stinging. After 1h we recorded whether the parasite was freely moving through the host nest or was under direct attack or killed by host workers. Behavioural analyses were conducted so that the experimenter was blind to the origin of the slavemaker.

Host colony aggression towards intruding parasite workers was measured as the proportion of colony members responding to the intruder. We ran a generalized linear model with the proportion of total responders as response variables and parasite prevalence$_{PARASITE}$, colony size and parasite prevalence$_{HOST}$ with two-way interactions as explanatory variables. We used the family parameter 'quasi-binomial' to control for overdispersion and checked a normal distribution of residuals graphically and statistically with Shapiro-Wilk normality test. If one of the interaction terms was significant, we kept the full model. Host colony response was noted as under attack if the parasite was immobilized or killed by the host colony or as free if the parasite was alive and not currently attacked by host workers at the end of the intrusion test. To

analyse host colony response, we used a generalized linear model (family binomial) and parasite prevalence$_{PARASITE}$, colony size and parasite prevalence$_{HOST}$ as explanatory variables. We started with a full model but due to the absence of any significant interactions, the model was reduced to a simple additive model. Statistical analyses were performed in R 3.4.1 (R Core Team, 2018).

Cuticular hydrocarbon recognition cues and parasite prevalence

In the host *T. longispinosus*, nine hydrocarbons are particularly relevant for nestmate recognition and explain most of the variance in aggression between non-nestmates (Jongepier and Foitzik, 2016a). Slavemakers including *T. americanus* carry less of these hydrocarbons on their cuticle compared to *Temnothorax* host species (Kleeberg *et al.*, 2017) and we analysed here whether slavemakers from high parasite prevalence sites carry less (or more) recognition substances on their cuticle. To this end, we analysed the cuticular hydrocarbon (CHC) profile of 7-12 parasite workers from our experiments (total N=40, supplementary material, Table T-2) from each of the four parasite populations using gas chromatography and mass spectrometry (Supplementary material-I, section S-1). This was done blind so the observers did not know the samples' origin. We calculated the total proportion of these recognition cues of all cuticular hydrocarbons (Supplementary material-I, Table T-3). Using a linear mixed-effects model, we tested whether this proportion is linked to parasite prevalence$_{PARASITE}$ and/or the slavemaker population, and removed non-significant factors until Akaike Information Criterion (AIC) was minimal, and included observer ID as random factor. Furthermore, we analysed the composition of the recognition cues depending on parasite prevalence$_{PARASITE}$ and slavemaker population using a permutational ANOVA (*adonis*, R package *vegan*).

RNA samples, extraction and sequencing

For the transcriptome analysis, we used 32 host colonies (Supplementary material-I, Table T-2), four from each site, which did not differ in colony size (Kruskal-Wallis test: $\chi^2 = 7.14$, df = 7, p = 0.41), that were a subset of the intrusion trials. Experiments were conducted with host origin randomized during each day and tested against social parasites from a randomly selected population. Each of the four focal host colonies per population encountered a parasite from a different population. Directly thereafter host worker(s) in direct contact or closest to the social parasite were picked and transferred to a vial, which was immediately dipped in liquid nitrogen (-196°C) and later transferred to the -80°C freezer. In some host colonies, none of the host workers responded with aggression to the parasitic intruder, in others many. Moreover, we found intra-colonial variation in that only some workers, the guards, focussed on the intruder. We expect differences in gene expression also between workers in their response varying within a colony. However, here we focussed on inter-population differences in gene expression in guards to a slavemaker intruder and to gain independent data we sampled only a single worker per colony for four colonies for each of the eight host populations.

To identify genes involved in host responses to social parasites, we focused on brain tissues for the transcriptome analysis. We prepared 32 RNA extractions from each of the 32 host brains (1 worker x 8 host populations x 4 parasite populations = 32 hosts). Ant brains were dissected on dry ice (Feldmeyer *et al.*, 2016). Brains were separately crushed in 50µL of TRIZOL for storage at -20°C before RNA extraction. RNA was extracted from brain samples separately using the RNAeasy mini extraction kit (Qiagen), and libraries were constructed and sequenced on Illumina HiSeq 4000 by Beijing Genomics Institute (BGI), Shenzhen, China, resulting in about 46 Mio, paired-

end 100 bp long reads per library. Sequence data was already cleaned by BGI by removing adapter sequences and low-quality reads. The data obtained from BGI was checked using FASTQC version 0.11.5 for read quality (Andrews *et al.*, 2010). Raw read data can be obtained in the Sequence Read Archive (SRA, BioProject ID PRJNA497176).

Sequencing and annotation of the *T. longispinosus* genome

A single monogynous *T. longispinosus* colony was used to provide all samples for genome sequencing. DNA was isolated from male pupae using the DNeasy Mini kit (Qiagen). A single male pupa was used for the construction of the three paired-end libraries (300, 500 and 800bp), and a pooled sample of eight male pupae for the mate-pair libraries with 2, 5 and 8Kb insert sizes. Library construction and sequencing of 150bp reads were conducted on an Illumina NextSeq 500 at StarSEQ, Mainz. Reads were assembled with AllPaths-LG (Butler *et al.*, 2008) (version r49967), yielding 3,987 scaffolds and 26,795 contigs, with a scaffold N50 of 514 Kb and a contig N50 of 30 Kb. In total, 92.3% of the conserved arthropod orthologs (BUSCO (Simão *et al.*, 2015a)) were present in the assembly.

A *T. longispinosus*-specific repeat library (see Supplementary material-II) identified 13.51% of the *T. longispinosus* assembly as repetitive elements. In total, 13,028 protein-coding genes were annotated using a 3-pass iterative MAKER (Holt and Yandell, 2011) workflow (version 2.31.8). Functional annotations were obtained based on BLAST searches against the Uniprot - Arthropod database (The UniProt Consortium, 2019) (release 2018 04; e-value: $1e^{-3}$) and Interproscan (Finn *et al.*, 2017) (version 5.24-63.0). For further details on genome assembly and annotation see the supplementary material-II. The *Temnothorax longispinosus* OGSv1.0 is accessible at GenBank (accession: PRJNA449506).

Expression analysis & functional annotation

Clean reads were aligned against the *T. longispinosus* OGSv1.0 genome using HISAT2 2.1.0 with default settings (Kim *et al.*, 2015). Read counts of the mapped sequences were obtained using HTSeq-count version 0.9.1 (Anders *et al.*, 2015). First, we controlled for parasite prevalence$_{PARASITE}$ and searched for genes differentially expressed based on parasite prevalence$_{HOST}$ and then we reversed the procedure. We used DESeq2 version 1.16.1 (Li, 2013; Gentleman *et al.*, 2004) to run these models, only taking genes as differentially expressed into account with an adjusted p-value of FDR<0.05. Since both parasite prevalence$_{HOST}$ and parasite prevalence$_{PARASITE}$ were continuous variables, the resulting lists of differentially expressed genes refer to as genes that are e.g. overexpressed in hosts facing more parasites from populations with higher or lower parasite prevalence. The log-fold change was measured in the unit of change of the continuous variable. To investigate modules of commonly expressed genes, we performed Weighted Gene Co-Expression Network Analysis (WGCNA) using the package WGCNA version 1.63 provided by Bioconductor (Langfelder and Horvath, 2008). The soft-threshold power was set to three according to the scale-free topology (Zhang and Horvath, 2005). 50 was chosen as the minimum number of genes in a module and the dissimilarity threshold was set to 0.25. We searched for functions of the different sets of genes associated with traits by performing a GO enrichment analysis. GO enrichment analysis was performed using topGO version 2.28.0, performing a Fisher's exact test on the different sets of genes compared to the whole genome using the weight01 algorithm (Tilford and Siemers, 2009). To see which pathways were enriched in the set of genes that showed a positive association to a trait, we performed KEGG pathway analysis. Pathway analysis was performed using the online tool http://www.genome.jp/kegg/tool/map_pathway1.html, results can be obtained in the supplement (Supplementary material-I, Table T-4).

Results

Behaviour

The proportion of workers responding to the intruder with aggression varied with the prevalence of the parasite population and colony size as well as their interaction (GLM: parasite prevalence$_{PARASITE}$: $t_{1,90}$ = -3.068, p = 0.003; colony size: $t_{1,89}$ = -2.715, p = 0.008; parasite prevalence$_{PARASITE}$*colony size: $t_{1,87}$ = 2.991, p = 0.004). In small colonies, a larger proportion of workers defended their nest against parasites from low parasite prevalence sites compared to larger colonies. In contrast, when host colonies encountered a parasite from a high prevalence population, in small colonies often none or a few of the workers detected and attacked the intruder while the proportion of attackers increased with colony size (Fig. 5-1a). Parasite prevalence on the host population and its interaction with the other variables did not affect the proportion of defenders in the host colony (GLM: parasite prevalence$_{HOST}$: $t_{1,88}$ = -5.236, p = 0.269; parasite prevalence$_{HOST}$*colony size: $t_{1,85}$ = -0.109, p = 0.335; parasite prevalence$_{HOST}$*parasite prevalence$_{PARASITE}$: $t_{1,86}$ = 2.746, p = 0.925). Moreover, the parasite's state i.e., whether it was ranging free or under attack by host defenders was also linked the prevalence of the parasite population and colony size, but not to its interaction (GLM; family=binomial, parasite prevalence$_{PARASITE}$: $z_{1,89}$ = 2.54, p = 0.011; colony size: $t_{1,88}$ = -2.27, p = 0.022; parasite prevalence$_{HOST}$: $z_{1,87}$ = 0.557, p = 0.577). Parasites from high prevalence communities are less often pinned or killed by host defenders than parasites from populations with a lower parasite prevalence (Fig. 5-1b). For example, we found 42% of the parasites from the Ohio site moved freely through the host nest, but only 8% of the parasites from Vermont. Larger host colonies were more likely to detect and immobilize or kill the intruding parasite, compared to smaller ones.

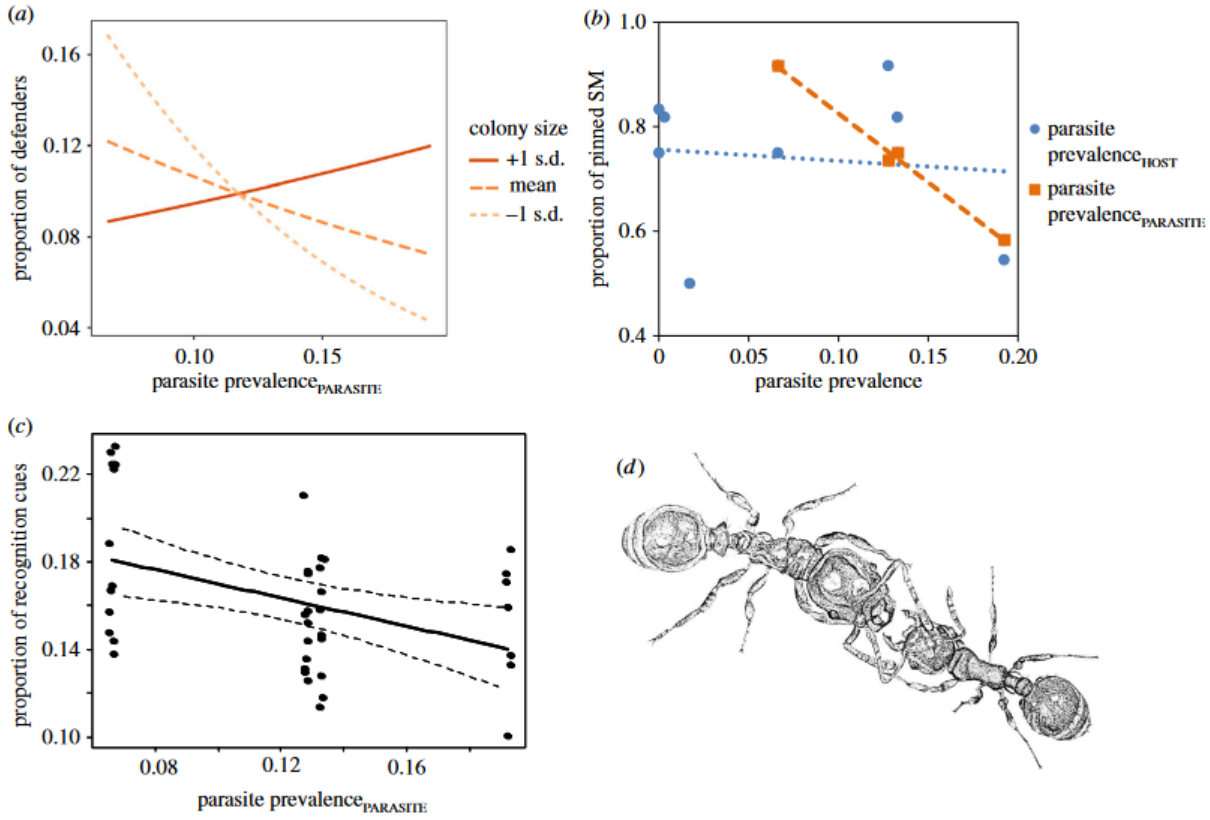

Figure 5-1 (**a**) Proportion of host workers involved in defence against the intruding slavemaker was affected by an interaction between the success of the slavemaking population (parasite prevalence$_{PARASITE}$) and host colony size. Lines represent three different colony size classes (large = mean +1 s.d.: solid line; midsized colonies = mean: long-dashed line; small colonies =mean -1 s.d.: short-dashed line). (**b**) The percentage of slavemaker intruders that were immobilized or killed by host defenders (proportion of pinned SM) depended on the success of the slavemaking population (parasite prevalence$_{PARASITE}$, brown squares, dashed line), but not on parasite pressure (blue circles, dotted line). (**c**) The total proportion of cuticular recognition cues in *T. americanus* in relation to its success. Each data point (n=40) represents the proportion of recognition cues in the CHC profile of one *T. americanus* worker (i.e., nine CHCs identified as relevant for nestmate recognition in *T. longispinosus*). The graph shows a linear regression line with a 95% confidence interval. (**d**) *T. americanus* slavemaker worker (left) interacting with a smaller *T. longispinosus* host worker (drawing by Inon Scharf).

Chemical recognition cues

Slavemakers from higher prevalence populations possessed fewer recognition cues compared to social parasites from populations with lower parasite prevalence (Fig. 5-1c). The proportion of recognition cues from all hydrocarbons decreased with increasing parasite prevalence (LMM: χ^2 = 8.053, df = 1, p = 0.0045). When parasite prevalence was accounted for, the slavemaker population had no further effect on the

proportion of recognition cues and was not retained in the final model. In a multivariate analysis, we show that both parasite prevalence and population of the slavemaker affected the composition (as opposed to the total proportion) of the recognition cues (ADONIS; parasite prevalence$_{PARASITE}$: F = 4.16, df = 1, p = 0.008; slavemaker population: F = 4.30, df = 2, p = 0.002). We detected differences between all pairs of populations (all F > 2.7, FDR-adjusted p < 0.048).

Gene expression analysis

Gene expression in the brain of host defenders was mainly influenced by parasite prevalence in the population of the slavemaker intruder (Fig. 5-2a) and much less so by parasite prevalence in the host population. Indeed, differential gene expression analysis identified only a single gene, which increased its expression with increasing parasite prevalence$_{HOST}$. This gene was annotated as armadillo repeat-containing protein 4 in *T. longispinosus*, which is known to be involved the in *Wnt* signalling pathway in *Drosophila* (Bejsovec, 2013). Instead, 278 genes increased their expression with increasing parasite prevalence of the social parasite population and an additional 175 genes lowered their expression with parasite prevalence$_{PARASITE}$ (Fig. 5-2b).

As some host colonies recognized, attacked or killed the intruding slavemaker and others ignored her, we additionally performed a gene expression analysis comparing brain gene expression of attacking vs. non-attacking hosts. We found 43 genes to be overexpressed in hosts that attack the parasite, 29.8 % of which overlapped with genes decreasing their expression with parasite prevalence$_{PARASITE}$, which was more than expected by chance (hypergeometrical test; p=1.6e-11) (Fig. 5-2b, Supplementary Files, Data-X1). Moreover, 222 genes were overexpressed in non-attacking hosts and 34.4 % of those overlapped with genes increasing their expression with parasite prevalence$_{PARASITE}$, which was more than expected by chance (hypergeometrical test;

p=4.2e-37). A heatmap of all the differentially expressed genes confirms that variation is linked to parasite prevalence $_{PARASITE}$ and host attack, but not to parasite prevalence $_{HOST}$ (Fig. 5-3). Furthermore, across all genes, the log-fold change depending on the prevalence of the parasite population was negatively correlated to the log-fold change in respect to attack *vs*. no attack (Supplementary material-I, Fig. F-1).

We contrasted our findings to a gene expression study based on whole-body transcriptomes (Tilford and Siemers, 2009) comparing New York *T. longispinosus* workers during a slave-raiding attack by *T. americanus* to host workers not involved in a raid. 22 genes overlapped between the differentially expressed gene lists of both studies, which amounts to 3.35% of all the genes found to be differentially expressed in the presented study (Supplementary material-I, Table T-5). In particular, we found five genes over-expressed in *T. longispinosus* defenders (Leucine-rich repeat-containing protein 49, Zinc finger MYND domain-containing protein 11, Phospholipase, Kinesin-like protein KIF14, Putative RNA-binding protein EEED8.10) to increase their expression with decreasing parasite prevalence$_{HOST}$. Conversely, five genes overlapped between hosts before raiding season and hosts facing social parasites from sites with higher parasite prevalence (elongation factor 1-alpha, ring canal kelch-like protein, serine/threonine-protein kinases 32B, PLK and SIK2).

A repetition of the analysis including host and parasite populations, instead of parasite prevalence$_{HOST}$ and parasite prevalence$_{PARASITE}$, showed that indeed origin of the parasite population explains changes in gene expression in the host better than host colony origin (Supplementary Files, Data-X2 and Data-X3).

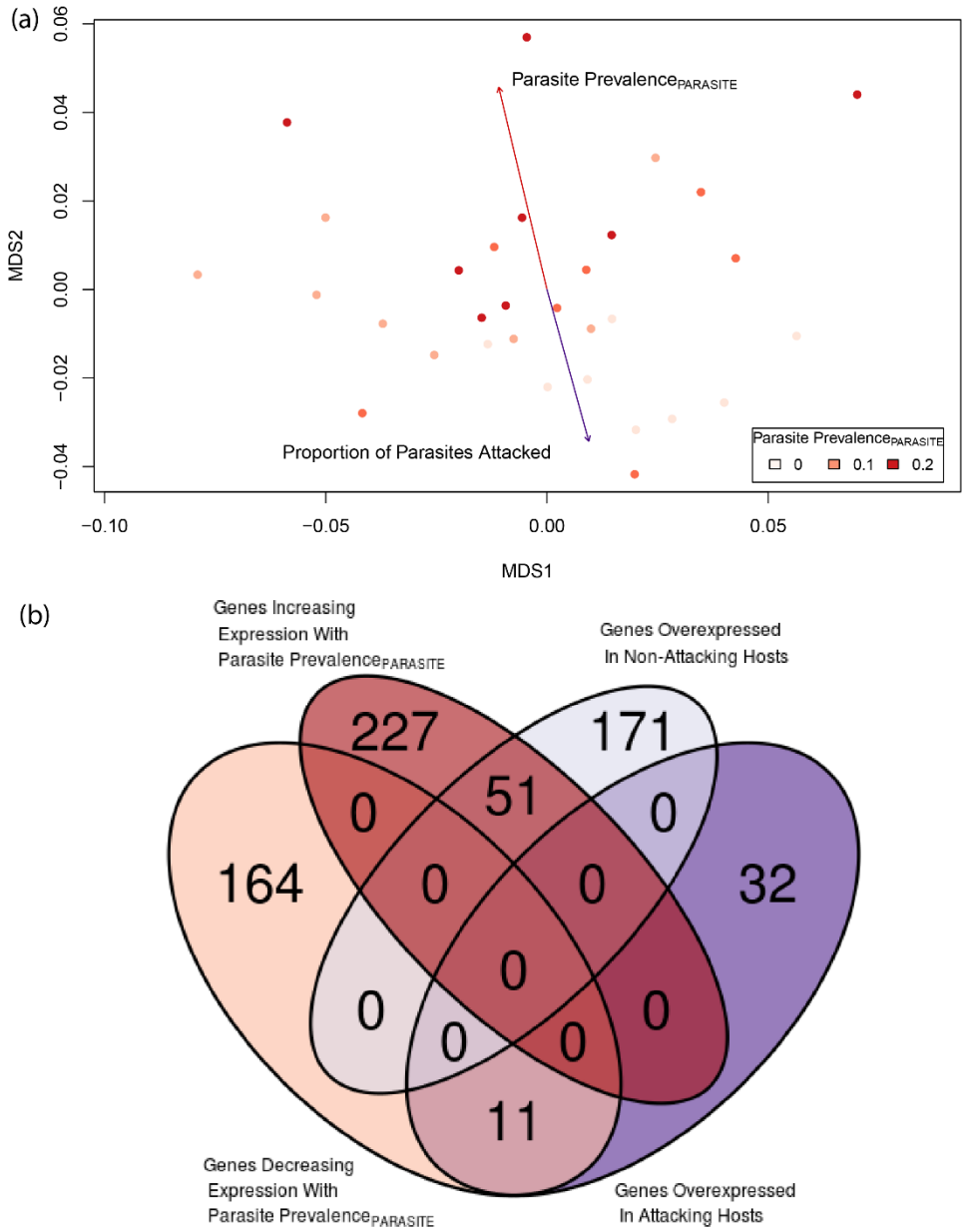

Figure 5-2 (**a**) Multi-dimensional scaling (MDS) plot showing sample distribution based on the variance in gene expression including all contigs. Different shades of red denote variation in the prevalence of the population of the parasite intruder (parasite prevalence$_{PARASITE}$). As parasite prevalence$_{PARASITE}$ was negatively linked to whether or not an intruding slavemaker was immobilized or killed during host colony intrusion, the respective arrows point in the opposite direction. Distribution of samples did not vary with parasite prevalence in the host colony's source population (see Results for details). Plot created using the R package vegan v. 2.5-2. (**b**) Venn diagram showing the number of genes differentially expressed between hosts that do or do not attack the intruding slavemaker (right, bottom and top) and between hosts facing more or less successful slavemakers (left, top, and bottom). Diagram created using the R package VennDiagram v. 1.6.20 (https://www.rdocumentation.org/packages/VennDiagram/versions/1.6.20)

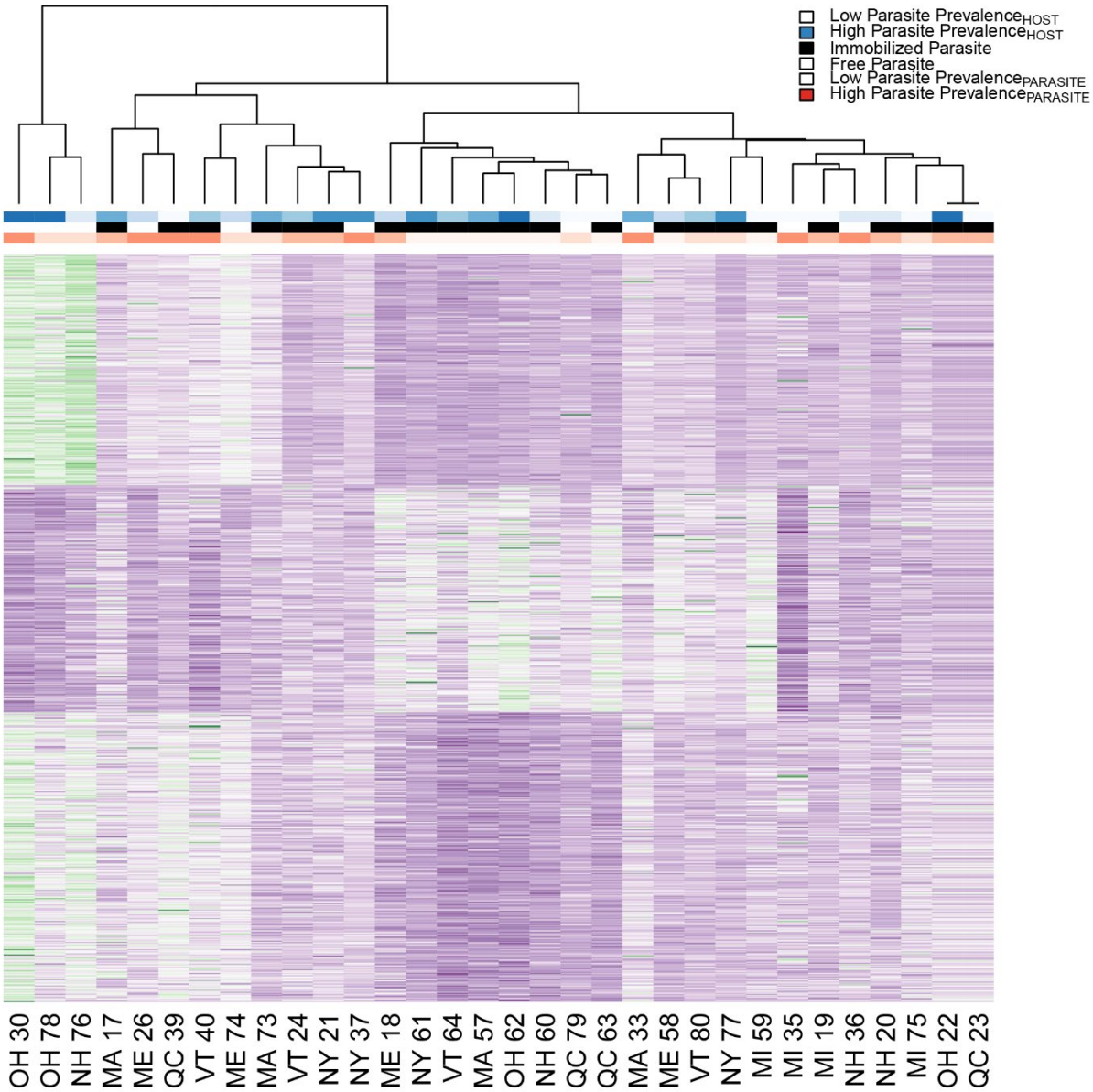

Figure 5-3 Heatmap based on the expression patterns found in all differentially expressed genes. Dendrogram showing relationships between samples based on similarities in gene expression. Colour bars above dendrogram show the parasite prevalence in the host population (blue), whether the slavemaker was immobilized/killed by the hosts (black) and the parasite prevalence of the population of the intruding slavemaker (orange). Heatmap created using R v. 3.4.4.

Weighted Gene Co-expression Network Analysis

The WGCNA grouped genes with similar expression patterns into 37 modules. Parasite prevalence$_{HOST}$ was unlinked to the eigengenes of any of these modules (Pearson correlation, n = 32, $p < 0.05$). In contrast, the eigengene values of two modules were significantly associated with parasite prevalence$_{PARASITE}$ of the intruder, with one showing a negative and one showing a positive correlation. The same two modules were also linked to whether host workers were attacking the parasite or not (Mann Whitney U tests, n=32, module 10: $p = 0.009$, module 32: $p = 0.02$). In addition, Module 9 was linked to attack *vs.* no attack and also tentatively associated with parasite prevalence$_{PARASITE}$.

Since genes central in networks are of special importance, we assessed the module membership of each gene in the co-expression network as a measure of network centrality. Module membership was calculated as the correlation between gene expression profile and module eigengene of each module (Langfelder & Horvath 2008). For the three modules correlated to at least one trait (parasite prevalence$_{PARASITE}$ and / or attack) network centrality was highly correlated to gene significance ($p < 0.0001$ for all cases). We also assessed whether differentially expressed genes are more central in their respective modules compared to all other genes, which we found for both genes overexpressed with increasing parasite prevalence$_{PARASITE}$ and overexpressed in non-attacking hosts compared to attacking ones (Supplementary material-I, Fig. F-2a, F-2b, F-3a and F-3b).

GO enrichment analysis

Enriched functions of genes both overexpressed with high and low parasite prevalence$_{PARASITE}$ were linked to “signal transduction” and “signalling” (Supplementary material-I, Fig. F-4). While genes overexpressed with higher parasite

prevalence$_{PARASITE}$ were enriched for “protein phosphorylation”, genes overexpressed with lower parasite prevalence$_{PARASITE}$ were enriched for “protein dephosphorylation”. Functions linked to an immune response like “activation of innate immune response” and “positive regulation of type I interferon production” were enriched in genes overexpressed in hosts confronted with parasites from sites with higher parasite prevalence.

We only found functions to be enriched in the WGCNA module of genes positively correlated with parasite prevalence$_{PARSITE}$ and negatively with host attacks, which were summarized as “DNA-mediated transposition”, including the *Wnt*-pathway (Fig. 5-4). The module negatively associated with parasite prevalence$_{PARASITE}$ (and positively to host attacks) showed functions linked to ubiquitin-dependent protein catabolism to be enriched, like “protein ubiquitination”, and epigenetic processes, such as “histone deacetylation”. This module also contained an enrichment of genes linked to “intracellular protein transport” and “positive regulation of autophagy”. These enrichments indicated that hosts that detect and attack intruding parasites undergo a major turnover in protein composition and changed their gene expression strongly and for longer term.

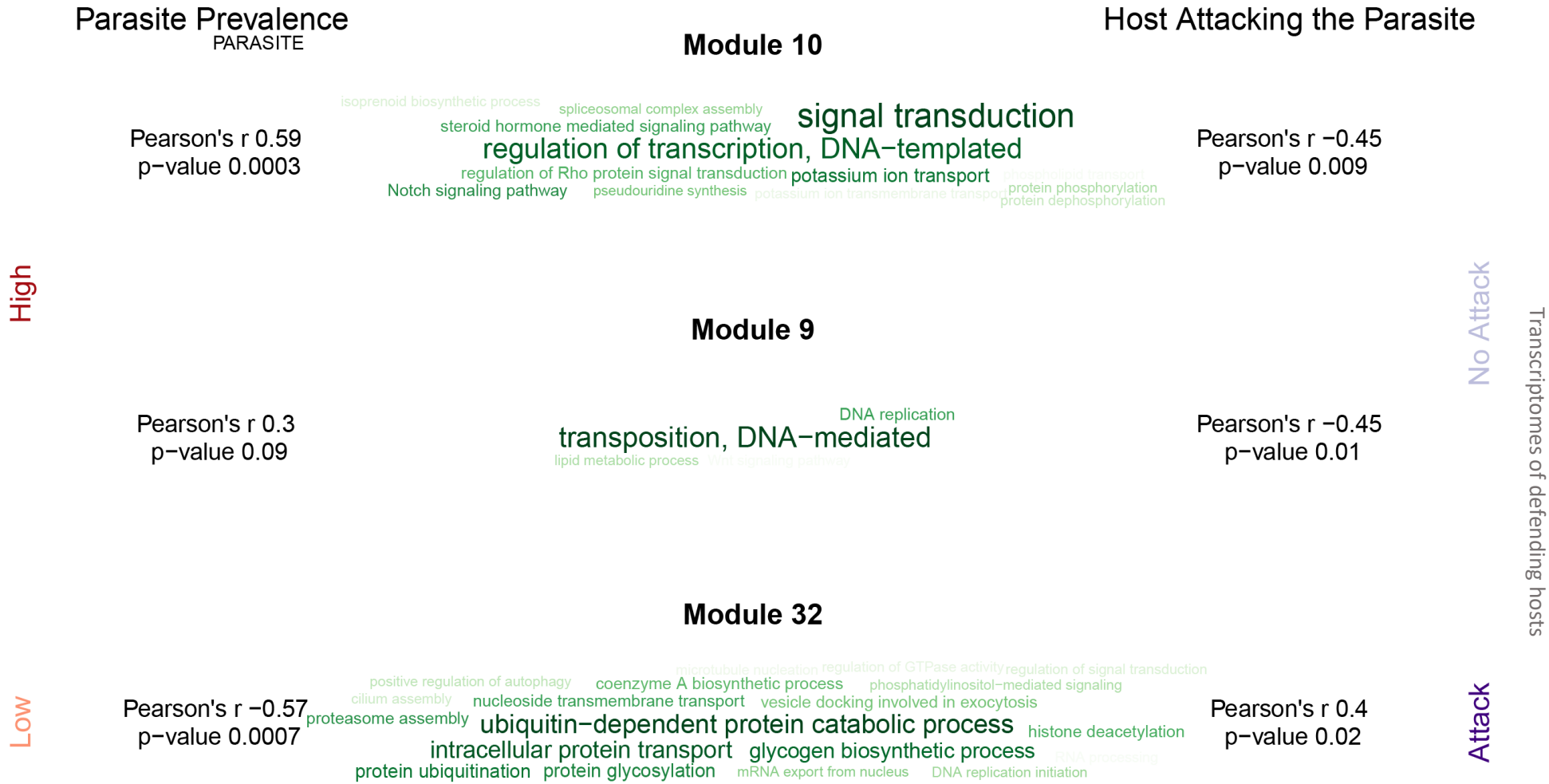

Figure 5-4 Enriched GO terms in three WGCNA modules which were significantly associated with prevalence in the parasite population and whether or not a slavemaker was attacked/killed by host defenders. Genes in modules were either positively or negatively linked to parasite prevalence$_{PARASITE}$ or under attack/no attack state of slavemakers, visualized using the R package tagcloud v. 0.6 (https://CRAN.R-project.org/package=tagcloud). The type size denotes the number of genes with this annotation (in green) found in the respective module.

Discussion

The behavioural responses of a host colony during the attack of an intruding parasite are critical for host fitness. If a parasite scout is allowed to return and recruit nestmates for a slave raid, most host brood will be lost, many workers will die and often the queen will be killed (Foitzik *et al.*, 2001). However, if hosts are able to detect and overwhelm the intruder, the host colony will remain unharmed. This behavioural interaction, therefore, drives the coevolutionary arms race between parasite and host, with parasites evolving ever better invasion strategies, while hosts improve detection mechanisms. We demonstrate here that the outcome of the parasite-host interaction depends much more on the expression of traits in the parasite than in the host. Parasite success of slavemaking ants can be measured directly as intrusion or raiding success, but in the long run, a successful social parasite population will increase in density and a higher fraction of hosts will be parasitized, that is parasite prevalence should go up. Therefore, a high parasite prevalence can be regarded as evidence for the ecological success of a parasite population (Jongepier *et al.*, 2015; Novak and Goater, 2013) as well as a sign of high parasite pressure on the host population.

In this study, we show that the likelihood that the attacker is subdued is linked to the success rate of the parasite population in the field and not to parasite pressure on host populations. This parasite success in turn is associated with a low expression of recognition substances on the cuticle of the parasite, allowing slavemakers to circumvent detection by the host. Moreover, this dependency on parasite success is clearly reflected in the brain gene expression patterns of the host. That is, how genes are expressed in host defenders' brains, does not depend on their own origin, but on the origin of the opponent, they are facing. This finding fits our studies revealing differences between parasite populations in behaviour, cuticular chemistry and

ecological success (Foitzik *et al.*, 2003; Brandt and Foitzik, 2004; Foitzik *et al.*, 2009). Yet the absence of an effect of parasite pressure on the host is surprising as previous work showed that host defence portfolios vary with parasite pressure (Jongepier *et al.*, 2014, 2015). However, these studies did not compare multiple parasite populations, but rather standardized parasite population to reveal differences among host populations. Our data here indicate that parasite origin overrides any behavioural or transcriptome differences between host populations.

Parasite success influences host behavioural response

Intruding social parasites have to be recognized by hosts as enemies and *T. longispinosus* colonies from all populations studied so far respond to *T. americanus* intruders with high levels of aggression (Kleeberg *et al.*, 2015; Scharf *et al.*, 2011). However, the number of host defenders was found to decline with parasite pressure on host populations, with hosts from highly parasitized sites shifting to flight rather than fight (Jongepier *et al.*, 2014). Albeit our current study included many, but not all the host populations studied before, we found that the proportion of host workers involved in nest defence did not depend on the geographic origin of the hosts or local parasite pressure. Rather, the fraction of workers involved in parasite defence depended on an interaction between colony size and the ecological success of the social parasite population. When parasites originated from low prevalence sites, the proportion of workers involved in defence decreased with colony size. This makes sense as social parasite cues might spread only locally in the nest and there is only limited space around the parasite for host workers to attack. Interestingly, this association with colony size was reversed against highly successful parasites. Here, we found a lower proportion of defending host workers in small colonies and a higher one in larger colonies. This is probably because successful parasites are rather able to undergo

detection by individual host workers and thus perform better against small colonies. Differences in host response to slavemakers of various *T. americanus* populations were quite strong, with parasites from the least successful Vermont population (parasite prevalence 6%) being nearly always attacked or killed (92%), while less than 60% of highly successful Ohio parasites (parasite prevalence 20%) were attacked in our experiments.

Parasite success is linked to chemical recognition substances

Avoiding detection by the host is essential for the ecological success of slavemakers. Former studies have shown that *Temnothorax* slavemakers, including our focal species, generally exhibit a cuticular hydrocarbon profile with lower proportions of hydrocarbons relevant for recognition in the host *T. longispinosus* (Kleeberg *et al.*, 2017). Here we demonstrate that interpopulation variance in this trait is further linked to parasite success. Slavemakers from high prevalence sites exhibit a cuticular hydrocarbon profile deprived of recognition cues, which should help to avoid host detection and aggressive attacks and therefore could explain their ecological success in the field. Instead, parasites from less successful populations carry more recognition substances and might therefore more easily induce host aggression. In addition, slavemakers from high prevalence sites might effectively manipulate a small number of hosts with their Dufour's gland secretion. Indeed, hosts from highly parasitized populations are more easily tricked to attack their nestmates by chemical manipulation by the Dufour's gland secretion than those from populations where social parasites are rare or absent (Jongepier *et al.*, 2015). In contrast, here we find that the behavioural responses of the host depend on the ecological success of the parasite population and not on parasite pressure of the host populations, indicating that differences in offensive traits, be they behavioural or chemical, mask any differences between host

populations. Similar inter-population variation in the efficacy of the Dufour's gland secretion to manipulate hosts into intra-colonial fights was found in the European social parasite *Harpagoxenus sublaevis* (Foitzik *et al.*, 2003).

Parasite success influences gene expression in the host

Gene expression analysis based on brain transcriptomes of defending host workers confirms our behavioural findings as it showed that more genes altered their expression depending on the ecological success of the intruding parasite than with parasite pressure on the host. Indeed, the expression of only a single gene co-varied with parasite pressure, when controlling for parasite success. In contrast, we found several hundred differently expressed genes linked to the ecological success of the parasite population, which indicates that host brain gene expression depends more on the origin of the opponent than on the geographic origin of the sampled worker. As our behavioural analysis showed that these effects were driven by whether or not hosts recognize intruding parasites, we analysed gene expression in host brains depending on whether these host workers attacked the intruder or not. Again, we identified hundreds of differentially expressed genes with host attack, which strongly overlapped with the analysis based on parasite success. Finally, some of our overexpressed genes were already detected to play a role in regulating host defences in *T. longispinosus* (Pamminger *et al.*, 2013) against *T. americanus* slave raids.

During the coevolutionary arms race between social parasites and hosts (Brandt *et al.*, 2005a), the parasites have perfected their raiding strategies, while hosts are under strong selection pressure to develop effective defence strategies (Foitzik *et al.*, 2001; Jongepier and Foitzik, 2016a, 2016b; Jongepier *et al.*, 2014; Foitzik *et al.*, 2009). As we had ample evidence, especially for the latter process, our initial expectation was that gene expression would also shift with parasite pressure. Our cross-fostering

experiment allowed us to disentangle parasite pressure on the host from parasite success and revealed that parasite success affects gene expression in the host much more than parasite pressure. The results of the WGCNA support the gene expression analysis: Here, we were able to find two modules positively and negatively correlated with parasite success respectively, but none was linked to parasite pressure. Overall, our analysis suggests that host responses and their gene expression in the brain depend on the type of intruder and especially on its traits, which are likely linked to its ecological success. An important social parasite trait is its cuticular hydrocarbon profile (Pamminger *et al.*, 2013), in particular, the expression of recognition substances, which we show here to be negatively linked to parasite success. In social insects and ants, in particular, the cuticular hydrocarbon profile is the main trait used to recognize foes (Guerrieri *et al.*, 2009).

Candidate genes and enriched functions linked to parasite success

Among the genes overexpressed in hosts encountering a successful parasite was glutamate [NMDA] receptor subunit 1, which was found to be overexpressed in the aggressive Africanized honeybee compared to the European honeybee pointing to a role in regulating aggressive behaviours (Alaux *et al.*, 2009). Additionally, a gene encoding an ionotropic glutamate receptor was also found to be upregulated across three species of *T. americanus* host workers defending their nest during slave raids (Alleman *et al.*, 2018). In honeybees, the expression of the *huntingtin* gene, which is one of our candidate genes for high parasite success, was linked to high-defensive bees (Hunt *et al.*, 2007). That we find overexpression of genes linked to aggression in hosts encountering successful parasites, which are less often attacked, is at first surprising. However, it could be explained by successful parasites exhibiting fewer recognition substances on their cuticle and possibly also manipulating hosts via the

Dufour's gland secretion into attacking each other. We did not note intracolonial attacks here, but an earlier study found that the degree by which host workers are manipulated by this chemical weapon to attack each other explains the survival of parasite intruders and their success in the field (Jongepier *et al.*, 2015). The GO enrichment of these genes showed many functions linked to signal transduction indicating a high neuronal activity in hosts encountering parasites from highly successful populations. A comparison of our results to a former study investigating raiding-specific expression differences in our two focal species (Alleman *et al.*, 2018) shows overlap in differentially expressed genes, suggesting that hosts, facing a successful parasite, share genes overexpressed in hosts not currently under attack by social parasites, while genes overexpressed during raids mostly overlapped with hosts facing less successful parasites. If highly successful parasites are indeed able to manipulate host behaviour to their favour, hosts would fail to express genes important for an effective nest defence, which is reflected in our results. Instead, less successful parasites induce aggressive host defences as shown in our behavioural data and cause the expression of defence genes. A few genes of the attack/ no attack analysis overlapped to the earlier raiding analysis, but no clear trend was visible here. Expression of genes linked to aggression does not necessarily imply that this aggression is addressed to the parasite, and this could be why we do not find a strong overlap between genes overexpressed during raiding defence and attacking hosts. The WGCNA results demonstrate that functions linked to histone modifications like "histone deacetylation" are enriched in a module negatively correlated with parasite success and positively to hosts attacking the intruder. Histone modifications, such as acetylation, methylation or phosphorylation regulate gene expression by facilitating or impeding the access of transcription factors to the DNA (Bell *et al.*, 2010). The consequential changes in gene expression can alter the phenotype, especially

behaviour. For example, histone acetylation was found to control the expression of genes linked to foraging in ants (Simola *et al.*, 2016). Also in our focal species, *T. longispinosus,* histone acetylation appears to play a role for behavioural flexibility*:* After removal of old workers, inhibition of histone acetyltransferase (HAT) facilitated the shift of young workers to foraging but impeded the reverse shift [Kohlmeier et al.c per com.]. Similarly, under HAT inhibition *T. longispinosus* colonies were unable to adapt their circadian rhythms to changes in the day/ night cycle (Libbrecht *et al.*, 2020). Therefore the upregulation of histone deacetylation genes following the encounter of slavemakers from less successful populations, which are most likely recognized and attacked, indicates long-term changes in gene regulation and expression supporting earlier work, that aggressive behaviour of *T. longispinosus* workers is altered over two weeks following a parasite encounter (Kleeberg *et al.*, 2014).

Interestingly, while our study reveals the importance of variation in gene expression for the interactions between parasites and hosts, differences in gene regulation were not found between host populations or depending on local parasite pressure on the host. Thus, there is no evidence for selection on regulatory regions in the host during host-parasite coevolution. Rather expression differences in the brain of host workers depended on the traits of the slavemaker they were facing and on the latter population of origin. If anything, gene expression in host worker brains thus could be interpreted as the *extended phenotype* of the parasite (Dawkins, 1982).

Conclusion

Parasite-host interactions are models for studying the dynamics of adaptation during coevolutionary arms races. Parasite prevalence can be shaped by many factors, including local adaptation of the host (Jongepier *et al.*, 2014; Jongepier and Foitzik, 2016a), climatic conditions and parasite virulence (Szöllosi *et al.*, 2011). Variation in

parasite traits important for the interaction was found lately to be quite important in avian brood parasites, especially when both host resistance and tolerance are taken into account (Medina and Langmore, 2016; Svensson and Råberg, 2010; Soler *et al.*, 2011). Despite previous studies revealing that host defence portfolios co-vary with parasite pressure in the *T. longispinosus*-*T. americanus* system (Jongepier *et al.*, 2014), we show here that the origin of the parasite determines the outcome of its interactions with the host. Indeed, the expression of genes in the brain of host defenders did not depend on parasite pressure or geographic origin of the host, but on the ecological success of the parasite, they are facing. This indicates that variation in parasite virulence is more important for the species interaction than reported variation in host defence traits.

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[removed for privacy purposes]

Author Contributions

R. K. and S.F. designed the experimental set-up and collected the ant colonies. R. K. conducted the behavioural experiments, chemical analyses and statistics. R. K. and M. S. analysed the gene expression data. Genome assembly and annotation was conducted by E. J. with support from B. F. and E. B.-B. F.M. contributed to the chemical analyses. R. K., M. S. and S. F. wrote a first draft and all authors revised it.

Supplementary Materials

Additional information on the methodology and more detailed informations about the results can be obtained from figshare:

Supplementary Material I containing sample informations, informations on CHCs and additional analyses performed including pathway analysis (https://doi.org/10.6084/m9.figshare.7539512.v1), Supplementary Material II containing additional information concerning the genome assembly used (https://doi.org/10.6084/m9.figshare.7539500.v1), Supplementary Data X2 containing the genes differentially expressed between host populations (https://doi.org/10.6084/m9.figshare.7539503.v1), Supplementary Data X3 containing the genes differentially expressed with parasite success and attack of the intruder (https://doi.org/10.6084/m9.figshare.7539506.v1), Supplementary Data X4 containing the genes differentially expressed between hosts facing parasites from different populations (https://doi.org/10.6084/m9.figshare.7539509.v1).

Raw RNA-Seq reads can be accessed from SRA under BioProject ID PRJNA497176 while the version of the genome assembly used for these analyses can be found under BioProject ID PRJNA449506.

Chapter 6

Tandem-running and scouting behaviour are characterized by up-regulation of learning and memory formation genes within the ant brain

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Abstract

Tandem-running is a recruitment behaviour in ants that has been described as a form of teaching, where spatial information possessed by a leader is conveyed to the following nestmates. Within *Temnothorax* ants, tandem-running is used within a variety of contexts, from foraging and nest relocation to – in the case of slavemaking species – slave raiding. Here, we elucidate the transcriptomic basis of scouting, tandem-leading, and tandem-following behaviours across two species with divergent lifestyles: the slavemaking *Temnothorax americanus* and its primary, non-parasitic host *T. longispinosus*. Analysis of gene expression data from brains revealed that only a small number of unique differentially-expressed genes are responsible for scouting and tandem-running. Comparison of orthologous genes between *T. americanus* and *T. longispinosus* suggests that tandem-running is characterized by species-specific patterns of gene usage. However, within both species, tandem-leaders showed gene expression patterns median to those of scouts and tandem-followers, which was expected, as leaders can be recruited from either of the other two behavioural states. Most importantly, a number of differentially-expressed behavioural genes were found, with functions relating to learning and memory formation in other social and non-social insects. This includes a number of up-regulated receptor genes such as a glutamate and dopamine receptor, as well as serine/threonine protein phosphatases and kinases. Learning and memory genes were specifically up-regulated within scouts and tandem-followers, not only reinforcing previous behavioural studies into how *Temnothorax* navigate novel environments and share information, but also providing insight into the molecular underpinnings of teaching and learning within social insects.

Keywords: gene expression, transcriptomics, learning, teaching, social parasitism, *Temnothorax*

Introduction

Individuals of a social group can acquire information through interactions with their group members (Caro and Hauser, 1992; Franks and Richardson, 2006; Grüter and Leadbeater, 2014), often occurring through social learning where the behaviours of one individual are observed and imitated by other members of the society. In most cases, the transmission of information is of mutual interest for sender and receiver. The widespread ecological success of social insects can be in part attributed to their efficient sharing of information (Hölldobler and Wilson, 1990). Information about resources or predators is often conveyed to nestmates and subsequently proliferated throughout the entire colony. For example, the waggle dance performed by honey bee workers benefits not only the individuals receiving the information – who are then able to more easily locate quality food sources – but also the colony as a whole for which the food is obtained (Frisch, 1967). The benefits of social information usage depend on many extrinsic and intrinsic factors (Grüter and Leadbeater, 2014), including the costliness of acquiring private information (Wray *et al.*, 2012), and the certainty of social information (Stroeymeyt *et al.*, 2017).

Many insects are good learners and capable of impressive long-term memory (Pascual and Préat, 2001). Learning in insects has primarily been studied in *Drosophila* flies and honey bees, both on behavioural (Papaj and Lewis, 2012; Menzel and Giurfa, 2001) and molecular levels. Learning and memory formation is associated with the activity of serine/threonine protein kinases and phosphatases in animals, including humans and insects (Mansuy and Shenolikar, 2006; Giese and Mizuno, 2013). These enzymes that transfer or remove phosphate groups to side chains of particular amino acids (serine/threonine or tyrosine) of target proteins are essential for neuronal plasticity. For example in *Drosophila melanogaster*, long-term memory formation is linked to the

activity of *Serine/threonine-protein kinase meng-po* (Lee *et al.*, 2018). Within *Drosophila* flies, social learning is commonly involved in reproduction, with female flies using social information for both determining mate choice (Mery *et al.*, 2009) as well as identifying suitable oviposition sites and substrates (Sarin and Dukas, 2009; Battesti *et al.*, 2012). Thus, while social learning has been shown in *Drosophila* (Danchin *et al.*, 2018), its scale and importance within a society increase tremendously in social insects; for example in honey bees with their "symbolic" dance language (Frisch, 1967). Thus, the sheer number and variety of mechanisms utilized by social insects for the transfer and exchange of information with nestmates make them of particular interest for the investigation of social learning (Dukas, 2007). Like in *Drosophila* flies, serine/threonine protein kinases are also important for learning in social insects (ex: Zhang *et al.*, 2015). Learning in honey bees is facilitated by the activity of biogenic amines (Scheiner *et al.*, 2006), important mediators of neuronal signalling in the central and peripheral nervous systems of many invertebrates. Biogenic amines such as dopamine, tyramine or serotonin appear to often have antagonistic or modulating effects on honeybee learning (Blenau *et al.*, 2000).

Ants form as complex societies as honey bees; indeed their colonies are often even more populous. However, in contrast to honey bees, ants use more chemical means to communicate. To date, very little is known about the molecular underpinnings of information transfer between nestmates in ants. For example, in many ant species with large colony sizes, a small number of neuromodulators and other candidate genes have been linked with trail-following: in the ant *Pheidole dentate*, trail-following behaviours are reduced in serotonin-depleted workers, suggesting that serotonin itself plays a role in modulating olfactory processes or motor functions associated with cooperative foraging (Muscedere *et al.*, 2012). Compared to recruitment via

pheromone trails, however, recruitment of nestmates through tandem-running is relatively understudied. This form of information transfer between nestmates – often found in ants with smaller colony sizes – likely evolved convergently in Ponerine and Myrmicines ants, and has been described within a number of taxa (Wilson, 1959; Hölldobler, 1974; Franks and Richardson, 2006; Franklin and Franks, 2012; Franklin, 2014; Möglich *et al.*, 1974). Tandem-running differs from trail-following in that, instead of scouts continuously laying pheromone trails for nestmates to follow and reinforce, individual scouts with information about a resource of interest recruit a small group of nestmates directly from the nest, which are then physically led to the resource.

With their small colony sizes, diverse lifestyles including slavery, and receptiveness to artificial environments, *Temnothorax* ants are ideal models for investigating tandem-running as a mode of information exchange between nestmates. Recruitment via tandem-running in *Temnothorax* is characterized by three distinct behavioural components: a scouting, a leading, and a following component (Möglich *et al.*, 1974). **Scouts** perform the highly risky task of searching for and reporting back the location of resources of interest such as food or nesting sites. Like in other social Hymenopterans, scouts are often the oldest, more experienced and thus most expendable individuals of the colony (Seeley, 1983), which face many dangers when exploring novel environments. Scouts that do locate a resource of interest then return to their home nest to recruit additional workers to the resource, effectively becoming **Leaders** of the ensuing tandem-runs. Tandem-run leaders physically guide one to a handful of nestmates – **Followers** – to the new resource (Wilson, 1959). While leading individuals tend to have first been scouts, those followers that reached the resource of interest via tandem-run can return to the home nest and become leaders by recruiting additional nestmates. Indeed, there is some evidence from *Temnothorax albipennis* that the

follower orients in a way that best facilitates the learning of the path taken to the destination, and afterwards is then able to effectively guide new nestmates (Franks and Richardson, 2006; Franklin and Franks, 2012). Additionally, previous experience as a tandem-leader or -follower appears to influence the propensity for tandem-running in *Temnothorax* (Franklin *et al.*, 2012), so that often a stable "oligarchy" of knowledgeable workers head nest relocations (Richardson *et al.*, 2018). Indeed, *Temnothorax* tandem leaders lead more and better tandem runs with experience (Glaser and Grüter, 2018) indicating that they learn over time to guide others to novel resources such as food, nest sites or host colonies.

Interestingly, within *Temnothorax*, tandem-running behaviour appears to have undergone contextual modification. Slavemaking species within this genus utilize a tandem-running-like behaviour for the purpose of raiding nearby host colonies and stealing their brood (Foitzik *et al.*, 2001). The slavemaking lifestyle has evolved multiple times independently within *Temnothorax*, with slavemakers species closely-related to their hosts (Emery, 1909; Beibl *et al.*, 2005; Kohlmeier *et al.*, 2017; Prebus, 2017). *Temnothorax longispinosus* is the preferred host of the most derived obligate slavemaker of that group, *T. americanus*. Like other *Temnothorax* species, *T. americanus* also utilizes tandem-running-like behaviour to recruit nestmates, in this case for raiding parties against host colonies (Buschinger and Winter, 1977; Pohl *et al.*, 2011). Workers of *T. americanus* do not show normal worker behaviour, as they neither forage nor take care of the queen or her brood (Wesson, 1890); though they do display reproductive division of labour among workers with only the older, infertile workers initiating or participating in slave raids (Pohl *et al.*, 2011). *T. americanus* colonies are entirely dependent upon enslaved workers for normal nest tasks, as the sole task of slavemakers is to invade nearby nests of host species in order to steal brood and

incorporate the newly-eclosed host workers into the slavemakers' workforce (Wilson, 1971; Hölldobler and Wilson, 1990; Pohl *et al.*, 2011; Alloway, 1979). In contrast to the slavemaking ants, workers of *T. longispinosus* show a clear age-dependent division of labour for tasks like nursing and foraging (Kohlmeier *et al.*, 2018), which is also visible by differentially expressed genes (Feldmeyer *et al.*, 2014; Kohlmeier *et al.*, 2019), and they continue performing these tasks even if enslaved by *T. americanus*.

Tandem-running behaviour has previously been described as a form of teaching, as it involves bidirectional feedback between leader and follower, where leaders must modify their behaviour in order to allow followers to keep up and thus learn the route to the new resource (Franks and Richardson, 2006; Richardson *et al.*, 2007). While tandem-running in *Temnothorax* is much slower than other forms of nestmate recruitment (Pratt, 2005), tandem-followers gain experience about the environment and learn the path to the target resource. Thus, costs in the form of a slower recruitment rate are associated with learning in tandem-running systems. Ultimately, both scouts and leaders possess information that may be transferred to nestmates; though the method of acquisition of this new information can differ depending upon whether the leader became so after scouting – i.e., by obtaining private information – or by obtaining social information through following (Möglich, 1978). Moreover, while scouts explore their environments alone, both leaders and followers engage in social interactions with nestmates.

The molecular underpinnings of tandem-running strategies in general - and whether they are molecularly similar between parasitic and non-parasitic lifestyles - have never been studied. As such, we seek to elucidate the molecular patterns underlying the contextually-distinct tandem-running behaviours of the slavemaking ant *Temnothorax americanus* and its non-parasitic host *T. longispinosus* and evaluate whether the

tandem-running behaviour of *T. americanus* during raiding and *T. longispinosus* during nest relocation share a common evolutionary basis. As a non-slavemaking lifestyle is the ancestral state for *Temnothorax*, a shared molecular pattern between slavemaker and host during scouting and tandem-running behaviours would indicate that slavemakers have co-opted ancestral genes previously associated with non-parasitic tandem-running behaviour; thus facilitating the evolution of novel raiding behaviours (Alleman *et al.*, 2018; Feldmeyer *et al.*, 2017). As gene expression within the brain can strongly influence behaviour in social insects (Whitfield *et al.*, 2003; Zayed and Robinson, 2012; Liang *et al.*, 2014), we first sought to determine the gene expression patterns within the brains of *T. americanus* and *T. longispinosus* during the tandem-running behaviours of each species. While scouting behaviour is relatively well studied, comparatively few studies explore differences in brain gene expression of scouts and workers focusing on other tasks (Ingram *et al.*, 2005). As tandem-leaders by necessity were either scouts or followers previously, and are engaged in active communication with nestmates, we expect this behavioural phenotype to possess the lowest number of uniquely associated genes. Moreover, we hypothesize that in scouts and followers, genes associated with learning in other social insects are up-regulated – like those involved in the production of biogenic amines or their receptors – as they are important for learning in both honey bees and fruit flies. Additionally, we expect to see genes up-regulated in *T. americanus* and *T. longispinosus* scouts that are associated with scouting or foraging in other social insects, like those involved in the production of amino acid neurotransmitters such as glutamate (Liang *et al.*, 2012). Ultimately, these findings should provide insight into not only the molecular underpinnings of tandem-running behaviour itself but into teaching and learning within social insects as well.

Material and Methods

Collection and maintenance of ant colonies

T. americanus and *T. longispinosus* colonies (11 and 8 colonies, respectively) were collected in the forests of the Edmund Niles Huyck Preserve in Rensselaerville, New York (42°31'41.0"N 74°09'38.8"W) during June 2016. Ant colonies were transported individually in Ziploc bags within their natural nest sites. Upon arrival in our laboratory, each colony was transferred into its plaster-floored nesting box containing a single slide-nest - into which the colony relocated. A slide nest is an artificial nesting site comprised of a small Plexiglas cavity sandwiched between two glass microscope slides. Colonies were then kept under a constant 25°C, 14L:10D light cycle and were fed twice weekly with honey and crickets.

Behavioural experiments and sampling

T. americanus and *T. longispinosus* behavioural experiments were carried out from the 29th of August to the 9th September, 2016. Both slavemaker and host colonies were provided with a plaster-floored arena 43cm x 28cm x 10cm in dimensions. The arena floor was kept damp for all colonies throughout the course of each experiment. Slavemaker and host colonies were tested under highly similar though lifestyle-specific conditions, selected specifically for this experiment to induce scouting and tandem-running: a) we induced nest-relocation in the non-parasitic host species *T. longispinosus* by opening the upper portion of their nest site and offering a new, empty nest-site into which the colony can relocate; b) *T. americanus* colonies were offered a *T. longispinosus* host colony as a potential raiding target during the raiding season of this species in summer (Fig. 6-1). Slavemaker and host workers were sampled during three separate behavioural states: a scouting state, a tandem-leading state, and a tandem-following state.

In order to sample individuals displaying these behaviours of interest in *T. americanus* slavemakers, one slide nest containing the slavemaker colony was placed at one corner of the arena and one slide nest containing a *T. longispinosus* host colony, with queen and brood, was placed at the opposite end of the arena. This setup affords slavemakers the chance to scout out and find the host colony, recruit additional individuals, and perform coordinated slave raids against that host colony.

Similarly, one slide nest containing a *T. longispinosus* colony was placed at one corner of the experimental arena, and an unused slide nest was placed at the opposite corner. To motivate *T. longispinosus* to perform their tandem-running behaviour, the upper slide of the occupied host colony was three-quarters removed, forcing the colony to seek out a new nesting site. In this experiment, we offered the host colony an unused nest in the opposite corner of the arena. Host individuals were sampled as **scouts** (single *T. longispinosus* searching at least 10cm from own nest entrance), as **leaders** of a relocation tandem-run (a worker leading one or more *T. longispinosus* workers at least 10cm from the home nest entrance), or as **followers** of a relocation tandem-run (following a *T. longispinosus* leader during a tandem-run). Slavemaker workers were sampled as **scouts** (a single *T. americanus* worker searching at least 10cm from own nest entrance), as **leaders** of a raiding party (a *T. americanus* worker leading one or more slavemakers at least 10cm from the home nest entrance), or as **followers** of a raiding party (following a *T. americanus* worker in a raiding party).

In both slavemaker and host experiments, workers observed performing a behaviour of interest were quickly placed into a 1.5mL centrifuge tube and then placed into liquid nitrogen for freezing. Sample tubes were then transferred from liquid nitrogen to a -80°C freezer for short-term storage. In total, 42 *Temnothorax* individuals were sampled: 21 *T. americanus* and 21 *T. longispinosus*, of which 30 samples were

sequenced (15 *T. americanus*, five samples per behavioural state from 11 unique colonies; and 15 *T. longispinosus*, also five samples per behavioural state from six unique colonies).

Subsequent dissection of *T. americanus* and *T. longispinosus* brains and extraction of RNA using the RNeasy Mini Extraction kit (Qiagen), was carried out before sending RNA samples to Beijing Genomics Institute (BGI) for library construction and sequencing 100 base pair (bp) paired-end reads on an Illumina HiSeq 4000. Our single-brain samples contained between 0.0073 µg and 0.0496 µg of RNA and a quality score of Level D, according to BGI's test result metric (Supplementary: Table SI1).

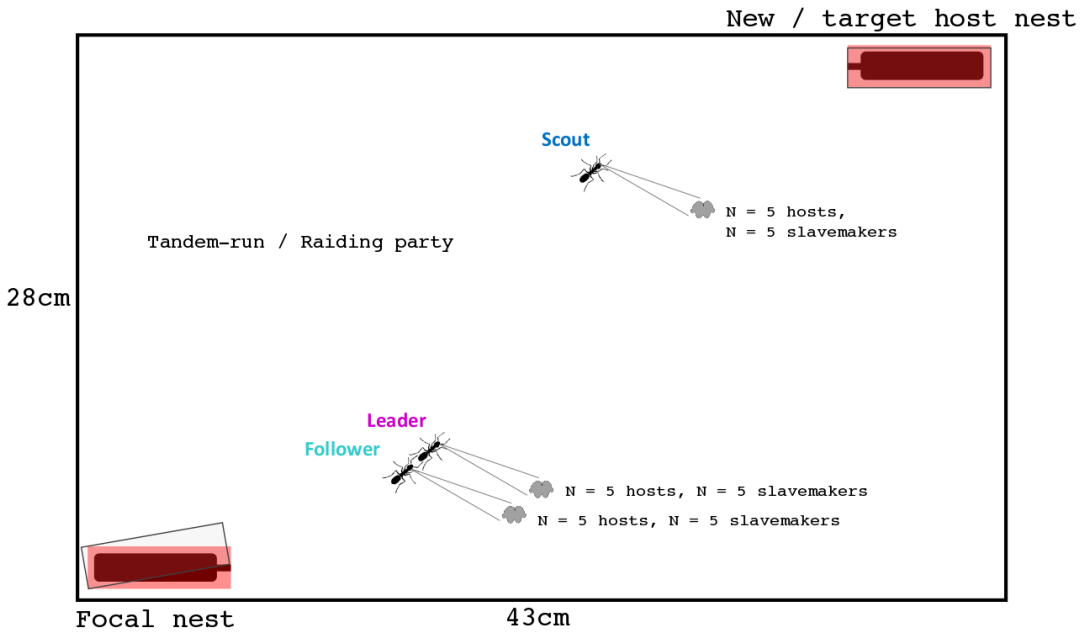

Figure 6-1 Experimental design for nest relocation/raiding trials and gene expression analyses. For each experiment, either a host (*Temnothorax longispinosus*) or a slavemaker (*T. americanus*) colony was positioned within its slide nest at one corner of the arena. In the opposite corner, either an empty nesting site for *T. longispinosus* or a host colony as raiding target was offered. To induce scouting for new nest sites in *T. longispinosus*, the upper cover of the old nest site was slid to the side. We sampled workers of both species during three distinct behaviours: scouting for new nesting sites or host colonies and leading or following tandem-runs/raiding parties. Sampled workers were flash-frozen in liquid nitrogen.

De novo transcriptome assembly

RNA-Seq resulted in approximately 20 million 100 bp paired-end reads per sample. Raw reads may be obtained from the NCBI SRA Run Selector under accession number

PRJNA503093. Removal of Illumina adapters and trimming of paired-end reads was carried out using Trimmomatic v0.32 (Bolger *et al.*, 2014). Afterwards, the trimmed reads were quality checked using FastQC v0.11.5 and sequences flagged as poor quality were removed (Andrews *et al.*, 2010). After trimming, 99.5% (328,117,222 in total) of the reads remained for *T. americanus* and 99.5% (330,634,890 in total) for *T. longispinosus* (Supplementary: Table SI2).

With the trimmed paired reads, we created one de novo transcriptome per species using the data of all states with Trinity v2.4.0 (Grabherr *et al.*, 2011) and the following non-default parameters: --SS_lib_type FR --min_contig_length 300 --full_cleanup --no_bowtie --bflyHeapSpaceMax 20G --bflyGCThreads 1. Annotation of the transcriptomes was carried out using *BLAST v2.6.0* (Altschul *et al.*, 1990) against the NCBI non-redundant protein invertebrate database (November 2016), with an E-value of 1e-5 and below. The quality of the resulting transcriptomes was determined using the script TrinityStats.pl included alongside Trinity (Grabherr *et al.*, 2011). Since we expected only a small number of contigs to differ in their expression between scouts, tandem-leaders and tandem-followers, we decided to implement several filtering steps a) reduce the probability of false positives, and b) remove low read counts, which likely represent noise. Therefore, as a first step, we ran TransDecoder v3.0.1 (Haas *et al.*, 2013) on both transcriptomes and only retained transcripts with open reading frames with a minimum length of 30 base pairs.

Analysis of gene expression

Reads were aligned to their respective filtered transcriptome using Bowtie (Langmead and Salzberg, 2012). Read count tables for both the *T. americanus* and *T. longispinosus* transcriptomes were produced with RSEM v1.3.0 (Li and Dewey, 2011) as implemented

in the align_and_estimate_abundance.pl script provided with Trinity and the following non-default parameters: --est_method RSEM --prep_reference --aln_method bowtie2.

For both *T. americanus* and *T. longispinosus*, we used the package DESeq2 v1.16.1 in order to identify differentially expressed contigs (Love *et al.*, 2014) (Supplementary: Script S1, Script S2). Before running DESeq2, we removed spurious reads by allowing only those contigs in the data set for which at least 10 reads mapped to at least four of our 15 samples. Additionally, we plotted the maximum Cook's distance as a function included alongside DESeq2 for all remaining contigs and removed those contigs with extreme expression variance as indicated by a maximum Cook's distance above 38. Thereafter, six pairwise comparisons between each of the behavioural groups were made in DESeq2 for each species: "Scout vs Leader", "Scout vs Follower", "Leader vs Scout", "Leader vs Follower", "Follower vs Scout" and "Follower vs Leader". The resulting p-values were adjusted for multiple testing using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) and only contigs with an adjusted p-value of 0.05 or less were further explored. Plotting the number of reads revealed that many contigs did not show clear read count differences between the behavioural groups. We, therefore, decided to include an additional filtering step to reduce the likelihood of false positives. We additionally ran a Kruskal-Wallis test on the raw count data of all differentially expressed contigs followed by a Dunn test correcting for multiple testing using the Benjamini-Hochberg procedure. We only explored those contigs further, which were detected both by DESeq2 as differentially expressed (FDR $p<0.05$) and the Kruskal-Wallis test (Dunn corrected $p<0.05$). Genes found to be up-regulated in each behavioural state in different pairwise comparisons were merged, and private versus shared DEGs were visualized in a Venn diagram using the R package VennDiagram v.1.6.17 (Chen and Boutros, 2011). We thus depicted genes that were a) responsible for the focal behaviour, in that they were up-regulated compared to at least one other

behaviour (referred to as "all up-regulated"), b) unique for the focal behaviour because they were only up-regulated in this behaviour and not in any of the others, and therefore of special interest for defining it on the molecular level (referred to as "privately up-regulated") and c) up-regulated in both other behaviours compared to the focal behavior which implicates that these genes are down-regulated in the focal behavior (referred to as "privately down-regulated"). Separation of differentially expressed contigs by behavioral state was visualized by carrying out a Principle Component Analysis (PCA) on transformed count data of all contigs as well as only the differentially expressed contigs using the function plotPCA provided by DESeq2. We also produced heatmaps to reveal expression variance between samples using only read counts of significantly differentially expressed contigs as input.

Identification of behaviour and learning candidate genes

The reference database used for BLAST reference before enrichment analysis was produced through a manual lookup of functional information within the online UniProt database (The UniProt Consortium, 2019). Genes from specific organisms that possessed functions of interest were compiled into a single list. We searched UniProt for genes possessing annotation information matching either "learning" and "memory", limiting those results via UniProt's default filter functionality to only those genes from organisms with labels "*Drosophila*", "*Apis*", "wasp", "cockroach", and "ant". Afterwards, we filtered this list for only reviewed entries and removed duplicates for genes that were found in different species. The protein sequences of the gene lists for each criterion were downloaded from UniProt. The resulting lists of sequences were used as blastx reference databases for the filtered transcriptome sequences of both species, with an E-value of 1e-5 and below. Since our transcriptome contained many isoforms which would map to the same candidate gene and therefore

falsify our enrichment results, we decided to only use Trinity 'genes' as a measurement for the number of hits both in the transcriptome as well as in the lists of differentially expressed contigs. Enrichment of these candidate genes in the differentially expressed genes was tested using hypergeometric tests with the functions phyper in R (R Core Team, 2018).

Weighted gene co-expression network analysis

We used Weighted Gene Co-expression Network Analysis (WGCNA) in order to track contigs with similar patterns of expression between behaviours within a species (Zhang and Horvath, 2005). Two separate WGCNA were performed, each in a block-wise fashion using all contigs from one of our two filtered transcriptomes (Supplementary: Script S3, Script S4). WGCNA for both *T. americanus* and *T. longispinosus* contig subgroups was performed with default parameters, except for the soft threshold (power = 4 for *T. longispinosus* and 10 for *T. americanus*), – which was chosen according to the approximated scale-free topology – minimum module size (50), and dissimilarity threshold (0.3) (Zhang and Horvath, 2005). Eigengene values of the resulting modules were extracted and a Kruskal-Wallis test was followed by a pairwise Wilcoxon test correcting for multiple testing used to determine whether individual modules were associated with specific behaviours.

GO enrichment and pathway analysis

Nucleotide contigs of both *T. americanus* and *T. longispinosus* transcriptomes were first transcribed into protein sequences with TransDecoder v3.0.1 (Haas *et al.*, 2013) before further Gene Ontology (GO) analyses. InterProScan v5.25-64.0 was run on the protein sequences in order to obtain GO terms and KEGG annotations for each contig (Jones *et al.*, 2014; Ashburner *et al.*, 2000; Ogata *et al.*, 1999). Finally, GO enrichment was performed using the package topGO v2.28.0 and the "weight01" algorithm,

executing a Fisher's exact test on the lists of "Biological Process" GO annotations of the differentially expressed contigs compared to the complete list of GO annotations of each respective transcriptome (Alexa and Rahnenfuhrer, 2018) (Supplementary: Script S5, Script S6). We used the KEGG-ID output of Interproscan to obtain the associated pathway information from the KEGG database (http://www.genome.jp/kegg/tool/map_pathway1.html; Ogata *et al.*, 1999). Pathways were extracted for contigs up-regulated in the three behavioural states of each species and afterwards, over-representation against the pathways of the whole transcriptome was tested using hypergeometric tests adjusting for multiple testing using Benjamini-Hochberg procedure using the functions phyper and p.adjust included in the R stats package (R Core Team, 2018).

Construction of orthologue sequence clusters

Finally, we constructed orthologue sequence clusters using OrthoFinder v1.1.8 (Emms and Kelly, 2015) with translated amino acid sequences of transcriptome contigs of both species in order to determine if orthologous genes were similarly expressed in like behaviours between *T. americanus* and *T. longispinosus*. We used two approaches: a) all contigs of the two transcriptomes in their entirety, and b) only the DEGs of one species against the transcriptome of the other species were compared. For our analysis, we were only interested in orthologue clusters containing one sequence of each species. Clusters, which contained one sequence per species and more than a single sequence in the other species were reduced to single-copy orthologous clusters based upon the highest BLAST score using an in-house R script (Supplementary: Script S7). Orthologue sequence clusters were used to cross-compare DEGs from *T. longispinosus* and *T. americanus.* Finally, a Kruskal-Wallis test on the read counts followed by pairwise Wilcoxon tests adjusting for multiple testing using the Benjamini-

Hochberg procedure was used to test whether gene expression patterns could be explained by the behavioural group.

Results

De novo assembly and annotation

De novo assembly of the trimmed paired forward and reverse reads using *Trinity* resulted in 294,117 transcripts for *T. americanus* and 256,533 for *T. longispinosus*, of which 290,644 contigs for *T. americanus* and 253,116 for *T. longispinosus* remained after filtering for open reading frames. The BLAST search against the non-redundant invertebrate database from November 2016 resulted in annotations for 57.74% (169,810 in total) of the contigs of the transcriptome from *T. americanus* and 59% (151,366 in total) of the contigs of the transcriptome from *T. longispinosus*. For both species, the top ten species in the BLAST annotations were from ants of the subfamily Myrmicinae, to which our focal species also belong. Mapping the trimmed reads back to the filtered transcriptomes showed similar back-mapping rates for both transcriptomes of 84.7% for *T. americanus* and 86.0% for *T. longispinosus*.

Analysis of gene expression

After the two additional filtering steps outlined above, 109,021 contigs remained for *T. americanus* and 102,603 for *T. longispinosus* (Supplementary: Table S1, Table S2). Of those, 201 were significantly differentially expressed (DEGs; FDR $p<0.05$ & Dunn corrected $p<0.05$) in *T. americanus* and 156 in *T. longispinosus* (Fig. 6-2, Supplementary: Table S3, Table S4). In *T. americanus*, the highest number of up-regulated genes occurred in scouts (80), followed by leaders (76). In *T. longispinosus,* followers showed the highest number of up-regulated genes (66), followed by scouts (61). The lowest number of up-regulated genes was found in followers of *T.*

americanus (69) and leaders of *T. longispinosus* (59). The number of differentially expressed genes (private or shared) did not differ between behavioural groups of both species contigs (Supplementary: Table SI3).

PCA plots of transformed read counts of the filtered transcriptomes from all contigs revealed no clear clustering of our samples according to behaviour, but a strong clustering according to colony ID (Fig. 6-3a and b). However, when PCA plots were generated based on differentially expressed genes only, samples were clearly clustered by behavioural phenotype (Fig. 6-3c and d). This was also apparent in the heatmaps based on DEGs revealing distinct expression patterns of scouts, leaders, and followers in both species, with leaders sharing many DEGs with followers as well as scouts (Fig. 6-4).

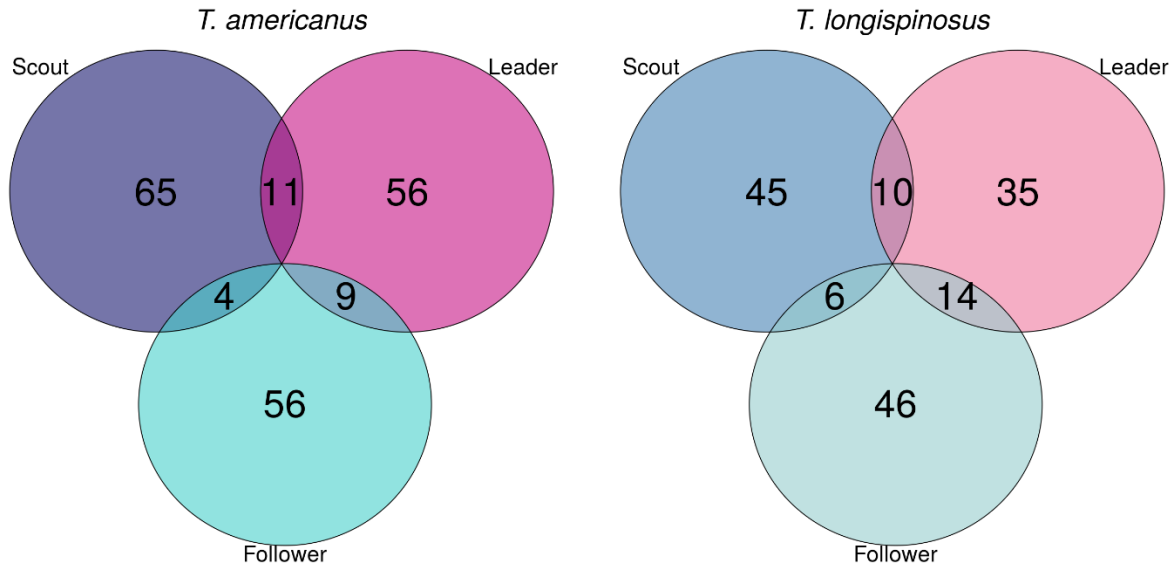

Figure 6-2 Venn diagrams outlining shared and private DEGs between the tandem-running behaviours of *T. americanus* (left) and *T. longispinosus* (right) depicting numbers of up-regulated contigs of specific behaviours.

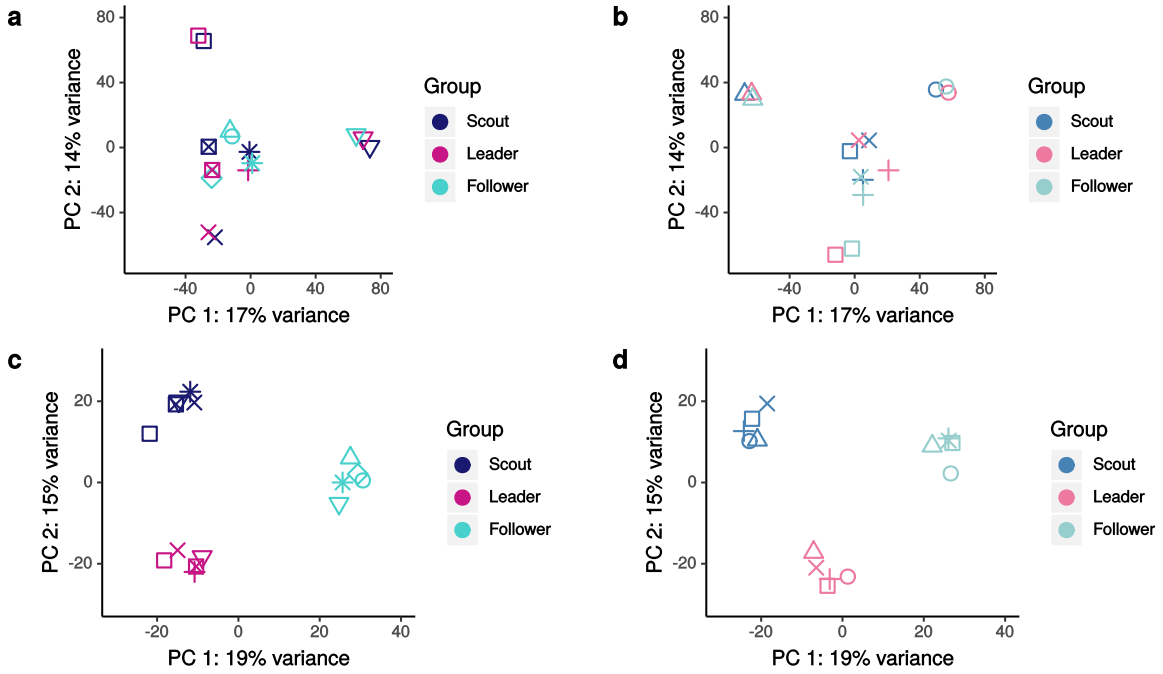

Figure 6-3 Principle Component Analysis plots outlining read count variance between sample individuals of each tandem-running behaviour in (**a**). the similarity between all genes of each *T. americanus* sample; (**b**) similarity between all genes of each *T. longispinosus* sample; (**c**) similarity between samples only based on DEG read counts of *T. americanus*; and (**d**) similarity between samples only based on DEG read counts of *T. longispinosus*. Within (**a**) and (**b**), we see no consistent grouping of samples according to behaviour, suggesting high gene expression similarity between the tandem-running behaviours of a species. However, differentially expressed genes (**c, d**) do cluster according to behaviour.

Identification of behaviour and learning candidate genes

Between 44 to 53 candidate genes were found in the lists of DEGs in all three behavioural states of both species. The number of candidates found per DEG list did not differ (X^2= 1.74, df=5, p=0.88). We found that DEGs of *T. americanus* scouts were enriched for functions linked to learning (Table 6-1). Other tests did not show significant enrichment, but we found some trends (p<0.1): DEGs of *T. americanus* scouts tended to be enriched for memory genes, as well as leaders in both species. Additionally, DEGs of followers of *T. americanus* tended to be enriched for functions linked to learning. Overall, we were able to identify several of our candidate genes from the gene expression analysis that aligned to one of the genes with known functions for learning and memory (Fig. 6-5, Table 6-2).

Weighted gene co-expression network analysis

Block-wise WGCNA yielded a relatively large number of modules for both *T. americanus* and *T. longispinosus*; 290 and 259, respectively. Of these modules, six of *T. americanus* and four of *T. longispinosus* were significantly associated with the focal behaviours after correction for multiple testing (Supplementary: Table S5, Table S6).

GO enrichment analysis

For both GO enrichment analyses of all up-regulated contigs of the three focal behaviours and the 10 WGCNA modules linked to behavioural phenotypes showed a large number of enriched functions both in *T. americanus* and *T. longispinosus* (Supplementary: Table S7, Table S8), of which we mention here only a few interesting ones. Our Gene Ontology enrichment analysis often yielded results, where significant enrichments were based on single or few genes of rare functions. As these functions might still be biologically meaningful, we decided to discuss them and show the number of genes driving the enrichment in parenthesis.

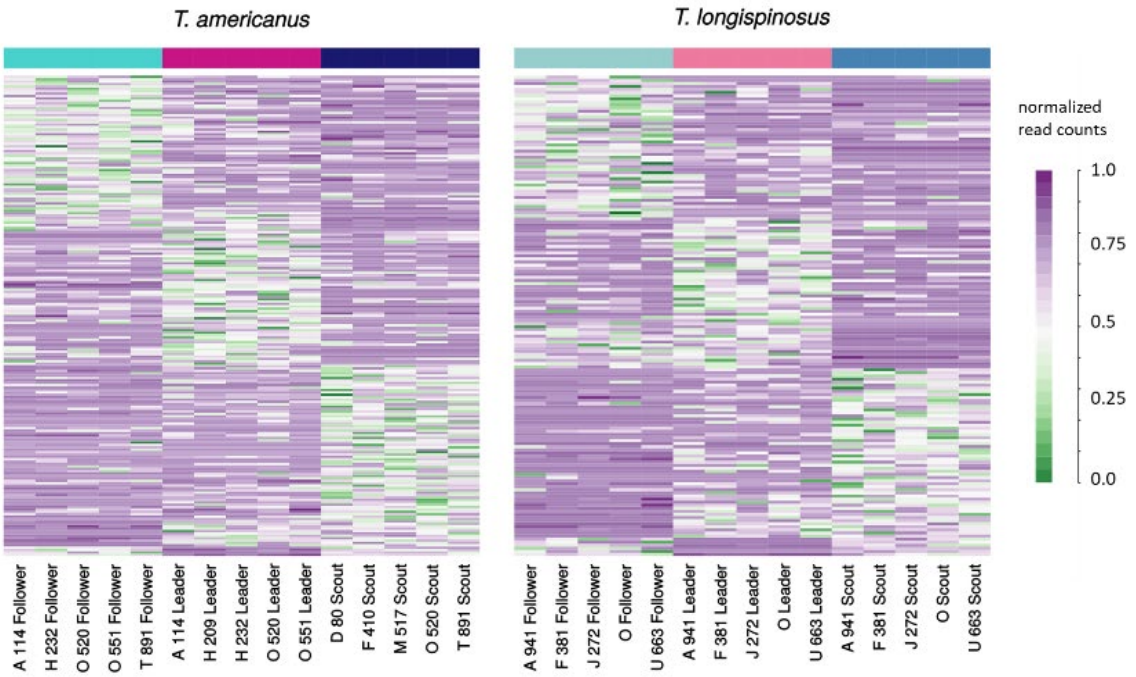

Figure 6-4 Heatmaps of all DEGs within *T. americanus* (left) and *T. longispinosus* (right). Identifiers at the ends of branches contain individual and colony IDs (e.g., O 520). Green indicates higher expression of a candidate gene and purple lower expression. Scouts in blue, leaders in pink and followers in turquoise.

Table 6-1 Statistical results of hypergeometric tests to test the enrichment of candidate genes in the lists of up-regulated contigs per behaviour in contrast to the filtered transcriptome using the function *phyper* provided in R (R Core Team, 2018).

Species (Genes in transcriptome)	Group	N of DEG in list	Function	Hits in Gene List	P-value
T. americanus (39989)	Follower	68	Learning	5	**0.05**
			Memory	6	0.10
	Leader	74	Learning	4	0.17
			Memory	7	**0.06**
	Scout	76	Learning	1	0.90
			Memory	4	0.49
T. longispinosus (39585)	Follower	63	Learning	3	0.32
			Memory	4	0.37
	Leader	59	Learning	3	0.28
			Memory	6	**0.07**
	Scout	60	Learning	5	**0.04**
			Memory	6	**0.07**

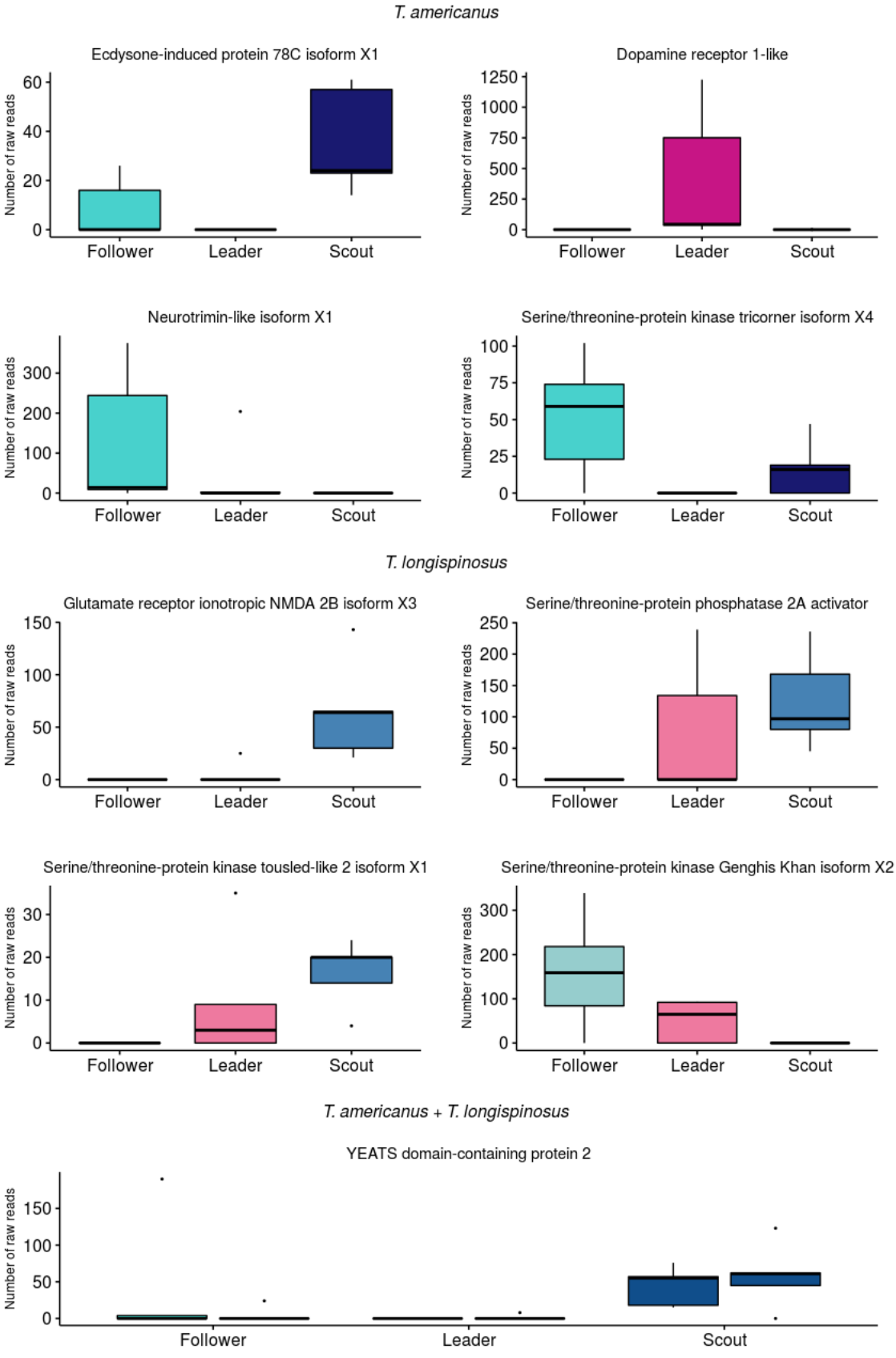

Figure 6-5 Boxplots showing the differences in raw read counts between behavioural groups of the discussed candidate genes.

In contigs up-regulated in scouts of *T. americanus*, the "steroid hormone mediated signalling pathway" (1) was found to be enriched. Additionally, in module 145 of *T. americanus,* whose expression pattern was significantly higher in scouts compared to leaders, the term "positive regulation of autophagy" (1) was enriched, while genes grouped in module 260, where expression was higher in scouts compared to followers, the function "regulation of cyclin-dependent protein serine/threonine kinase activity" (2) was enriched (Figure 6-6). GO functions enriched in modules with higher expression in both leaders and followers compared to scouts included "regulation of histone acetylation" (1), "serotonin biosynthetic process" (1) and "response to pheromone". A module with the highest expression in followers was enriched for "histone H4-K20 trimethylation" (1).

Scouts of *T. longispinosus* up-regulated genes enriched for the function "immune response" (1), while "histone deacetylation" (1) and was enriched in genes up-regulated in leaders. In module 203, which was higher expressed in scouts compared to leaders and followers the GO term "positive regulation of TOR signalling" (1) was enriched (Figure 6-6). Additionally, the term "G-protein coupled receptor signalling pathway" (4) was enriched both in a module with the strongest expression in followers, while a similar term "adenylate cyclase-modulating G-protein coupled receptor signaling pathway" (1) was enriched in a module highly expressed in leaders.

Pathway analysis

Searching for pathways present in the up-regulated contigs of followers, leaders, and scouts yielded more results for *T. longispinosus* than for *T. americanus* (Supplementary: Table S9, Table S10). One of the pathways that only occurred in a single behaviour was "calcium-signalling pathway", which was significantly enriched in followers of *T. longispinosus.*

Gene expression comparison based on orthologous clusters

In order to compare gene expression patterns between species, we performed an orthologue/homologue analysis upon the complete transcriptomes of both species. Using Orthofinder, we identified 181,685 orthogroups, which comprised 49% of all contigs used as input. Of these, 12,149 orthogroups were single-copy, thus with one contig per species. An additional analysis using a custom R script – designed to obtain additional single-copy orthogroups from non-single-copy orthogroups – resulted in an additional 13,950 single-copy orthogroups, bringing the final number of single-copy orthogroups up to 26,099 (Supplementary: Table S11). However, after inter-species comparison, none of the genes differentially expressed in one species was found to also be differentially expressed for the same behaviour of the other species. Additionally, we created in the same way as described above single-copy orthogroups between the differentially expressed contigs of one species and the transcriptome of the other. This resulted in a total number of 136 single-copy orthogroups for *T. americanus* and 119 for *T. longispinosus* (Supplementary: Table S12, Table S13). Based on this set of orthogroups, seven genes were identified, which were DE in both species. However, only one gene showed the same expression pattern and was up-regulated in scouts, the YEATS domain-containing protein 2 (Table 6-3). With this one exception, homologous gene expression analyses revealed that different genes were associated with our focal behaviours in the two species.

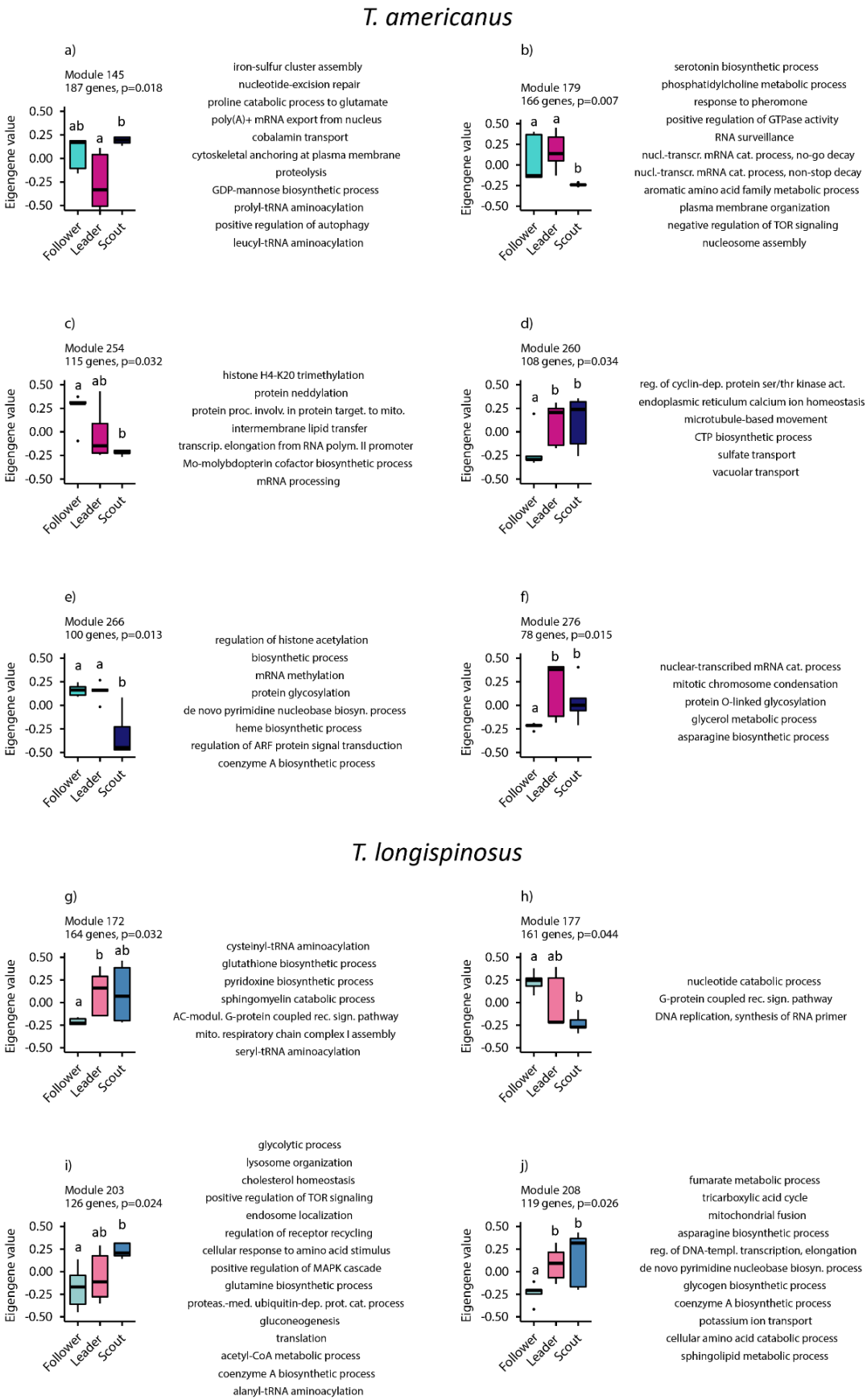

Figure 6-6 GO enrichment of modules significantly differing in eigengene values according to behaviour for *T. americanus* (**a–f**) and *T. longispinosus* (**g–j**). Discussed functions are printed in bold. Boxplots on the left indicate the effect of group, displaying the p-value of the Kruskal–Wallis test and the significance of Wilcoxon test pairwise comparisons using compact letter display.

Discussion

The original function of tandem-running behaviour – which has been described previously as a form of animal learning (Franks and Richardson, 2006; Richardson *et al.*, 2007; Leadbeater *et al.*, 2006) – is the recruitment of nestmates to resources such as food or new nesting sites. This behaviour is most commonly utilized in ants with small colony sizes and is typified by the behavioural recruitment of individual workers, which learn landmarks during the tandem-run to find their way to important resources (Pratt *et al.*, 2001). However, the adoption of alternate lifestyles by an ant species might necessitate the repurposing of tandem-running behaviour. Slavemaking *Temnothorax* species utilize a tandem-running behaviour during slave raids upon host colonies. We show here that in the slavemaking species *T. americanus* and its preferred host *T. longispinosus*, scouting, leading, and following behaviours are associated with the up-regulation of a small set of genes within the brain, which clearly differ between species and are often found to be associated with learning and memory formation in other insects.

Learning and memory functionality underlies tandem-running behaviour

The relatively low number of behaviour-specific DEGs can be explained by the fact that tandem-running behaviour in *Temnothorax* ants is exhibited by workers of the same behavioural caste and similar age. Scouts, followers, and leaders are the oldest and most experienced workers of a colony, as they engage in the riskier outside tasks (Negroni *et al.*, 2016; Kohlmeier *et al.*, 2018).

As outlined previously, tandem-run leaders could have been either scouts or followers prior to becoming a leader, whereas scouts will rarely become followers and likewise followers will rarely become scouts. Consequently, we expected leaders to exhibit the lowest number of uniquely expressed genes and to share more co-differentially

expressed genes with either scouts or followers. Our findings support these expectations to a certain extent, as indeed in both species leaders have the lowest number of differentially expressed genes and share more genes compared to the co-differentially expressed genes between scouts and followers (Figure 6-2). Yet these patterns did not statistically deviate from the other two behavioural types. However, further evidence on the intermediate state of leaders comes from our WGCNA results. In the ten modules significantly associated with behaviour, the leaders always group with either scouts or followers; whereas the eigenvalues of scouts differed from followers in eight of the ten cases (Figure 6-6).

Among DEGs associated with specific behaviours, many were found to be involved in learning and memory in other insects, as indicated by enrichments of these functions. Scouts, in particular, up-regulated numerous learning and memory genes, which was expected as scouts more so than leaders and followers have to gather spatial information to navigate back to their nests. Ecdysone-induced protein 78C isoform X1 is such a learning gene up-regulated in *T. americanus* scouts that was linked to long-term courtship memory in *Drosophila* (Ishimoto *et al.*, 2009). Another important learning gene is glutamate receptor ionotropic, NMDA 2B isoform X3, which was up-regulated in *T. longispinosus* scouts compared to followers (Table 6-1). In an earlier study, we already found glutamate receptor NMDA to be up-regulated in the brain of *T. longispinosus* workers, at that time in response to an attacking slavemaker (Kaur *et al.*, 2019). Our findings are in line with previous investigations into honey bees, in which genes related to glutamate signalling were up-regulated in scouts (Liang *et al.*, 2012). Additionally - and also within honeybees - glutamate signalling genes were found to be differentially expressed between both food source- and nesting site-scouts compared to their recruits (Liang *et al.*, 2014). Moreover, inhibition of the expression of

a subunit of the NMDA receptor in the honey bee brain resulted in the impairment of memory formation (Müßig *et al.*, 2010). The up-regulation of both the glutamate receptor and ecdysone-induced protein 78C isoform X1 support our initial prediction that learning functionality is indeed important for *Temnothorax* when exploring novel environments. Followers additionally show social learning, as they receive social information from leaders. In this context, the gene neurotrimin-like isoform X1 - which was found to be up-regulated in slavemaker followers - is interesting, as neurotrimin-knockdown mice show deficits in emotional learning (Mazitov *et al.*, 2017).

Biogenic amines also play a key role in learning, memory, and exchange of information within insects (Scheiner *et al.*, 2006; Blenau *et al.*, 2000; Kim *et al.*, 2007; Berry *et al.*, 2012; Awata *et al.*, 2016). Here we show that dopamine receptor 1 was up-regulated in the leader phenotype of *T. americanus* compared to both scouts and followers. While octopamine increases sensitivity for sensory inputs and thus improves learning, other biogenic amines including dopamine often have antagonistic effects on learning, at least in honey bees. Indeed, as leaders already know the way to the resource of interest, while both scouts and followers are in the process of learning, the expression pattern that we detected can be explained by an upregulation of learning functionality in scouts and followers relative to leaders.

Several serine/threonine-protein kinases and phosphatases candidate genes were up-regulated in *T. americanus* followers and *T. longispinosus* scouts and followers, both characterized by the learning of novel environments (Table 2). Serine/threonine protein kinase or phosphatase activity is required in learning and memory formation in mammals (Mansuy and Shenolikar, 2006; Rahman *et al.*, 2012a).

Enriched functions related to learning, memory, and lifespan

Amongst functions of the genes differentially expressed in both species, we find some related to learning and memory, matching our initial expectations. Post-translational processes like "regulation of histone acetylation" (1) seem to play a role in followers and leaders of the slavemaker, a process that when reduced by blocking the histone acetyltransferase (HAT) showed impairment of memory formation in the honey bee (Merschbaecher *et al.*, 2016, 2012). Additional enrichment of up-regulated genes in followers for "histone H4-K20 trimethylation" (1), another epigenetic mechanism that was linked to memory formation in *A. mellifera* (Biergans *et al.*, 2012; Lockett *et al.*, 2010), suggests that especially followers in *T. americanus* are learning. Genes up-regulated in leaders of the host on the other hand were enriched for "histone deacetylation" (1), an epigenetic mechanism contrary to histone acetylation. Interestingly, also deacetylation is linked to memory as inhibition results in impaired aversive memory in honey bees (Lockett *et al.*, 2014). A study in *D. melanogaster* showed that both up-regulation as well as knockdown of histone deacetylase impairs memory (Fitzsimons *et al.*, 2013), suggesting that a certain amount of histone deacetylation is necessary for memory formation.

Biogenic amines are not only important for learning (Sitaraman *et al.*, 2008), but also play a role in other behaviours like foraging (Schulz and Robinson, 1999) and trail following in insects (Muscedere *et al.*, 2012). Therefore it is not surprising that we find the "serotonin biosynthetic process" (1) to be enriched in both followers and leaders in *T. americanus*. Followers and leaders of *T. longispinosus* were enriched for similar terms: "G-protein coupled receptor signalling pathway" (4) and "adenylate-cyclase modulating G-protein coupled receptor signalling pathway" (1), which can both be linked to learning (Dolezelova *et al.*, 2007; McGuire *et al.*, 2005; Livingstone *et al.*, 1984).

In addition, we found that scouts of slavemakers and hosts differed in the expression of genes linked to the TOR signalling pathway, which is linked to lifespan in *D. melanogaster* (Kapahi *et al.*, 2004). Scouts of the host species up-regulated genes linked to functions like "positive regulation of TOR signalling" (1) and "acetyl-CoA metabolic process" (1), indicating a negative effect on lifespan, presumably because scouts are the oldest workers, facing high extrinsic mortality, and thus do not need to invest in body repair and maintenance. In the closely related ant species *T. rugatulus*, a similar pattern was observed with "negative regulation of the TOR pathway" being enriched in young queens in comparison to old queens (Negroni *et al.*, 2019), while in the termite *Cryptotermes secundus* a gene associated with the TOR pathway was up-regulated in young compared to old queens (Monroy Kuhn *et al.*, 2019). Interestingly, slavemaking scouts up-regulate functions like "positive regulation of autophagy" (1) a process that increases lifespan in *D. melanogaster* and *C. elegans* (Mason *et al.*, 2018; Meléndez *et al.*, 2003). Our finding could also be linked to the fact that scouts face the risk of injury by hosts during raids and thus upregulate repair mechanisms.

Orthology between slavemaker and host

While *T. americanus* and *T. longispinosus* do show similar tandem-running behaviours during raiding and nest emigration, respectively, homology analysis only revealed a single gene up-regulated in scouts of both species, namely YEATS domain-containing protein 2. This protein belongs to a family with links to chromatin modification and transcription (Schulze *et al.*, 2009; Wang *et al.*, 2008). While enrichment analyses of the DEGs revealed epigenetic/histone associated functions to be overrepresented in leaders and followers, this epigenetic regulator appears as a single candidate commonly differentially expressed in scouts of both species. This pattern is corroborated by the general picture of the differentially expressed genes between

these two species, which indicates that they mainly employ different genes to carry out the different behaviours. We find a similar pattern in a recent study comparing the transcriptomes of three slavemaker species during raiding behaviour and outside raiding season with three host species during nest defence and when not under attack (Alleman *et al.*, 2018), and the same holds true for genes with signatures of positive selection (Feldmeyer *et al.*, 2017). This indicates species-specific selection pressures on *T. longispinosus* and *T. americanus*, possibly due to their divergent and antagonistic lifestyles.

However, even within a single species – the honey bee – brain gene expression patterns differed between scouts when sampled in two different contexts: searching for food sources and nest emigration (Liang *et al.*, 2014). Indeed, this evidence that brain gene expression can differ radically between contexts with seemingly minor behavioural differences is intriguing, as behavioural differences are even more pronounced when contrasting scouting and tandem-running behaviours between *T. americanus* and *T. longispinosus*. For example, for slavemakers raiding includes confrontation, and they often recruit multiple nestmates – slavemaker and slave alike – for raiding parties; suggesting species-specific adaptations. Hence, we examine here two separate species with two contextually-unique tandem-running behaviours. Thus, any differences in gene usage between these two species could be due to one or both of these factors. The genetic toolkit hypothesis applied to behaviour states that the similarities in behaviour even between different species is due to the differential expression of common, highly conserved, and structurally similar toolkit genes like the *foraging* gene in *D. melanogaster* and *Amfor* in *A. mellifera* (Toth and Robinson, 2007). Indeed, in *Polistes* paper wasps, regulation of genes associated with foraging and provisioning behaviours appear to be conserved across lineages (Toth *et al.*, 2010). However, a more recent study on the different castes of honey bees, social wasps, and

ants showed that - across these distinct social insect lineages - functions and pathways, rather than genes, are conserved (Berens *et al.*, 2015a). Thus, the extent to which genes or functions are conserved across lineages does appear to vary and is potentially lineage-specific.

Framed by these previous findings, we neither find gene overlap nor functional similarity between the raiding behaviour of the slavemaker *T. americanus* and tandem-running associated with colony relocation in the non-slavemaking *T. longispinosus*. Since the common ancestor of all *Temnothorax* was non-parasitic (Beibl *et al.*, 2005), this would seem to suggest that modern *Temnothorax* slavemakers such as *T. americanus* utilize a genetically-distinct behaviour from traditional non-slavemaking tandem-running. Whether this is the result of heavy modification of existing tandem-running pathways induced by selective pressures resulting from a parasitic lifestyle or an entirely new behavioural phenotype remains unclear; however as *Temnothorax* ants rely less on pheromone trails (Basari *et al.*, 2014), any mechanism for leading nestmates to important resources would likely be under strong selective pressures.

Conclusion

Here we elucidate which genes underlie scouting, leading, and following behaviour within the tandem-running-like behaviours of two *Temnothorax* ant species: the slavemaking *T. americanus* and its primary, non-parasitic host *T. longispinosus*. A number of genes were identified within these behaviours that have previously been linked to functions such as learning, memory, or foraging in other insects like *Apis mellifera* and *Drosophila melanogaster*. Especially intriguing is the dopamine receptor expressed in leaders of *T. americanus* and the glutamate receptor ionotropic up-regulated in scouts of the host, genes with clear learning and memory functionalities. Our findings in part support previous behavioural studies demonstrating that tandem-

running is a process of learning, and subsequently passing that learned information on to nestmates (Richardson *et al.*, 2007; Caro and Hauser, 1992). Further RNAi knockdown of genes involved in learning/teaching and found to be differentially expressed between the behaviours herein examined would be a logical next step for understanding the role genes and pathways play in slavemaking and non-slavemaking tandem-running behaviour.

Relatively low numbers of DEGs within each tandem-running behaviour indicates that the differential expression of only a few genes is required to alter *Temnothorax* behaviour. However, we were unable to identify DEG orthologues between the comparable tandem-running behaviours of *T. americanus* and *T. longispinosus*. Whether this lack of orthology in externally-similar behaviours is due to the context under which the behaviours are utilized, or due to species-specific selective pressures, is unclear. At present, however, the raiding behaviour of the slavemaker *T. americanus* and the nest relocation tandem-running behaviour of *T. longispinosus* do appear to have very little overlap in gene expression, suggesting lifestyle-driven divergent evolution between these two *Temnothorax* species. Indeed, even within-species, externally similar behaviours like searching for food and nest immigration in honeybees can have very different gene expression patterns (Liang *et al.*, 2014). Among *Temnothorax*, enslaved hosts will also engage in raiding activity alongside their slavemakers, providing us with a unique opportunity to investigate brain gene expression in *T. longispinosus* followers both during raiding and tandem-running for nest relocation. This should shed additional light on whether behavioural context or species-specificity underlies the differences in gene expression between raiding and relocation tandem-running behaviour and thus bring us one step closer to understanding the underlying molecular uniqueness of raiding behaviour in ants.

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Author Contributions

A. A. and S. F. were jointly responsible for the concept and design of the work. Ants were collected by A. A. and S. F. Data were primarily collected by A. A., and analyzed by M. S. B. F. contributed to the bioinformatics analysis. Manuscript drafted primarily by A. A. and was critically revised by all authors.

Supplementary Materials

Additional material can be retrieved from the following Dryad repository https://doi.org/10.5061/dryad.b412jp5 which contains the following: Trinity RNA sequence assemblies/ transcriptomes for both *T. americanus* and *T. longispinosus*, filtered RNA sequence assemblies/ transcriptomes for both species, blast annotation of transcriptomes for both species, InterProScan annotation of both *T. americanus* and *T. longispinosus* transcriptomes, analytical scripts, expression matrices, lists of differentially expressed contigs for both species, lists of contigs in modules of WGCNA for both species, GO enrichments of gene lists for both species, pathway enrichments of gene lists for both species and lists of Orthogroups for both species. Remaining RNA sequences deposited into NCBI SRA under BioProject ID PRJNA503093

Chapter 7

Comparative analyses of caste, sex, and developmental stage-specific transcriptomes in two *Temnothorax* ants

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Abstract

Social insects dominate arthropod communities worldwide due to cooperation and division of labour in their societies. This, however, makes them vulnerable to exploitation by social parasites, such as slave-making ants. Slave-making ant workers pillage brood from neighbouring nests of related host ant species. After emergence, host workers take over all nonreproductive colony tasks, whereas slavemakers have lost the ability to care for themselves and their offspring. Here, we compared transcriptomes of different developmental stages (larvae, pupae, and adults), castes (queens and workers), and sexes of two related ant species, the slavemaker *Temnothorax americanus* and its host *Temnothorax longispinosus*. Our aim was to investigate commonalities and differences in group-specific transcriptomes, whereupon across-species differences possibly can be explained by their divergent lifestyles. Larvae and pupae showed the highest similarity between the two species and upregulated genes with enriched functions of translation and chitin metabolism, respectively. Workers commonly upregulated oxidation-reduction genes, possibly indicative of their active lifestyle. Host workers, but not workers of the slavemaker, upregulated a "social behaviour" gene. In slavemaker queens and workers, genes associated with the regulation of transposable elements were upregulated. Queens of both species showed transcriptomic signals of anti-ageing mechanisms, with hosts upregulating various DNA repair pathways and slavemaker queens investing in trehalose metabolism. The transcriptomes of males showed enriched functions for quite general terms realized in different genes and pathways in each species. In summary, the strong interspecific commonalities in larvae, pupae, and workers were reflected in the same enriched Gene Ontology (GO) terms. Fewer commonalities occurred in the transcriptomes of queens and males, which apparently utilize different pathways to achieve a long life and sperm production, respectively. We found that all

analyzed groups in this study show characteristic GO terms, with similar patterns in both species.

Introduction

The ecological success of social Hymenoptera is based on tight cooperation and an efficient division of labour in their colonies. Reproduction is monopolized by one or a few female individuals (the queens), whereas all other colony members (the workers) take care of the brood and the queen, build and defend the nest, and forage for food (Hölldobler and Wilson, 1990), but never reproduce. The altruistic behaviour of workers can be exploited by social parasites, such as slave-making ants.

Freshly mated ant queens typically found new colonies independently or may be re-adopted by their natal colony. In contrast, young slavemaker queens usurp a nest of another, closely related ant species ("Emery's rule" (Emery, 1909)). They have to drive away or kill the resident queen and, in many species, all adult workers. Host workers emerging from the conquered brood take care of the slavemaker's own offspring (Buschinger, 1986; D'Ettorre and Heinze, 2001). Slavemaker workers have lost the ability to nurse the brood and forage and thus rely entirely on the help of their enslaved workforce. To replenish or increase this workforce, they conduct raids on host colonies to steal brood. Young slave-making workers also participate in reproduction and lay unfertilized male-destined eggs even in the presence of the queen (Foitzik and Herbers, 2001a; Franks and Scovell, 1983; Brunner *et al.*, 2005; Heinze, 1996), while host workers typically reproduce only in the queens' absence (Bourke, 1988). As fertility is often positively associated with longevity (Monroy Kuhn and Korb, 2016) and egg-laying queens and workers live longer than their nonreproductive nestmates (Heinze and Schrempf, 2012; Kohlmeier *et al.*, 2017; Tsuji *et al.*, 1998), workers of slave-making ants might be more long-lived than host workers. Hence,

though typically closely related, slave-making ants and their hosts differ substantially in their life histories.

The morphological and life history differences between slavemakers and their hosts are likely reflected in different patterns of gene expression. Here, we compare gene expression of different castes, developmental stages, and sexes of the slave-making ant, *Temnothorax americanus* (Emery, 1895) (formerly *Protomognathus americanus*) and its primary host *Temnothorax longispinosus* (Roger, 1863). The aim of our study is to determine the phenotype-specific expression patterns between castes and sexes and to compare them between species showing different lifestyles — slavemaker and host. Within the ant subfamily Myrmicinae, the Formicoxenus-species group (sensu Blaimer *et al.*, 2018) is a hot spot for the evolution of slave-making, with at least four independent origins within the genus *Temnothorax* alone (Alleman *et al.*, 2018; Beibl *et al.*, 2005). Our two focal species have been well-studied as a model for an ongoing coevolutionary arms race between social parasites and hosts (Foitzik *et al.*, 2001; Jongepier *et al.*, 2014; Kaur *et al.*, 2019). For example, analyses of brain gene expression in *T. americanus* and *T. longispinosus* workers during scouting and tandem running revealed a divergent molecular regulation of seemingly similar behaviour in the two related species (Alleman *et al.*, 2019).

The aim of the present study is to identify characteristic expression patterns of larvae, worker pupae, workers, queens, and males and to search for commonalities and differences between the two ant species *T. americanus* and *T. longispinosus*. On the one hand, we contrast here whole-body gene expression of ants with divergent morphological and behavioural phenotypes, so that the transcriptomic signatures should clearly differentiate between these groups within each species. On the other hand, larvae of both species are expected to overexpress growth-related genes, and

pupae should commonly overexpress genes associated with tissue reconstruction and cuticular synthesis (Dubrovsky, 2005). Queens of the two species are predicted to show strong transcriptomic correlates of reproduction and longevity (Negroni *et al.*, 2019). Due to their different life histories, worker and queen transcriptomes might show less interspecies commonalities in gene expression. The short-lived males are expected to strongly invest in sperm production and less in anti-ageing mechanisms.

Material and Methods

Collection and colony maintenance

Colonies of the ant species *T. americanus* and *T. longispinosus* were collected in June 2016 at the E.N. Huyck Preserve, Rensselaerville, New York, USA (42°31′43.8″N, 74°09′44.7″W), from which collection permits were obtained. After transfer to the laboratory, ant colonies were kept in three-chambered nest boxes with a plaster floor and a nest site consisting of a Plexiglas® frame sandwiched between two microscope slides covered by red foil. The ants were maintained in a climate chamber at artificial summer conditions at 23°C with a 12 hr day/12 hr night cycle until sampling in July and August 2016. The ants were fed with crickets and honey twice a week. Fresh water was available ad libitum.

Extraction, sequencing, and de novo assembly

RNA extraction of all samples was conducted in November 2016 using the RNeasy mini extraction Kit (Qiagen, Inc.). For each species, we analyzed a medium-sized larva, an unpigmented worker pupa, a male, an adult worker, and a fertile queen from three different colonies each (N = 3). We extracted RNA from whole bodies, as we had no prior assumptions as to which tissue differed most strongly in gene expression between our groups. To determine the sex of the larvae, we tested heterozygosity. For

T. longispinosus, the test shows clearly that all larvae are female. For *T. americanus*, the samples show a heterogeneous pattern of heterozygosity, for example, lowest heterozygosity in two of the queens; thus, we cannot clarify whether the larvae in this species are male or female.

Library construction and sequencing were conducted at BGI Hong Kong on an Illumina HiSeq 4000. For rRNA depletion, a poly-A selection was conducted. With a read length of 100 bp, the libraries resulted in a total of 40 million paired-end reads (4 GB) for each sample. The quality of raw reads was assessed using "FastQC" v0.11.4 (Andrews *et al.*, 2010), and Illumina adapters were cut from all sequences using Trimmomatic v0.32 (Bolger *et al.*, 2014). We conducted a de novo transcriptome assembly for both species using Trinity v2.4.0 (Grabherr *et al.*, 2011), including the paired forward and reverse sequences of the 15 samples for each species. For *T. longispinosus*, the transcriptome had a total length of 275,441,418 bp, equivalent to 62,775 Trinity "genes" or 160,971 Trinity transcripts with a GC content of 42.32%. The transcriptome of *T. americanus* had a total length of 267,319,768 bp, equivalent to 86,416 Trinity "genes" or 228,156 Trinity transcripts with a GC content of 42.45%. The reads of the samples mapped back to their corresponding transcriptomes with an average back mapping rate of 82.27% (for *T. longispinosus*) and 66.17% (for *T. americanus*).

Differential gene expression and enrichment analyses

To investigate the clustering of samples according to the expression profiles of the 20,000 most variant genes, we used WGCNA v1.64-1 (Langfelder and Horvath, 2008), a package of the software R (R Core Team, 2018). Paired forward and reverse reads were mapped to the corresponding transcriptomes using Bowtie2 v2.3.5 (Langmead and Salzberg, 2012), and read counts were estimated using RSEM v1.3.0 (Li and Dewey, 2011). To identify genes upregulated in a specific developmental stage or caste, we

calculated the differentially expressed genes (DEGs) in a pairwise comparison within each species using the R package DESeq2 v1.14.1 (Love *et al.*, 2014) as embedded in Bioconductor v3.4 (Gentleman *et al.*, 2004). DESeq2 models read counts assuming a negative binomial distribution using generalized linear models with logarithmic link to access the log2 fold change between two conditions of one factor or even designs including more than one factor. For our pairwise comparisons, we used the Wald test as implemented in DESeq2 to obtain genes that differed significantly in their expression between two groups of the only factor in our design, developmental stage/caste. This was done for all possible 20 pairwise comparisons, only keeping genes with a positive log fold change and an FDR adjusted p-value < 0.05. We then merged the lists of genes that were upregulated in the same developmental stage or caste compared to any of the other groups (see Supplement S1 and S2) and created a Venn diagram of the resulting five sets using the webtool provided on bioinformatics.psb.ugent.be/webtools/Venn. This allowed us to extract the genes that were only upregulated in a single developmental stage or caste, in the following referred to as "privately upregulated," as well as the genes that were upregulated in all but one developmental stage/caste, which we here refer to as "privately downregulated" in the latter. To compare the number of DEGs across species, we had to take the transcriptome size into account, which was larger in *T. americanus*.

The nucleotide sequences of both transcriptomes were translated into amino acid sequences using TransDecoder v. 3.0.1 (https://github.com/TransDecoder). We classified these sequences into families and predicted domains by running Interproscan v. 5.27-66.0 (Quevillon *et al.*, 2005) on the amino acid sequences. For functional annotation, we performed a GO enrichment analysis with topGO v. 2.28.0 (Alexa and Rahnenfuhrer, 2018) using Fisher's exact test to test for enrichment of GO

terms in the sets of privately up-or downregulated genes compared to the whole transcriptome of the focal species. We only took terms into account, which had at least 10 annotations in the transcriptome (node size 10). We illustrated the GO terms in each gene set, which had a p-value < 0.05, in a word cloud, adjusting the font size according to the p-value (see Supplement S3 and S4)(font size adjustment = −1 × log (p-value)) using the R package tagcloud v. 0.6 (Weiner, 2015).

Analysis of orthologous genes

First, we investigated whether the privately differentially expressed genes of the several groups show sequence similarity, that is, correspond to the same gene between species. Therefore, we ran Orthofinder v2.2.3 (Emms and Kelly, 2015) to find upregulated orthologous gene sequences. Only orthogroups with exactly one sequence per species were considered for downstream analyses (from now on referred to as single-copy orthologs). These single-copy orthologs were used to compare gene expression patterns between species for each developmental stage and caste.

Additionally, we performed a separate gene expression analysis by obtaining single-copy orthologs between the two transcriptomes and afterwards again running DESeq2 including phenotype and species as well as their interaction in the model. Afterwards, we compared the expression of genes inside each caste between species again in pairwise comparisons. We then performed GO enrichment analyses on the resulting gene lists for the genes upregulated in *T. longispinosus* and the genes upregulated in *T. americanus* separately.

GO enrichment was again performed as described before, but since only the orthologous genes were used for the gene expression analysis, GO enrichment analysis was also only performed with the entirety of orthologous genes as

"universe." Each orthogroup was associated with two sequences, one in *T. americanus* and one in *T. longispinosus*. Although the sequences in each orthogroup should be very similar and therefore yield the same GO annotations, there were some minor differences in the annotations of the orthologs for both species. Therefore, the annotated GO terms were merged so that in case of a conflict both the GO terms of *T. americanus* as well as the ones of *T. longispinosus* were used for this gene as "universal" annotations.

Results

Clustering of transcriptomes

Samples of the host species *T. longispinosus* cluster according to developmental stage, caste, and sex, with larvae being the outgroup to all others (Figure 7-1a). The queen and worker groups are grouped together, and so are the males and worker pupae. Clustering was much less clear in the slavemaker *T. americanus*. Here, only the worker pupae and males clustered in one group each. Instead, samples of *T. americanus* were grouped into two major branches, one containing the worker pupae, workers, and queens, the other all males, larvae, and one odd worker (Figure 7-1b). Removing the latter worker from the analysis did not change the allocation of the other specimens. The separation of the two major branches was deeper than in *T. longispinosus*, suggesting stronger differences between males and larvae compared to all the other samples of *T. americanus*.

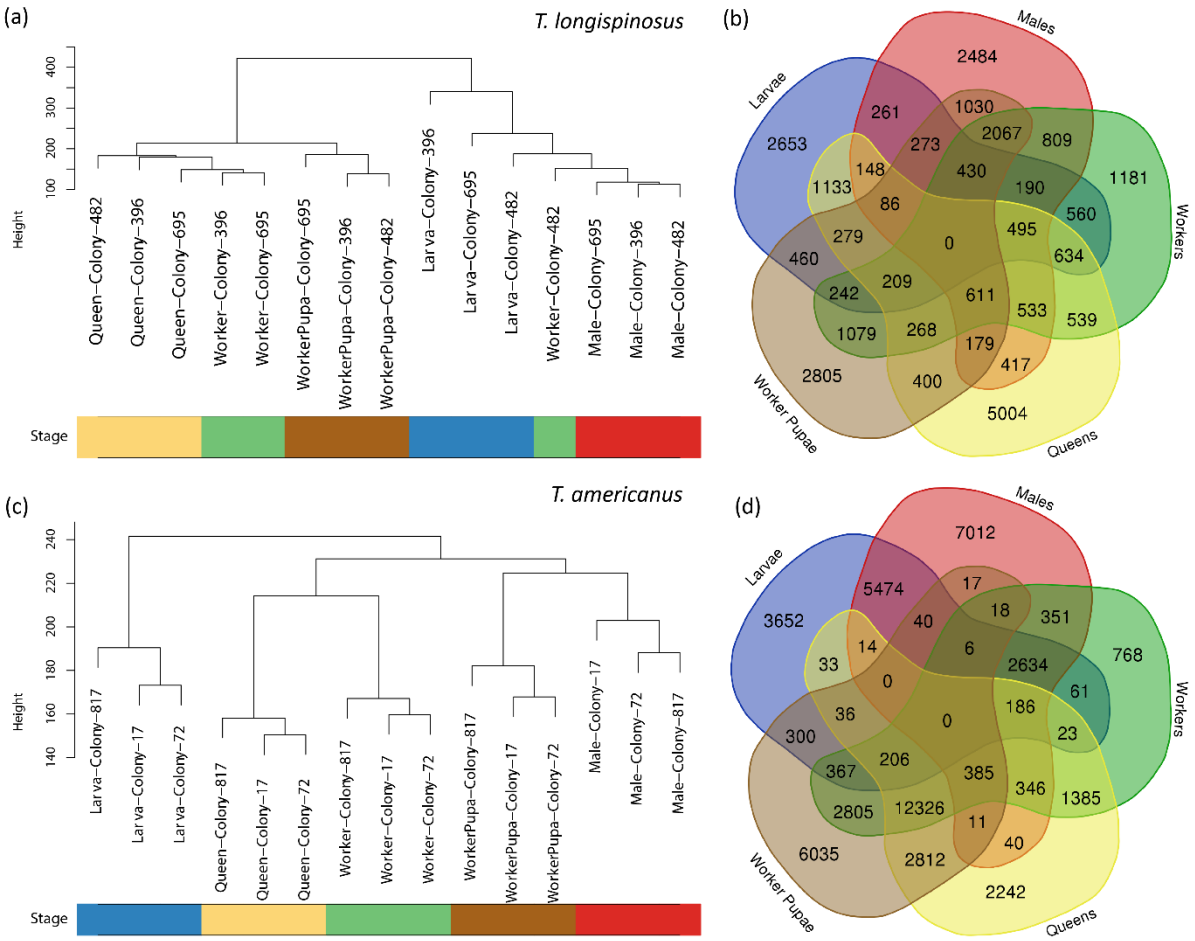

Figure 7-1 Gene expression differences between groups of individuals in the slave-making ant *Temnothorax americanus* and its main host *Temnothorax longispinosus*. Dendrograms were created using UPGMA with the Euclidean distance between all genes of the samples as input for (**a**) *T. longispinosus* and (**c**) *T. americanus*. Venn diagrams show the number of genes overexpressed with an adjusted p-value < 0.05 in each phenotype and the overlap between these calculated using Wald test pairwise comparisons as implemented in DESeq2 for (**b**) *T. longispinosus* and (**d**) *T. americanus*.

Gene expression analysis

In our within-species analyses, we found a total of 27,459 (17.06%) unique genes to be upregulated in at least one caste of *T. longispinosus*. The largest number of privately upregulated genes was found in queens (5,004, i.e., 3.1% of all transcripts), while workers had the lowest number of privately upregulated genes (1,181, 0.7%). Queens and workers co-upregulated 3,289 genes (2.0%), while worker pupae and larvae shared only 1,979 (1.2%) of the upregulated genes.

In *T. americanus*, a total of 49,585 (21.73%) genes were upregulated in at least one caste. Again, workers exhibited the lowest number of private DEGs (768, 0.3%), which was significantly less than in *T. longispinosus* ($\chi2 = 297.78$, $df = 1$, $p < 0.00001$). *Temnothorax americanus* queens had less than 1.0% of uniquely upregulated genes (2,242), that is, significantly less than the DEGs detected in host queens ($\chi2 = 2,333.2$, $df = 1$, $p < 0.00001$). The highest overlap of upregulated genes occurred among all female specimens, that is, workers, worker pupae, and queens, which commonly upregulated 12,326 genes (5.4%). This is a much higher overlap than that between the respective castes in *T. longispinosus* (268 genes (0.17%); $\chi2 = 8,260.5$, $df = 1$, $p < 0.00001$).

Besides other differences in the number of upregulated genes in the different groups between species, we found more DEGs among developmental stages, castes, and sexes in *T. americanus* (including shared genes) than in *T. longispinosus* ($\chi2 = 3,786.9$, $df = 1$, $p < 0.00001$).

The analysis of the downregulated genes gave no further information about group-specific functions as the resulting terms were very general. Therefore, this study concentrates on the upregulated genes only.

Enrichment analyses

GO enrichment analysis revealed that genes of the same functionality were differentially expressed in larvae, worker pupae, and workers of the two species (Figure 7-2). In larvae, this included genes with the functions “translation” and “chitin and carbohydrate metabolism,” which are both indicative of growth. The most outstanding gene function in worker pupae of both species was “chitin metabolic process,” pointing to the production of the cuticle as a major process during the pupal

stage. In addition, genes with "cell adhesion functions" were upregulated in pupae of both species. Differentially expressed genes in workers of the two species were dominated by the function "oxidation-reduction process." Workers are more active than the other groups and this possibly explains this interspecies commonality in gene expression in workers. We found the enriched term "social behaviour," based on one gene of *Gp9*-like pheromone binding proteins, only among the DEGs of workers of the host *T. longispinosus*.

The two most prominent GO terms in *T. longispinosus* queens, "DNA recombination" and "DNA repair," might be associated with their longevity and fecundity. Similarly, *T. americanus* queens upregulated genes linked to trehalose metabolic processes and other sugar-related catabolic and metabolic processes. *Temnothorax americanus* queens also upregulated genes linked to response to oxidative stress, because either they suffer from increased oxidation or, more likely, prevent damage from oxidative stress, which also plays a role in ageing (Finkel & Holbrook, 2000). Both queens and to a lesser extent workers of *T. americanus* upregulated genes (21 genes in total) with the functionality "DNA integration," which is strongly associated with the activity of transposable elements.

Predominant GO terms in *T. longispinosus* males were "ATP synthesis-coupled proton transport" and diverse "biosynthetic processes," while GO terms in *T. americanus* males included "protein phosphorylation" and "intracellular transport." Interestingly, neither queens nor males shared many genes and gene functions across species, indicating species-specific gene expression in reproductives.

Analyses of orthologs

We first tested whether the same genes were responsible for group-specific gene expression in both species by searching for an overlap between the differentially expressed genes of one species and the orthologs of these differentially expressed genes in the other. With less than 5%, this overlap was generally small, but it was higher in the pupal and larval stages (mean 2.7%) than in the adult stages (1.2%; Figure 7-3).

Our orthology analyses revealed 30,758 orthologous genes in the two species, on which we based the following comparisons. A dendrogram of the samples of the two species based on these orthologs revealed three clusters, the outgroup being the larvae, males, and one odd worker of *T. americanus* (Figure 7-4). Samples of the same developmental stage, caste, or sex never clustered with representatives of the other species, but always with members of their own species. The topology of the dendrogram within species resembled that of the species-specific analyses (Figure 7-1a and c).

Figure 7-2 Results of the GO enrichment analyses based on genes uniquely overexpressed in each of the five groups (larva, worker pupa, worker, queen, and male) of the slave-making ant *Temnothorax americanus* and its main host *Temnothorax longispinosus*. GO enrichment was calculated using topGO using the weight01 algorithm and comparing GO annotations of biological processes of the lists of uniquely overexpressed genes with the ones of the whole transcriptome using Fisher's exact test. Displayed are only terms with a p-value below 0.05; font-size negatively correlates with p-values.

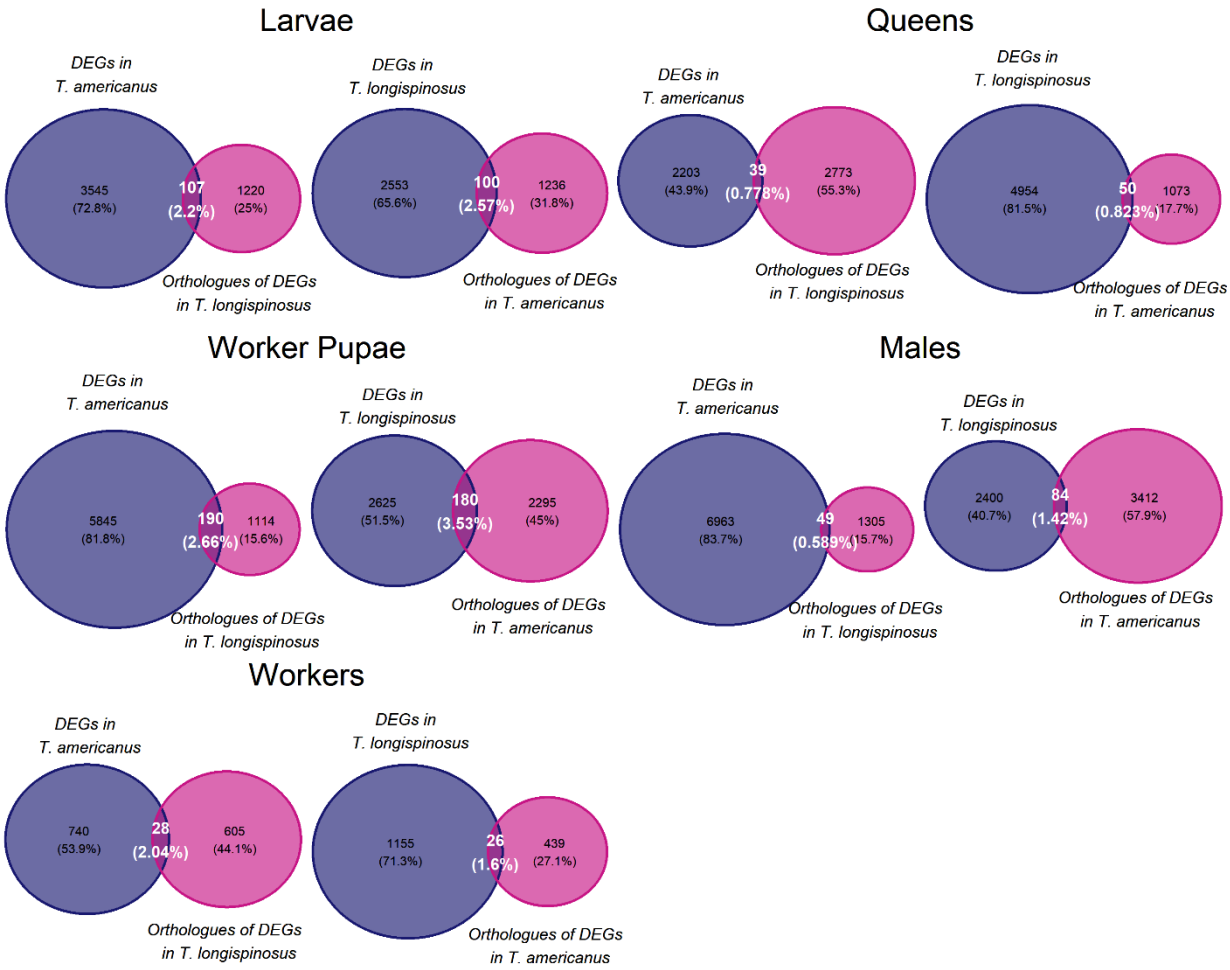

Figure 7-3 Overlap of overexpressed genes across species. Orthologous genes of uniquely overexpressed genes were searched for in the respective other species, and these were compared to the actual uniquely overexpressed genes in this other species. The figures on the left show the number of unique DEGs found in one phenotype in the slavemaker *Temnothorax americanus* (in purple), and the orthologs of the unique DEGs of its host, *Temnothorax longispinosus* in pink. The intersections are those genes found differentially expressed in both species of the respective group. The figures on the right show the unique DEGs of *T. longispinosus* in pink and the respective overlap of orthologous genes of unique DEGs in *T. americanus*.

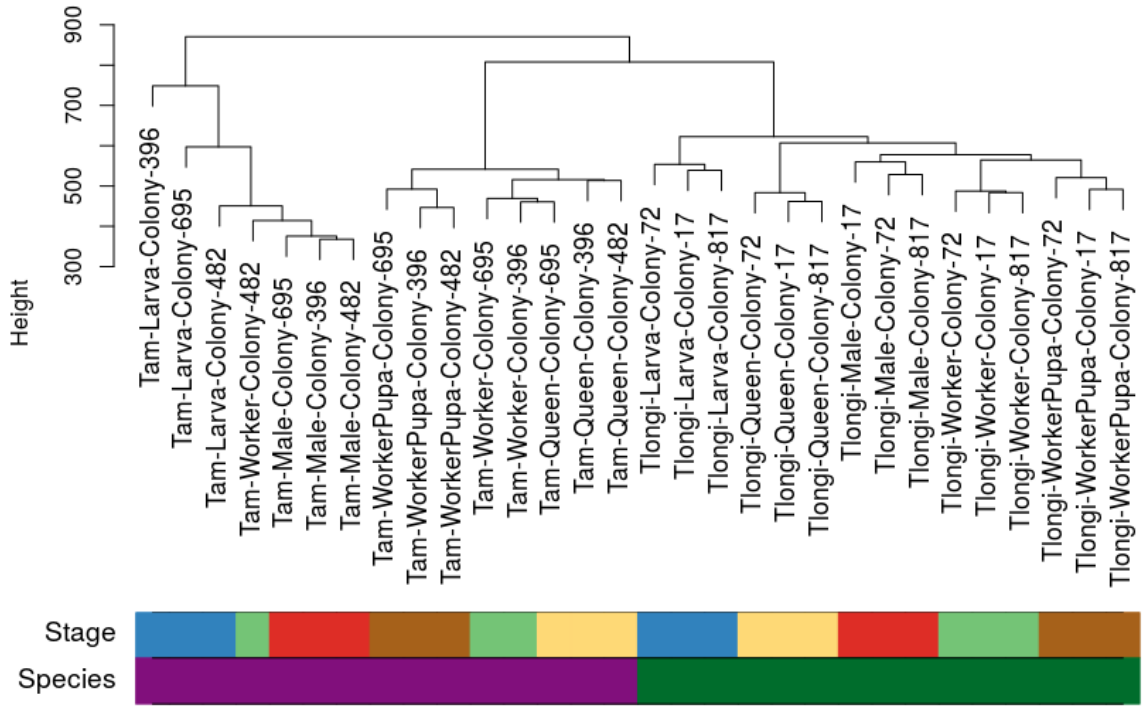

Figure 7-4 Dendrogram of samples of the slave-making ant *Temnothorax americanus* and its main host *Temnothorax longispinosus* based on single-copy orthologous genes only. Clustering was performed using UPGMA of Euclidean distances between orthologous genes of samples of both species.

For single-copy orthologous genes upregulated in the host *T. longispinosus* compared to the slave-making ant *T. americanus* and vice versa, we found the smallest difference in the number of DEG between *T. longispinosus* and *T. americanus* workers (245, 0.8%). Indeed, only 76 orthologous genes were found to be upregulated in the host *T. longispinosus*, in which workers take over the normal worker tasks, compared to *T. americanus*, in which workers mainly focus on raiding and reproduction. More differences in gene expression were found between *T. longispinosus* and *T. americanus* queens, which differentially expressed 1,916 genes (6.23%), and especially between males, for which we found the strongest transcriptomic differences (3,103 genes, 10.09%).

The GO enrichment analysis based on differentially expressed genes between species revealed that all castes, sexes, and developmental stages of *T. longispinosus* upregulated genes with "cellular metabolic process" functionality compared to the same stages in *T. americanus* (Figure 7-5). In *T. americanus*, conversely, we found that all groups but the queens upregulated genes linked to "translation."

Whereas the genes upregulated in *T. longispinosus* queens involved cellular metabolism, DNA repair and RNA processing functionalities, those upregulated in *T. americanus* were largely linked to "response to oxidative stress." Similarly, sensory processing genes were upregulated in slavemaker queens. In addition to an upregulation of "cellular metabolic process" genes, host workers showed an upregulation of genes associated with anti-ageing or body maintenance functionalities, such as "nucleotide excision repair" and "glutathione metabolic process." *T. americanus* workers instead upregulated more genes involved in translation, protein folding, and transport, which might be indicative of *T. americanus* workers being reproductively active.

Figure 7-5 Results of the GO enrichment analyses based on orthologous genes overexpressed between the slave-making ant *Temnothorax americanus* and its main host *Temnothorax longispinosus* in the five groups (larva, worker pupa, worker, queen, and male). Gene lists were created using only the single-copy orthologous genes between *T. americanus* and *T. longispinosus* as input for DESeq2, using phenotype and species as well as their interaction as explanatory variables and only taking genes with an adjusted p-value below 0.05. Displayed are only terms with a p-value below 0.05. Font size negatively correlates with p-values.

Discussion

Transcriptomic characteristics of developmental stages, castes, and sexes

The transcriptomes of the different developmental stages, castes, and sexes were characterized by gene functionalities important for their respective life phase and/or style. Whereas genes upregulated in larvae and pupae were mainly involved in growth and tissue buildup (e.g., chitin metabolism), those of workers reflected their physical activities (e.g., oxidation-reduction process). Furthermore, enriched functions in queens highlighted the importance of high fecundity and long lifespan. For example, the dominant GO term in *T. longispinosus* queens, DNA repair, is associated with anti-ageing strategies (Hart *et al.*, 1979; Lucas *et al.*, 2016). Similarly, in *T. americanus* queen transcriptomes were enriched for known longevity pathways, but interestingly different ones. For example, the 2nd most enriched functionality, "trehalose metabolic processes," is involved in the regulation of lifespan and resistance to stress in the red flour beetle (Xu *et al.*, 2013). Trehalose also plays a role in the insulin-signalling pathway, which affects insect lifespan (Broughton and Partridge, 2009; Broughton *et al.*, 2005; Tatar *et al.*, 2001).

"DNA integration," a GO term enriched in the transcriptomes of both *T. americanus* queens and workers, is associated with the activity of transposable elements. Transposable elements might counterbalance the negative effects of reduced genetic diversity by increasing genetic variation (Giraud *et al.*, 1997; Serrato-Capuchina and Matute, 2018). For example, they appear to facilitate the adaptation of invasive ant species to novel environments (Schrader *et al.*, 2014). In obligate social parasites, such as *T. americanus*, the effective population size is considerably smaller than in their hosts (Seifert, 2007). Transposable elements might help the slavemaker to catch up in the arms race with their hosts.

In males, which do not engage in colony activities, but focus on mating and reproduction, the enriched terms point to general cellular functions, which at present are difficult to interpret.

Commonalities and differences in gene expression between species

According to the genetic toolkit hypothesis, specific phenotypic traits are typically controlled by a conserved set of genes across species (Toth and Robinson, 2007; Toth *et al.*, 2010). Nevertheless, other studies showed that molecular pathways or functionalities rather than genes are conserved in evolution (Berens *et al.*, 2015a). Our analyses point to the latter: Even within the more similar early developmental stages, only less than 3% of the genes were commonly overexpressed in *T. americanus* and *T. longispinosus*, even though in larvae and pupae the same dominant functions were enriched. The more pronounced interspecies similarities during development and in workers compared to reproductives suggest a high degree of conserved molecular pathways in these groups. In bumblebees, gene expression was also more conserved during early development, while adult transcriptomes diverged more (Harrison *et al.*, 2015). In both *Temnothorax* species, larval transcriptomes were characterized by an upregulation of translation genes. This was expected, as protein biosynthesis is an important process during larval growth. Larvae and pupae of both species upregulated genes associated with chitin metabolism, reflecting the importance of the formation of the cuticle during development. Pupae also showed an upregulation of genes with cell adhesion functions, which play a role in the development of different cell types and are essential for epithelial tissue development (Harris, 2012; Rio, 1993). Workers of both species upregulated genes linked to oxidation-reduction processes relative to all other castes and developmental stages, supporting an earlier finding in *T. longispinosus* (Feldmeyer *et al.*, 2014). In female reproductives of both species, we found strong

signals of anti-ageing mechanism, even though different pathways were utilized. Whereas *T. longispinosus* queens mainly prevent DNA degradation, *T. americanus* queens invest in trehalose metabolism. Moreover, the orthology analysis revealed that slavemaker queens also invest more than host queens in genes linked to the response to oxidative stress. Why queens of the two species are using different molecular pathways to reach a long life span will be investigated in future studies.

Species-specific expression profiles

T. americanus and *T. longispinosus* differ in numerous traits, all of which might be reflected in divergent gene expression patterns. Nevertheless, gene functions that differ between *T. americanus* and *T. longispinosus* across castes might also provide first insights into the evolution and the consequences of the life history of slave-making ants. *T. longispinosus* queens and workers upregulated genes linked to cellular metabolic processes. Host ants might have a higher metabolism, which would match the constantly higher activity level of nonparasitic ants. *T. americanus* upregulated genes linked to translation in all castes except queens. The upregulation of translation genes might reflect the production and maturation of eggs in the ovaries of *T. americanus* workers, which are much more fecund than host workers (Foitzik and Herbers, 2001a).

Conclusion

Despite their close relatedness, the two ant species *T. americanus* and *T. longispinosus* show divergent lifestyles (Beibl *et al.*, 2005; Feldmeyer *et al.*, 2017). In agreement with our expectation, our gene expression analyses revealed similarities across the two species in molecular pathway characteristics for larval and pupal transcriptomes. Though adult workers of *T. americanus* and *T. longispinosus* show distinct behaviors

and morphologies, their transcriptomes are characterized by similar gene functions. Queens, however, differed strongly in gene expression and functionalities between *T. americanus* and *T. longispinosus*. Yet they commonly invested in anti-aging processes, but utilized different molecular pathways. Future studies involving more slavemaker and host species will reveal whether the differences in gene expression between the two species are idiosyncratic or associated with the shift from a free-living to a socially parasitic lifestyle.

Acknowledgments

[removed for privacy purposes]

Author Contributions

C.G.: Conceptualization (lead); data curation (lead); formal analysis (lead); methodology (supporting); writing – original draft (lead). M.S.: Formal analysis (lead); methodology (supporting); visualization (lead); writing – original draft (supporting). E.J.: Conceptualization (lead); writing – original draft (supporting). E.B.-B.: Conceptualization (lead); Writing – original draft (supporting). B.F.: Formal analysis (supporting); methodology (supporting); writing – original draft (supporting). J.H.: Conceptualization (lead); writing – original draft (lead). S.F.: Conceptualization (lead); writing – original draft (lead).

Supplementary Materials

The following supplementary materials are available online at https://doi.org/10.1002/ece3.6187:

Table S1: Differentially expressed genes from pairwise comparisons between different developmental stages, castes and sexes in *T. americanus*, Table S2: Differentially expressed genes from pairwise comparisons between different developmental stages, castes and sexes in *T. longispinosus*, Table S3: Enriched GO terms in uniquely overexpressed genes in different developmental stages, castes and sexes from intraspecific comparison of *T. americanus*, Table S4: Enriched GO terms in uniquely overexpressed genes in different developmental stages, castes and sexes from intraspecific comparison of *T. longispinosus*.

Raw reads can be accessed from SRA under BioProject ID PRJNA606685.

Chapter 8

Parasite presence induces gene expression changes in an ant host related to immunity and longevity

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Based on:

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Abstract

Most species are either parasites or exploited by parasites, making parasite-host interactions a driver of evolution. Parasites with complex life cycles often evolve strategies to facilitate transmission to the definitive host by manipulating their intermediate host. Such manipulations could explain phenotypic changes in the ant *Temnothorax nylanderi*, the intermediate host of the cestode *Anomotaenia brevis*. In addition to behavioural and morphological alterations, infected workers exhibit prolonged lifespans, comparable to that of queens, which live up to two decades. We used transcriptomic data from cestodes and ants of different castes and infection status to investigate the molecular underpinnings of phenotypic alterations in infected workers and explored whether the extended lifespan of queens and infected workers has a common molecular basis. Infected workers and queens commonly upregulated only six genes, one of them with a known anti-ageing function. Both groups overexpressed immune genes, although not the same ones. Our findings suggest that the lifespan extension of infected workers is not achieved via the expression of queen-specific genes. The analysis of the cestodes' transcriptome revealed dominant expression of genes of the mitochondrial respiratory transport chain, which indicates an active metabolism and sheds light on the physiology of the parasite in its cysticercoid stage.

Keywords: host lifespan; transcriptomics; host-parasite interaction; *Temnothorax nylanderi*; *Anomotaenia brevis*; extended phenotype; Hymenoptera

Introduction

Parasitism — life by exploiting resources from other living organisms — is one of the most fascinating life-history strategies in nature and is used by the majority of species on earth (Windsor, 1998). As a parasite's fitness is often based on its ability to infect and potentially manipulate a host, selection has led to the evolution of diverse and sophisticated infection or transmission strategies of parasites. In particular, parasites with complex life cycles have developed a variety of strategies to facilitate trophic transmission from the intermediate to the definitive host. They can achieve this by altering their host's morphology, physiology, or behaviour to their benefit (Thomas *et al.*, 2010). However, not all changes in hosts observed upon parasite infection are the result of direct manipulation by the parasite; some alterations evolved as host defences, others are simply the by-product of infection, benefitting neither parasite nor host (Thomas *et al.*, 2005; Cézilly *et al.*, 2013). Some intriguing examples for host alterations are found in insects that serve as intermediate hosts, such as the neotropical ant *Cephalotes atratus* when infected by the nematode *Myrmeconema neotropicum*. In addition to changes in behaviour, infected ants also display a striking alternate morphology, a berry-red abdomen, which attracts birds, the definitive hosts (Poinar and Yanoviak, 2008; De Bekker *et al.*, 2018). Phenotypic alterations observed in infected insect hosts can include an extended host lifespan. For example, infected female *Tenebrio molitor* beetles, intermediate hosts of the rat tapeworm *Hymenolepsis disminuta*, show an increased survival of 40% (Hurd *et al.*, 2001). A similar phenomenon is found in mice, where the parasitic nematode's release of the molecule ES-62 extends the lifespan of this mammalian host by 70 days likely due to the anti-inflammatory properties of the secretion (Crowe *et al.*, 2020). Some parasites use secretions to manipulate their host's behaviour such as the hairworm *Paragordius tricuspidatus*, which elicits water-seeking behaviour in its cricket host by releasing

proteins of the Wnt family directly into the head (Biron *et al.*, 2006; Martin *et al.*, 2015). Other parasites hijack the immune system like the parasitic wasp *Cotesia congregate,* which elicits an elevated octopamine level in the hemolymph of its host *Manduca sexta* similar to what is observed after an immune challenge (Adamo, 2013, 2005). Additional proposed methods of host manipulation include energetic drain by scavenging nutrients or the direct damage of organs by the parasite infecting host tissue (Lafferty and Shaw, 2013).

As these manipulations often result in the death or at the very least in fitness reduction of the intermediate host, detection of parasites via innate immunity is important. Insect hosts, for example, have evolved diverse defence and evasion mechanisms including phagocytosis, nodule formation, the encapsulation and later melanization of parasites and behavioural defences such as social immunity (Gillespie *et al.*, 1997; Cremer *et al.*, 2018). On the other hand, parasites evolved strategies to evade and/or suppress the immune responses of their hosts (Schmid-Hempel, 2009). Thus, studying the differences between infected and non-infected individuals might not only assist in gaining a deeper understanding of the immune responses of the host but moreover shed light on possible strategies of the parasite to suppress or evade the hosts' immune system. An intriguing example of a parasite infection altering the host's phenotype is represented by our focal host-parasite system, the parasitic tapeworm *Anomotaenia brevis*, and its intermediate host, the cavity-dwelling ant *Temnothorax nylanderi*. Eggs of the cestode *Anomotaenia brevis are* transmitted from the definitive host, a woodpecker, to the intermediate ant host via ingestion of infected bird faeces during the ants' larval stage (Plateaux, 1972). Within the ant, the cestode eggs probably first develop into oncospheres as shown in tapeworms with comparable life cycles like *Hymenolepis diminuta* (Shostak, 2014). The oncospheres then access the

hemocoel by rupturing the gut using hooks where they develop into cysticercoids, a larval stage of the parasite. In fact, infected workers can be parasitized by one to 72 cysticercoids (Scharf *et al.*, 2012a; Sistermans, pers. comm.). The complex life cycle of the parasite is completed when woodpeckers feed upon parasitized ant colonies that reside in acorns and sticks on the floor of Western European forests. The cysticercoids develop into adult cestodes, which then attach to the bird's gut (Péru *et al.*, 1990).

On average 19% of the adult workers, but never the queen, are infected in parasitized colonies (Scharf *et al.*, 2012a). Parasite infection induces multiple alterations in the phenotype of the social host. Firstly, infected ants exhibit a strikingly light, less sclerotized cuticle compared to their brownish nestmates (Trabalon *et al.*, 2000). Secondly, the behaviour of infected workers is altered. They show reduced activity and flight behaviour and remain mostly in the centre of the nest on the brood pile (Trabalon *et al.*, 2000). Moreover, they receive more care from their nestmates and are fed more often (Scharf *et al.*, 2012a). Lastly, the physiology of infected ants is affected too. For example, infected workers possess a higher reproductive potential than uninfected workers do, and they lay eggs when the queen is removed or dies (Beros *et al.*, 2019; Feldmeyer *et al.*, 2016). Moreover, infected colonies raise a higher proportion of intercastes (Scharf *et al.*, 2012a), that is worker-queen intermediates, maybe because young queens are smaller in size (Gabrion *et al.*, 1976). Also, the survival of infected workers is increased compared to their uninfected nestmates (Beros *et al.*, 2015). Indeed, long-term analyses show that more than half of all infected workers survive at least three years such that their survival during this time span does not differ from that of queens, who can live for up to two decades in this species (Plateaux, 1986; Beros *et al.*, 2021). Still, it is currently unknown what the average and maximal

lifespan of infected workers is, albeit anecdotal evidence reveals that they can become seven years old (pers. communication A. Buschinger).

Previous work on brain gene expression in infected and uninfected workers of *T. nylanderi* revealed expression changes linked to the infection status of the individual and the colony (Feldmeyer *et al.*, 2016). These changes included the downregulation of genes, like actin, myosin, and tropomyosin, associated with muscular functions and the upregulation of a few genes from longevity pathways. No clear upregulation of immunity genes was found, most likely due to the tissue type investigated—the brain. Here we focus on transcriptomic shifts in the ant hosts' abdomen, where the cysticercoids reside and which contains fat-body tissue, involved in the regulation of immunity and longevity in social insect queens (Negroni *et al.*, 2019). Using transcriptomic data of abdomens from ants of different castes — the re-productive queens and the usually non-reproductive workers — and varying individual and colony-level infection status, we have the following objectives: Firstly, we want to identify genes that exhibit strong expression changes induced by infection and which may explain aspects of the phenotypic alterations observed in infected workers. Those differentially expressed genes could either represent defence mechanisms of the host against its endoparasite, for example by melanization, they could be by-products of infection such as a more active metabolism, or transcriptomic changes actively induced by the parasite to alter the hosts' phenotype. Secondly, we are interested in whether the extended lifespans observed for both infected workers and uninfected queens have a common molecular underpinning. Upregulation of the same genes linked to longevity pathways could suggest that the parasite hijacks the ants' phenotypic plasticity to induce the expression of queen genes as it was previously shown in a parasitized wasp (Geffre *et al.*, 2017). Lastly, we will study gene expression

in cestode cysticercoids themselves to provide the first information on the parasites' transcriptome, gaining insights into whether the parasite is potentially synthesizing and actively secreting proteins into the host. Looking at transcribed genes in the parasite can only be the first step, proteomic studies have to follow to reveal which types of proteins, how many are indeed released, and what their function could be. Nevertheless, our transcriptome study investigating gene expression both in the host and in the parasite will allow a deeper understanding of the molecular underpinnings of this host-parasite system.

Materials and Methods

Collection and sampling of ant colonies

Ant colonies were collected in October 2017 and 2018 from the Lenneberg forest near Mainz, Germany. Ants were transported to the Johannes Gutenberg University Mainz, relocated to slide nests composed of a plexiglass cavity sandwiched between two microscope slides, and placed in three-chambered nesting boxes (see Supplementary Information Figure S1). Until sampling, the ants were kept at 22 °C and fed only with honey twice weekly, as feeding crickets could contaminate the gut content. Water was provided ad libitum. In November 2017, we selected four parasitized colonies and sampled four infected and four uninfected workers from the brood pile as separate groups. Infected workers are mostly found directly on the brood pile despite showing less brood care activity (Scharf *et al.*, 2012a). Infection status was preliminarily assessed by the colour of the cuticle, which is yellow in infected workers in contrast to the typical brown colour of their uninfected nestmates (Trabalon *et al.*, 2000). Important to note is that in parasitized colonies, infected workers can also occur with a normal brown cuticle, and without dissection or analysis of gene expression, infection status cannot be determined with certainty (Scharf *et al.*, 2012a). Therefore, we

confirmed infection status later on by mapping the reads of each sample against the cestode transcriptome. The gaster of the ants was detached directly in front of the postpetiole and four abdomens per group (infected workers and uninfected workers) were pooled into 100 µl of Trizol to reduce variance between samples. Abdomens were directly crushed in Trizol and RNA was extracted using the RNAeasy mini extraction kit (Qiagen, Hilden, Germany) following the standard protocol. Sequencing of 75 bp single-end reads was performed on an Illumina NextSeq500 at the Institute of Molecular Biology (IMB, Mainz, Germany) in Mainz aiming for 15 Mio reads per sample to be able to detect strongly expressed genes. To investigate gene expression in queens in *T. nylanderi*, the entire procedure was repeated exactly one year later in 2018, with four unparasitized colonies (i.e., lacking yellow, infected workers) which did not differ in colony size from the parasitized ones (Wilcoxon, W = 3, p = 0.2). We sampled the only queen of each colony as well as four workers located directly on the brood pile whose abdomens were pooled again. With this, we were able to compare gene expression between queens and workers without the year of sampling as a confounding factor. As queens also usually reside on the brood pile, the location inside the nest was the same for all our four groups. This resulted in 16 samples (Figure 8-1, see Supplementary Information Table S6). Unparasitized colonies differed in size from 59–132 workers, indicating that queen age might also differ. *T. nylanderi* is a strictly monogynous species—meaning that colonies are founded and consequently headed by a single queen—so colony size is strongly linked to colony age (Keller and Passera, 1990).

Differential expression analysis in ants

To remove all remaining cestode RNA and other putative contaminant sequences from all samples, we used FastQScreen with the newly assembled and filtered cestode

transcriptome, sequences from humans, *Escherichia coli*, vectors, and adapters (Wingett and Andrews, 2018). Detailed information about the assembly of the cestode transcriptome can be found in the Supplementary Information. The infection status of worker pools from parasitized colonies was determined by identifying those four samples with a high percentage of cestode RNA (between 14 and 21%, see Supplementary Information Table S7).

RNA-Seq reads were trimmed using Trimmomatic (version 0.39) in single-end mode using the non-default trimming parameters: TRAILING 3, LEADING 3 and SLIDINGWINDOW 4:15 (see Supplementary Information Table S8) (Bolger *et al.*, 2014). The quality of reads before and after trimming/filtering was assessed using FastQC (Andrews *et al.*, 2010). Filtered reads were mapped against the genome assembly of *T. nylanderi* using HISAT2 v2.1.0 (Kim *et al.*, 2015) with the parameter --dta as preparation for the genome-guided assembly. The resulting BAM files were sorted and indexed using Samtools v0.1.19 (Li *et al.*, 2009) and used to create a genome-guided transcriptome assembly using StringTie v1.3.6 (Pertea *et al.*, 2015). To extract transcript sequences, we ran gffread v0.11.4 on the merged GTF file. We checked the transcriptome quality using TransRate v1.0.3 (see Supplementary Information Table S9) (Smith-Unna *et al.*, 2016). Predicted amino acid sequences of the transcripts were retrieved using TransDecoder v5.5.0 (Haas *et al.*, 2013) and functionally annotated using InterProScan v5.46-81.0 (Jones *et al.*, 2014). The following analyses were conducted in R v3.6.1 (R Core Team, 2020)ve. We performed the differential expression analysis on the gene level and for this used the gene count matrix produced by StringTie. Thus, in the following "gene" refers to genes assigned by StringTie. To remove low read counts we only kept those genes that were mapped by at least five reads in at least three samples.

We conducted a Principal Component Analysis (PCA) using read counts for all genes and tested the association between infection status (infected/not infected) to each of the principal components (PC) using the Wilcoxon test. For those PCs that significantly reflected differences in infection status ("infection PCs" hereafter), we identified top-loading genes and assessed their functional annotation. Hereto, the transcripts belonging to these genes were annotated using blastx v2.9.0 against the NCBI non-redundant invertebrate protein database (downloaded: 18.03.19), only considering hits with an E-value below 10^{-5} and taking for each transcript the hit with the highest bit score (Altschul *et al.*, 1990). We assigned functions to genes that were represented by more than one isoform transcript by only considering the longest isoform (see Supplementary Material). Enrichment analysis of the genes contributing to the "infection" PCs was performed using topGO v2.36.0 (Alexa and Rahnenfuhrer, 2018). We used the weight01 algorithm and the Kolmogorov-Smirnov test to test for enrichment of functions of genes that were either positively or negatively associated with the "infection" PCs. By using the loading of each gene as a score, we were able to differentially weigh the functions of genes depending on their contribution to the PC. Only functions with $p < 0.05$ were considered as significantly enriched.

Gene expression was contrasted (1) between the infected and uninfected workers from parasitized colonies and (2) between queens and workers from unparasitized nests. For both comparisons, we used Wald's test implemented in DESeq2 v1.24.0 (Love *et al.*, 2014). We only considered contigs with an adjusted p-value below 0.05 as significantly differentially expressed. The differentially expressed genes were checked for components of the melanization cascade in *Drosophila melanogaster* (see Supplementary Information Table S10) as we were interested in candidate genes for a potential immune response of the ant towards the cestode. Melanization is a common

defence against endoparasites in insects such as *Drosophila melanogaster* and thus we decided to investigate especially genes contributing to the melanization cascade (Carton and Nappi, 1997). Functional enrichment of these differentially expressed genes (DEGs) was performed as described above only this time using a Fisher's exact test to test for an overrepresentation of functions in our lists of overexpressed genes compared to all genes. In addition, we searched the BLAST hits of our differentially expressed genes against reviewed entries in the UniProt database from humans, mice, and *Drosophila* (The UniProt Consortium, 2019). Afterwards, we identified specific terms that are associated with longevity, fecundity, stress, and immunity (see Supplementary Table S11) in the lists of differentially expressed genes. We tested whether the groups differed significantly in the number of genes associated with these terms by using a χ^2-test comparing the number of genes that are associated with this function to the number of genes that lack this functionality.

Expression of cestodes in ant abdomens

In addition, we identified genes that were expressed by the cestodes inside the focal infected workers which were used for the analyses described above as these also included cestode tissue (see section above). Therefore, we extracted reads from infected ants, which mapped one or multiple times against the newly assembled cestode transcriptome. Bowtie2 v2.3.5 (Langmead and Salzberg, 2012) was used for mapping the filtered and trimmed reads against the cestode transcriptome and RSEM v1.3.1 (Li and Dewey, 2011) for transcript quantification to see which genes the cestodes express inside the ant's abdomen.

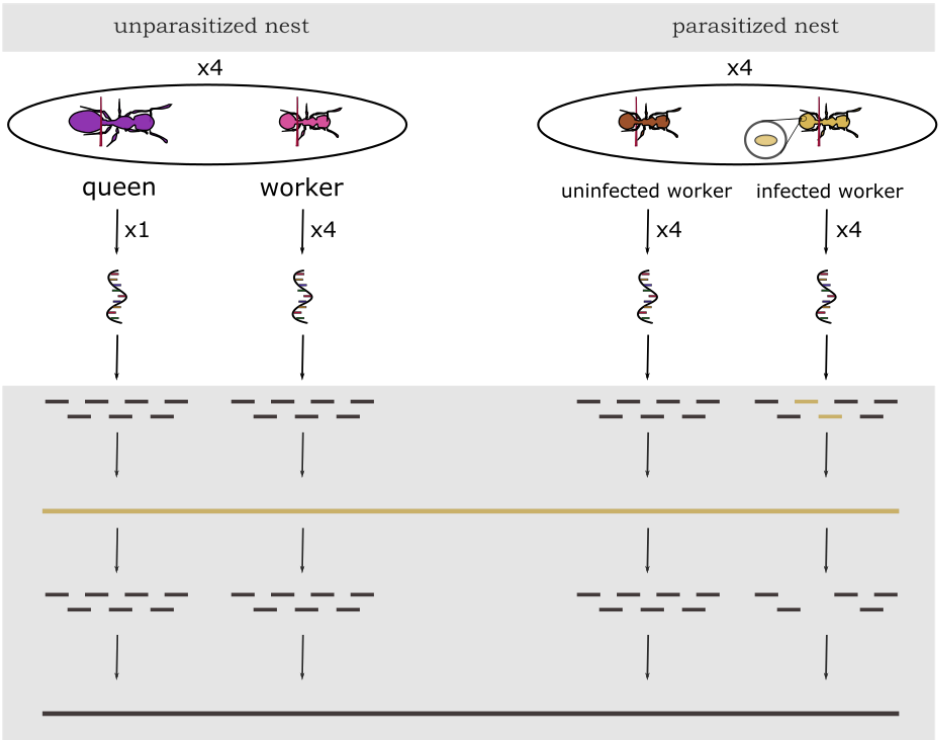

Figure 8-1 Experimental design of sampling and schematic analysis of gene expression data in *T. nylanderi* ants with different caste (workers or queens) and infection status. From each of the four unparasitized nests (left side) four workers were pooled per colony. Queen samples from unparasitized colonies consisted of only one individual. From each of the four parasitized nests (right side) four uninfected workers and four infected workers were pooled into two separate samples. Bottom grey part represents bioinformatic workflow: After sequencing, all samples were filtered for cestode RNA and other putative contaminants (yellow) and afterwards, all ant sequences were mapped against the genome of *T. nylanderi* for further analyses (black).

Results

Gene expression in *T. nylanderi*

Principal Component Analysis

We sequenced the RNA of abdomens from infected and uninfected ants from parasitized nests as well as from queens and inside workers from unparasitized nests. Sequencing resulted in ~15 million 75 bp single-end reads per sample with an average GC content of 43.6% and a mean coverage between 13 and 18x (see Supplementary Information Table S8). These RNA reads were filtered for cestode RNA as cysticercoids are located in the ants' abdomen. Moreover, we filtered the transcriptome to only contain transcripts with an open-reading frame at least 150 bp long and the gene count

matrix for only those genes having at least 5 counts in at least 3 samples. After filtering, a total of 15,578 genes remained for further analysis. To gain insights into the significance of infection status on the general gene expression pattern, we conducted a PCA based on the overall read counts of these genes and tested the influence of infection status (infected or not) on each of the 16 principal components. Only PC2 and PC16 were found to be significantly influenced by infection status (Wilcoxon, PC2: $W = 1$, $p = 0.002$; PC16: $W = 0$, $p = 0.001$). As the latter explained less than one percent of the variance, we will focus on PC2 explaining 15% of the variance. Our samples clustered according to their group and infection status on PC2 (Figure 8-2a). Only the queens diverged from this pattern and showed the highest variance. As queen samples were derived from a single individual and not a pool of four individuals, it is not surprising they showed the most within-group variance. While the queens from colonies 1 and 3 clustered near workers from the same nest, the queen samples from colonies 2 and 4 clustered between the infected workers and their nest-mates. These latter two queen samples likely originated from older colonies, as their colonies contained more workers (117 and 132 workers vs. 59 and 76 workers). We were especially interested in genes contributing to PC2, where presumably older queens and infected workers clustered together, as these might correspond to age/longevity. Amongst the ten genes with the highest loadings on PC2, we found one encoding for transferrin, which is known to play a role in the innate immune system (see Supplementary Information Figure S2). The gene with the highest loading was encoding bromodomain-containing protein 4-like isoform X1 in *Vollenhovia emeryi*, important in epigenetic memory in humans (UniProt). The enriched Gene Ontology (GO) terms in genes contributing mostly to PC2 in both directions were mostly linked to oxidation-reduction processes and metabolism (Figure 8-3). Genes negatively

correlated to infection status were also enriched for “fatty acid biosynthetic process” and “lipid transport”.

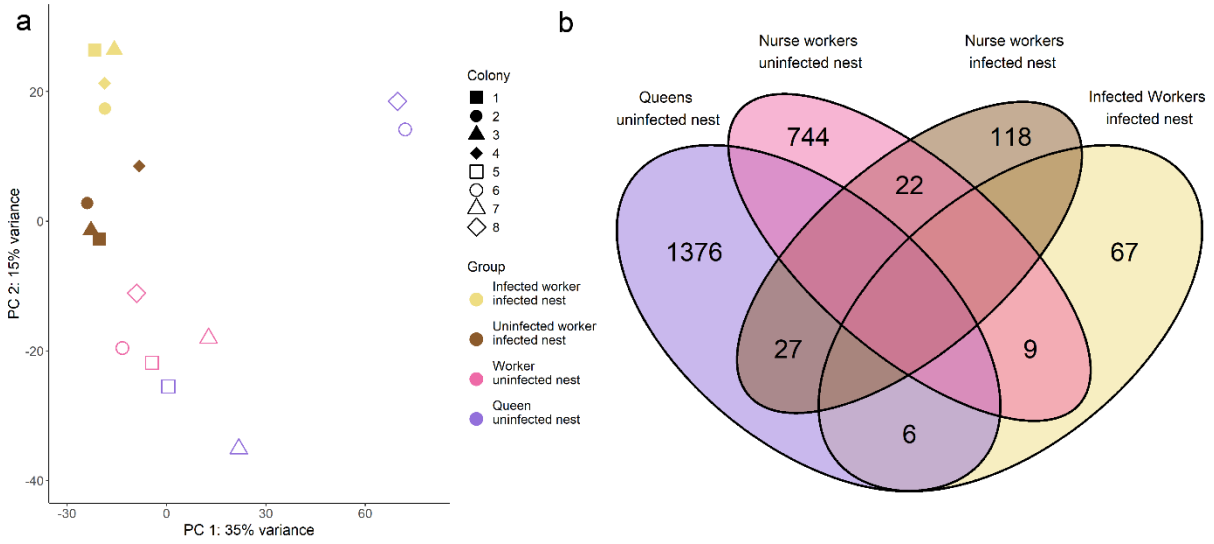

Figure 8-2 (**a**) Principal component analysis based on all genes (**b**) Venn diagram depicting the number of upregulated genes in pairwise comparisons between groups of the same nest and their overlap. Overlap, which is zero by default as the two groups compared do not share up-regulated genes, is left blank.

Figure 8-3 GO analysis of terms enriched in PC2 with positive loadings/positively correlated with PC2 (left in green) and negative loadings/negatively correlated with PC2 (right in red). Font sizes of individual terms are scaled by the negative natural logarithm of the p-value of the Kolmogorov-Smirnov test.

Differential Gene Expression Analysis

We found a total of 249 differentially expressed genes when comparing gene expression between infected and uninfected workers from parasitized nests. Of those, 82 genes were upregulated in infected workers, while in contrast 167 genes were upregulated in uninfected workers. Amongst the ten genes upregulated in uninfected workers with the lowest adjusted p-value and a BLAST annotation, we found two genes encoding for vitellogenin receptors (see Supplementary Information Figure S3). In contrast, a gene encoding mucin-2-like was found to be upregulated in infected workers (see Supplementary Information Figure S4 as well as S5 and S6 for genes upregulated in the other groups). When looking at the annotations from UniProt, we identified amongst those genes two encoding proteins that had functional and/or GO annotations including melanization or the melanosome: carboxypeptidase B-like isoform X2 and lysosomal-trafficking regulator isoform X1. In infected workers, genes with functionality in isoprenoid biosynthetic processes and phospholipid transport as well as the perception of smell were enriched, yet these enrichments are based on single genes. Their uninfected nestmates showed upregulation of genes related to metabolism (Figure 8-4a).

Between queens and uninfected workers from the same nest, we found a total of 2184 genes to be differentially expressed, 1409 of them upregulated in queens and 775 in workers. Enriched functions in nurses from unparasitized nests were often stress-related, while queens upregulated more genes related to DNA replication and protein synthesis (Figure 8-4b).

The overlap between the genes differentially expressed between infected workers and their nestmates and the genes differentially expressed between queens and their workers comprised a total of six genes including one encoding carboxypeptidase B-like

isoform X2 (Figure 8-2b, Supplementary Information Figure S7). Using a hypergeometric test based on the total number of differentially expressed genes this overlap was not significantly more than expected by chance (hypergeometric test: $p = 0.309$). Text mining for specific functionalities of DEGs revealed that infected workers upregulate more genes related to immunity than their uninfected nestmates ($\chi^2 = 4.35$, $p = 0.037$; Figure 8-5a) as queens ($\chi^2 = 8.93$, $p = 0.003$) do, in addition to genes related to longevity and fecundity (longevity: $\chi^2 = 20.47$, $p < 0.00001$; fecundity: $\chi^2 = 30.79$, $p < 0.00001$; Figure 8-5b). To ensure that the high variability of the queen samples (see Principal Component Analysis) does not lead to the detection of false positives, we repeated the gene expression analysis for queens and their workers, by taking colony size as a batch effect into account (see Supplementary Information Figures S8–S11 and Supplementary Material). We compared those results to our previous analysis and focus our discussion only on those findings that are consistent between both analyses. In both analyses, we only found little overlap between genes upregulated in queens and infected workers, the overlap in both cases comprising the gene encoding carboxypeptidase B-like isoform. Moreover, in both analyses, we detected a strong representation of genes linked to immunity in infected workers and queens.

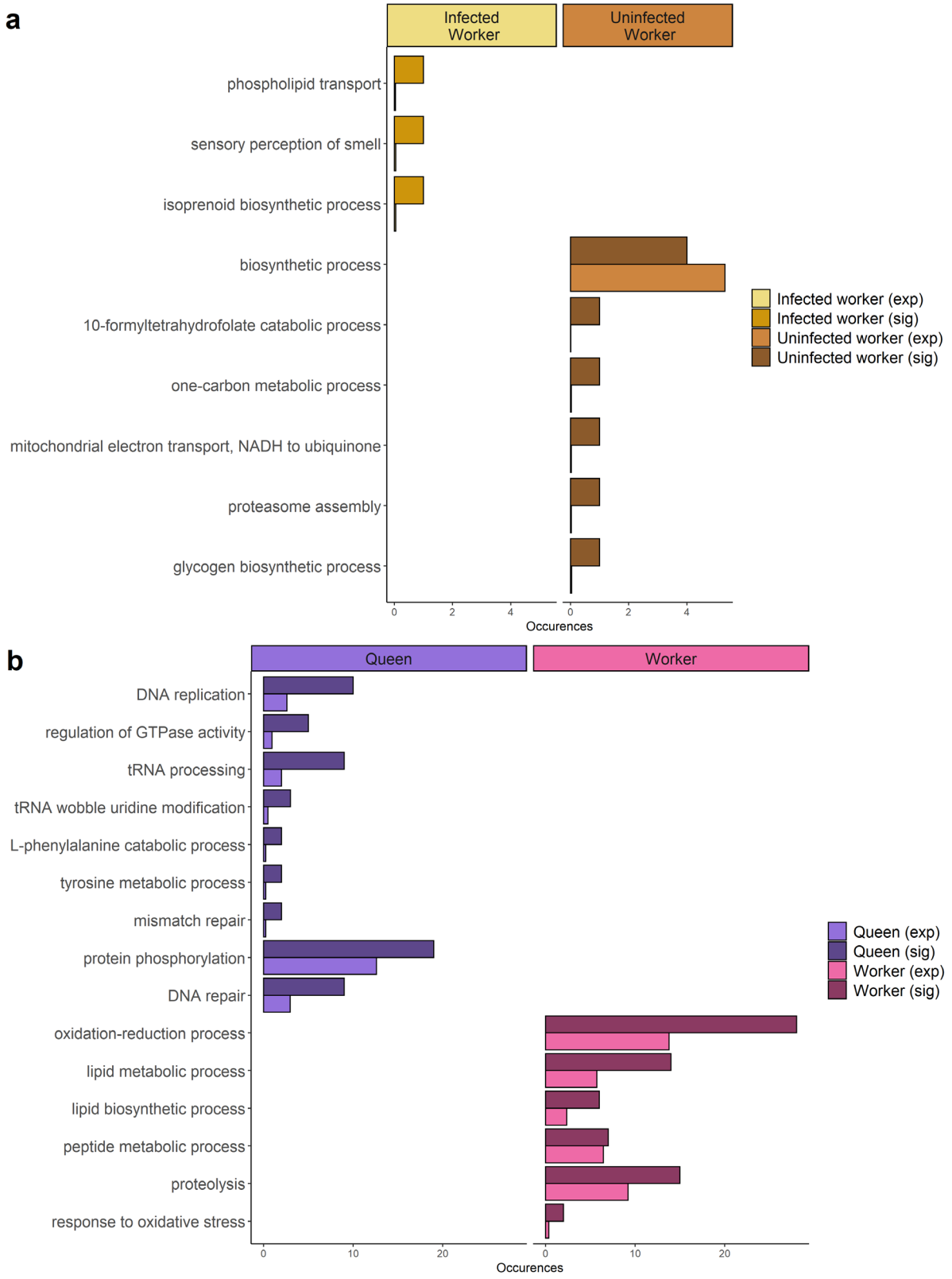

Figure 8-4 Bar plots depicting the significantly enriched Gene Ontology terms upregulated in (**a**) ants from parasitized nests: infected workers (left panel) and uninfected workers from parasitized nests (right panel). (**b**) ants from unparasitized nests: queens (left panel) and workers (right panel). Number of genes annotated with the specific term found in the candidate gene list are depicted (sig) as well as the number expected in a list of this size based on the annotation of the whole transcriptome (exp).

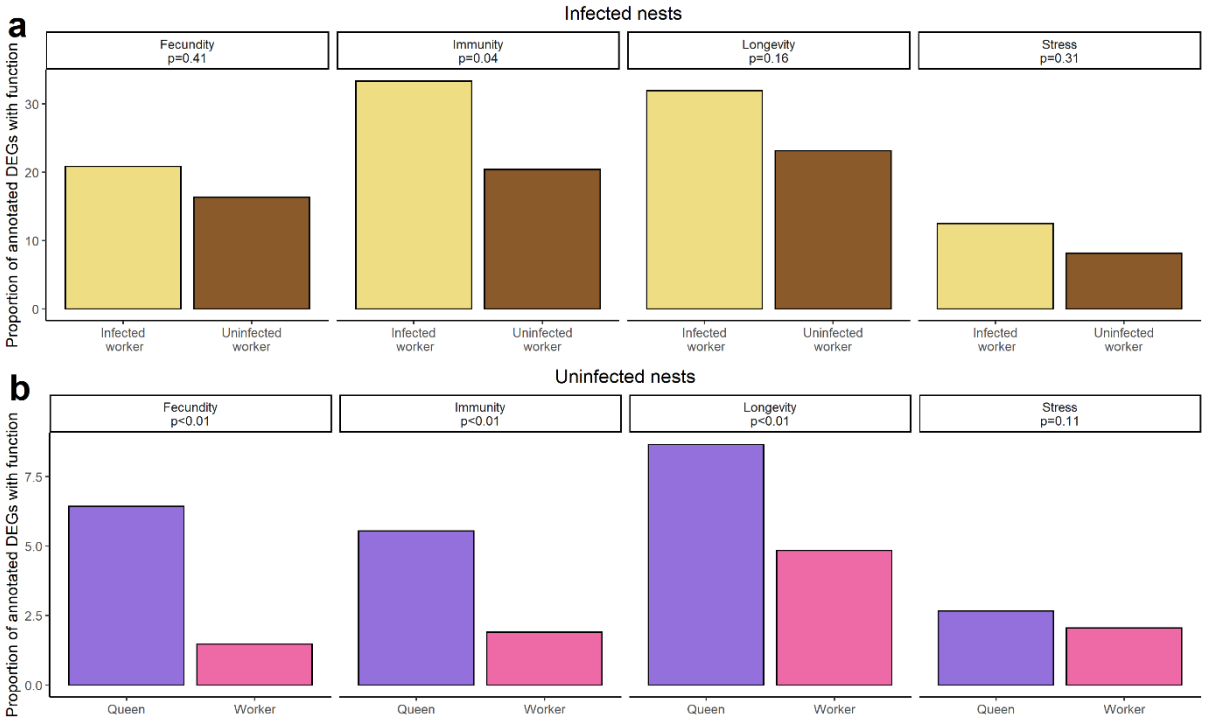

Figure 8-5 Results of text mining approach of terms related to fecundity, immunity, longevity, and stress based on a UniProt search of upregulated genes in (**a**) infected workers (yellow) and their uninfected nestmates (brown) of infected nests. (**b**) Queens (pink) and workers (purple) of uninfected nests. p-value of χ^2-test and the according function are given in boxes.

Cestode transcriptome

We collected a total of 329 cysticercoids of the cestode *A. brevis* from 34 infected workers out of 18 different colonies, sequenced their RNA and assembled a first transcriptome using Trinity (Haas *et al.*, 2013). The final cestode transcriptome after all filtering steps consisted of 90,096 contigs with a mean length of 1079.01 bases and a GC content of 47%. Cestode specificity was confirmed as 94.65% of the contigs had the best BLAST matches against other species from the phylum of Platyhelminthes. The overall alignment rate of the filtered and trimmed reads against the filtered transcriptome was 95.80%. Using BUSCO we were only able to detect 24.1% of nematode orthologs in the cestode transcriptome (single-copy: 2.8%, duplicated: 21.3%, fragmented: 1.0%, missing: 74.9%) (Simão *et al.*, 2015b). Analyzing the genes, which are highly expressed by the cestodes, we found that 97.89% of all reads

originated from a gene encoding the cytochrome c oxidase subunit I, represented by in total 20 contigs (see Supplementary Information Table S5). Looking at the most prominent GO terms occurring in the cestode transcriptome, we did not find that those related to transport or specific metabolic networks were more prominent compared to the transcriptome of the ant (see Supplementary Information Figure S8). Also, when analyzing the cestode transcripts from the ant abdomen (backmapping rate: 96.33–96.78%), we found cytochrome c oxidase subunit 1 (CO1) transcripts to be the most abundant, but a much smaller fraction of the reads were assigned to the CO1 gene (11.28–14.55%). Additionally, the subunits II and III were detected as the second and third most expressed transcripts in the transcriptome of the cestode as well as in the samples from the ant abdomens, but to a lesser extent (0.12–1.01%). The ten most expressed transcripts for each dataset are reported in the Supplementary Information (Table S5).

Discussion

Interactions between parasites and hosts have led to fascinating cases of phenotypic alterations in hosts, many of them modulated to facilitate transmission to final hosts. Ants of *T. nylanderi* infected with the cestode *A. brevis* show a variety of morphological, physiological, and behavioural alterations compared to their nestmates. We analyzed the transcriptomic changes underlying these alterations to shed light on the molecular basis of the interactions between host and parasite. We observe gene expression changes in infected workers and propose three different not mutually exclusive causes for these: Firstly, a response of the host workers against the parasite. Secondly, they might indicate possible manipulation strategies of the parasite. Lastly, transcriptomic shifts might represent a mere by-product of parasite infection, for example, due to the higher physiological costs. By analyzing the cestode

transcriptome, we provide the first molecular data for this cestode and identify putative mechanisms of host manipulation.

Infected workers upregulate genes involved in immunity

Our study revealed genes related to immunity to be upregulated in infected workers compared to their nestmates. A previous study focusing on ant brains based on the same host-parasite system (Feldmeyer *et al.*, 2016) found many differentially expressed genes in infected workers compared to uninfected ones, but no general overexpression of immune genes or genes similar to the ones overexpressed in our study. The difference is likely based on the different tissues, as the abdominal fat body is largely responsible for the immune response in insects (Tsakas and Marmaras, 2010). As described above the observed changes in gene expression can be explained by multiple hypotheses that are not mutually exclusive: They might represent an immune response of the host workers against the parasite. Invertebrates including Hymenopterans largely rely on innate immune responses to defeat endoparasites — albeit there is some immune memory (Konrad *et al.*, 2012; Ferro *et al.*, 2019; Gillespie *et al.*, 1997). An important innate immune response is the encapsulation or melanization of parasites by hemocytes as seen in *Drosophila* flies parasitized by parasitoid wasps (Carton and Nappi, 1997; Castillo *et al.*, 2011), which requires phenoloxidases or lectins (Rizki and Rizki, 1990; Keebaugh and Schlenke, 2012). The parasite, *A. brevis*, lives as cysticercoid larvae in the ant's hemocoel, and thus might be directly threatened by encapsulation, melanization, or other immune defences of the host (Schmidt *et al.*, 2001). Interestingly, dissections have so far revealed no evidence for an immune response towards the cestode, such as encapsulation or melanization. Of course, this does not rule out the possibility that certain hosts may successfully mount immune responses to inhibit or remove parasites. On the

transcriptomic level, we found two genes upregulated in infected workers, which encode for proteins that have melanization functionalities. While carboxypeptidase is known to be involved in the melanization and immune response in *D. melanogaster* flies (Settle *et al.*, 1995; Chang *et al.*, 2004), the other candidate gene encoded a lysosomal-trafficking regulator, which also plays a role in immune defence (Rahman *et al.*, 2012b). But why did we find an upregulation of immune genes related to melanization when there is no histological evidence for such an immune reaction towards the cestode? One possible explanation could be the upregulation of multiple mucin genes in infected workers. Mucins are known to be a host defence against helminths (Hasnain *et al.*, 2017), and were also shown to protect larvae and eggs of parasitoids from being encapsulated by preventing the adhesion of hemocytes (Yin *et al.*, 2018). Upregulation of mucins in infected workers might indicate that parasite infection induces the production of mucins in the host, which in turn could prevent the encapsulation and melanization response. Although convergent evolution could occur, we rule out that these transcripts originate from the cestode itself as best BLAST hits of the mucins were found in ants, not cestodes and this was additionally confirmed by running blastn on the respective genes using default parameters against the nucleotide collection (nt) database. To investigate the link between mucin production and melanization response in hosts further experiments are needed. These could include blocking mucin production in the host to test whether this results in encapsulation of the parasite. Furthermore, the observed changes in gene expression might represent a reaction not directed towards the cestode. Due to their lighter sclerotized cuticle, for example, infected workers might be more susceptible to other pathogens such as fungi and viruses, thus making an upregulated expression of immune genes necessary (Barnes and Siva-Jothy, 2000; Leger *et al.*, 1988; Reeson *et al.*, 1998; Wilson *et al.*, 2001). Our last proposed explanation for the underlying results in infected ants is a

possible manipulation by the parasite. Activation of the immune system might be a route to modulate other characters of the ant by the cestode like behavior or morphology as shown for example in *Manduca sexta* (Dunn *et al.*, 1994; Adamo, 2005). Amongst the enriched functions, we found sensory perception of smell to be enriched in infected workers. In ants, perception is important for social behavior. In *Solenopsis invicta* for example, a single nucleotide polymorphism in a gene encoding an odorant-binding protein determines whether colonies accept only a single, or multiple queens (Ross and Keller, 1998). Thus, the observed change in genes responsible for perception of smell might indicate a modulation of behaviour. Whether this represents an active manipulation of behaviour by the cestode through induction of the immune response or simply a by-product of infection, requires further testing. Additional studies could compare the expression of the corresponding gene in infected workers, infected workers with a killed cestode, immune-challenged workers and healthy workers. Moreover, future studies should aim for a higher sequencing depth to also detect more subtle gene expression changes.

Molecular underpinnings of longevity in infected workers and queens

Infected workers in contrast to their uninfected nestmates show increased survival not different from that of the queens (Beros *et al.*, 2015). During their elongated life, infected workers are mostly inactive and do not contribute to colony life (Scharf *et al.*, 2012a; Charbonneau *et al.*, 2017). Infected ants do not reproduce in the presence of the queens and thus are unlikely to increase their direct fitness by living longer. Moreover, on a colony level, the reduction in lifespan of uninfected workers in parasitized colonies likely outweighs the increase in lifespan of infected workers, since on average 19% of the workers are infected in parasitized colonies (Scharf *et al.*, 2012a). In addition, infected ants show a low activity level, contributing little to the usual worker

chores such as brood care and foraging for food. By contrast, one may speculate that the longevity of infected individuals might be adaptive for the parasite. For the cestode to be transmitted to its final host, a woodpecker has to pick at the stick the colony resides in and pick up an infected individual. Thus, the prolonged life of the ant host might increase the chances to be transmitted to the final hosts before the natural death of its intermediate host. Inducing the expression of queen-specific genes in infected workers would represent a cost-efficient strategy for the parasite and thus should be adaptive. If lifespan is manipulated by the parasite, we would expect infected workers to upregulate similar longevity-related genes as the queen. Thus, our first step was to characterize the transcriptomes of queens from unparasitized *T. nylanderi* colonies by contrasting them to uninfected workers from the same nest. This comparison showed the highest number of differentially expressed genes. In part, these transcriptomic differences might not only reflect variation in the regulation of fecundity and ageing but simply tissue composition in the gaster of queens and workers with queens exhibiting for example much larger ovaries relative to workers. Previous studies on *T. longispinosus* contrasting transcriptomes of queens and workers of different fertility status or developmental stages also showed highly divergent gene expression profiles in queens (Feldmeyer *et al.*, 2014; Gstöttl *et al.*, 2020). Moreover, queens of *T. longispinosus* upregulate genes with functions in DNA replication, which is similar to what we find here for *T. nylanderi* (Gstöttl *et al.*, 2020). This is in accordance with comparative work showing that functionalities are conserved in castes of different species (Berens *et al.*, 2015a). When looking at the overlap between genes upregulated in queens and infected workers, we found one promising candidate, the carboxypeptidase B, which in *D. melanogaster* is encoded by the silver gene and positively associated with increased lifespan (Carnes *et al.*, 2015). However, generally speaking, we did not find more overlap than expected by chance in genes upregulated

in infected workers with upregulated genes in queens. In part, this might be explained by differences in tissue composition and age between groups, which we could not control for as colonies were freshly sampled from the wild and experimental infection of nests is not established, or the fact that workers regulate longevity differently than queens. There is evidence for the latter in the closely related species *T. rugatulus* where fecund workers with extended life expectancy also seem to express different genes than queens (Negroni *et al.*, 2019, 2021a). Moreover, the lifespan extension of infected workers might be explained solely by social aspects. Infected workers were shown to be less active compared to their nestmates (Scharf *et al.*, 2012a). This might result in a lower production of reactive oxygen species and prolonged lifespan as shown in the housefly *Musca domestica* (Yan and Sohal, 2000). Additionally, since infected workers do not engage in foraging outside the nest, they have lower extrinsic mortality similar to queens which also stay inside the nest (Scharf *et al.*, 2012a; Kirkwood, 1977; Keller and Genoud, 1997). As a second line of evidence, we investigated genes with a high contribution to PC2, which grouped the two older queens with the infected workers and thus is linked to longevity. Two of the top genes seem to be involved in immunity. The gene with the highest contribution was encoding a bromodomain-containing protein, an epigenetic reader (Supplementary Information Figure S2), with a role in inflammation and cancer in humans (Ghoshal *et al.*, 2016). Transferrin is a key player in iron metabolism, and previous work in insects suggests that it is also involved in immune response (Iatsenko *et al.*, 2020). It was also one of the few genes involved in immunity found to be upregulated in the brains of infected workers (Feldmeyer *et al.*, 2016). In general, both queens and infected workers expressed more genes related to immunity than their nestmates. Immunity and longevity are tightly interlinked, but which of the two represents the cause or

consequence of the prolonged lifespan remains to be investigated (Aurori *et al.*, 2014; Xia *et al.*, 2019).

Overexpression of Cytochrome C Oxidase Subunit I in the cestode

Overall, our newly assembled transcriptome of the cestode in the cysticercoid stage only contained 24% of genes usually found in nematodes, the phylogenetically closest phylum with an ortholog set available. This is to be expected as during this life stage only a part of genes is expressed. Moreover, the parasitic lifestyle itself might also result in gene loss and thus genome reduction as shown in other systems (Spanu *et al.*, 2010; Tsai *et al.*, 2013; Zhou *et al.*, 2009). When analyzing the transcriptome of the cestode *A. brevis*, we found that the majority of reads belong to a single gene, encoding the cytochrome c oxidase subunit I, which represents the terminal part of the mitochondrial respiratory electron transport chain producing ATP. The strong overexpression of a single gene might additionally explain the observed incompleteness of our assembled transcriptome, leaving less coverage for other genes. We propose different non-exclusive explanations for such a strong overexpression of this gene: (1) Energy metabolism. Studies on other tapeworms show that the main energy resource is carbohydrates including trehalose and glycogen, which are absorbed from the hemolymph of the host and metabolized by aerobic respiration (Tsai *et al.*, 2013; Novak *et al.*, 1993). Moreover, preliminary data suggest that the parasite releases proteins into the abdomen of the ant, and for these transport processes, energy is required, which might be acquired using aerobic respiration (Butter, pers. comm.). (2) Prolongation of cysticercoid lifespan. In *C. elegans*, overexpression of cytochrome c oxidase was observed in dauer larvae as well as in long-lived adults (Ruzanov *et al.*, 2007). The cysticercoid stage resembles in part the dauer state of nematodes like *C. elegans*. As the duration of the life stage inside the

intermediate host can vary, cysticercoids might overexpress genes related to lifespan extension during this waiting period. Furthermore, the expression of CO1 is positively linked to longevity as the expression of cytochrome c oxidase declines as a function of age and in *Drosophila* this decline in expression can cause a shortened lifespan (Klichko *et al.*, 2014; Kwong and Sohal, 2000). (3) Stress response. Cytochrome c oxidase is involved in an organism's response towards oxidative stress probably via the handling of reactive oxygen species (ROS) as dysfunction causes an increase in ROS (Srinivasan and Avadhani, 2012). During dissections, the removal from their intermediate host might have triggered oxidative stress in cysticercoids, which resulted in the observed overexpression of CO1 to be able to cope with it. Moreover, oxidative stress could be the result of high ROS production in the host associated with an immune response, as shown in *D. melanogaster* (Nappi *et al.*, 1995). (4) Exposition to oxygen. Due to our dissection protocol cysticercoids were exposed to a high level of oxygen compared to their previous environment inside the ant. Cytochrome c oxidase catalyzes electron transport towards molecular oxygen and its activity is correlated with oxygen levels (Chandel *et al.*, 1996). Thus, the extreme upregulation of CO1 might be due to our dissection procedure which exposed cestodes to an unnaturally high oxygen level. Usually, the cysticercoid is removed from the ant when eaten by their final host, and exposure to oxygen might signal to the cestode that it needs to progress into its next life stage. Thus, overexpression of CO1 might be adaptive in the context of metamorphosis and establishment in the final host.

Conclusion

We were able to explain some of the physiological differences between infected workers and their nestmates through the expression of certain genes, including candidate genes related to melanization and immunity. Although there was only

minimal overlap in genes upregulated in infected workers and queens, some of these genes are known to be involved in longevity in other insects and thus represent interesting candidates for future studies. Characterization of the cestode transcriptome revealed a strong expression of a gene involved in mitochondrial electron transport, indicating a high energy consumption by the parasite. We are currently conducting proteomic studies to investigate whether they can explain the elongated lifespan of infected workers. Moreover, we hope that in the future a sequenced genome will be available for *A. brevis*, which will allow us to gain more insights into the molecular underpinnings of the host-parasite interaction.

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[removed for privacy purposes]

Author Contributions

Conceptualization, S.B., F.B. and S.F.; Formal analysis, M.S., E.J. and B.F.; Methodology, M.S., L.K. and B.F.; Resources, E.J. and S.F.; Writing—original draft, M.S. and S.F.; Writing—review & editing, L.K., S.B., F.B., E.J., B.F. and S.F. All authors have read and agreed to the published version of the manuscript.

Supplementary Materials

The following are available online at https://www.mdpi.com/2073-4425/12/1/95/s1: Additional tables and figures referenced in the manuscript as well as detailed methods regarding the assembly of the cestode transcriptome.

Figure S1: Expression of top four genes contributing to PC2 in the Principal Component Analysis, Figure S2: Top ten genes according to adjusted p-value that had a functional

BLAST annotation upregu-lated in uninfected nestmates compared to infected workers of the same nest, Figure S3: Top ten genes according to adjusted p-value that had a functional BLAST annotation upregulated in infected workers compared to their uninfected nestmates, Figure S4: Top ten genes according to adjusted p-value that had a functional BLAST annotation upregulated in nurse compared to queens, Figure S5: Top ten genes according to adjusted p-value that had a functional BLAST annotation upregulated in queens compared to nurses, Figure S6: Genes overlapping between DEGs upregulated in queens and infected workers, Figure S7: Ten most prevalent GO terms found in the cestode transcriptome and the ant transcriptome, Figure S8: Overlap between differentially expressed genes upregulated in queens after controlling for colony size as batch effect and the genes upregulated in infected workers, Figure S9: Genes and their expression in overlap between differentially expressed genes upregulated in queens after controlling for colony size as batch effect and the genes upregulated in infected workers, Figure S10: Results of text mining approach of terms related to fecundity, immunity, longevity and stress based on a UniProt search of upregulated genes when controlling for colony size as batch effect, Table S1: Sampling information for *Anomotaenia brevis* cestodes, Table S2: Databases used for filtering of cestode RNA reads, Table S3: Read Statistics for *A. brevis*, Table S4: Assembly statistics for the filtered *A. brevis* transcriptome, Table S5: Top 10 expressed genes in the transcriptome of intact *Anomotaenia brevis* cestode pool as well as their proportion in the cestode transcripts from infected workers abdomens, Table S6: Colony information for colonies used for RNA-Seq samples of *T. nylanderi*, Table S7: Sample information for RNA-Seq samples of *T. nylanderi*, Table S8: Number of reads for samples of *T. nylanderi* before and after filtering and trimming, Table S9: Assembly statistics for the transcriptome of *T. nylanderi*, Table S10: Proteins involved in the melanisation pathway in *Drosophila melanogaster*, Table S11: Search terms

used for word search analysis based on the functional annotation using the UniProt database.

Additionally, the count matrix, the list of DEGs together with their UniProt annotation, the results of the GO enrichment, the transcriptomes, and the analytic scripts are provided as Supplementary Material separately (doi:10.17632/7jvdd7jwks.1). Raw reads can be accessed from SRA under BioProject ID PRJNA673150.

General Discussion

The goal of this dissertation was to explore the molecular mechanisms underlying the plastic behaviours and life strategies of ants and how these may be exploited by parasites. Next-generation sequencing and bioinformatics technologies now enable sequencing and analysis of transcriptional changes underlying plastic phenotypes. In my dissertation, I used transcriptomic data from RNA-Sequencing experiments to study plastic responses in ants, by comparing gene expression between individuals.

The following synthesis is divided into two parts: The first part discusses how the social environment impacts transcriptional responses. To answer this question, I examined the transcriptomic changes induced by social interactions with conspecifics, the complete absence of these interactions, or interactions with social parasites. Comparison of the transcriptomic response of queens of the same species with different morphologies revealed that gene expression changes depending on the social environment, as individuals are likely adapted to their social niche (**Chapter 1**). Similar to other systems, loss of social interactions over a varying period of time affected both behaviour and gene expression in the brains of the ants (**Chapter 2**). Social parasites are closely related to their host species, and in parasite nests, the two species live together, with hosts directing their helper behaviour toward the parasites. Thus, these interactions can be considered social, even so, they do not increase the fitness of host workers. While there is much evidence at the behavioural and physiological level that the presence of social parasites affects host characteristics, less is known about the underlying molecular processes that drive these shifts (**Chapter 3**). In my work, I found that the presence/absence and the number of parasites in the social environment determine both host defensiveness and gene expression in the brain (**Chapters 4 and 5**). Overall, my results point to an important role of the social environment in determining an individual's phenotype, and further implications are discussed below.

The second part of my dissertation addresses the question of how the evolution of parasitism in ants may have been facilitated by the redirection of plastic traits. I examined how phenotypic plasticity in the host species might have enabled the evolution of social parasitism in ants and whether an endoparasite uses phenotypic plasticity which evolved to allow caste differentiation to extend the lifespan of its host. In addition to comparing transcriptomes between individuals of the same species, for two of the chapters, I additionally used orthologue analysis to compare whether the same genes are responsible for similar phenotypes between species. My evidence suggests that the evolution of social parasitism is not facilitated by the reuse of already existing genes in the non-parasitic ancestor (**Chapters 6 and 7**). Moreover, the extended phenotype of an endoparasite, the prolonged lifespan of its intermediate host, seems to not be realized by regulating the expression of already existing caste-specific genes in the host (**Chapter 8**). Thus, my work provides no evidence that the evolution of parasitism was promoted by the reuse or redirection of host species genes in the systems studied. In the end, I propose additional experiments to further unravel whether redirection of phenotypic plasticity plays a role in the evolution of social parasitism in ants.

The influence of the social environment on gene expression

It has long been known that intraspecific social interactions are important for group-living species, from mammals to insects, and that they strongly influence the fitness of an individual (Formica *et al.*, 2012; Silk *et al.*, 2003). Not surprisingly, the most extreme change in the social environment an organism can experience, social isolation, has been shown to have multifactorial effects on animals, including changes in immunity, gut microbiome and gene expression, and behaviour (Cruces *et al.*, 2014; Donovan *et al.*, 2020; Chapter 2; Bibancos *et al.*, 2007). But which molecular mechanisms allow for

these changes to be realized immediately following changes in the social environment? Two possible mechanisms might contribute to these responses in general: they might either result in the release of cryptic genetic variation which is usually canalized or genotypes might react differentially to changes in the social environment in the form of phenotypic plasticity (Bailey and Moore, 2018). How phenotypic plasticity is involved in the response towards social fluctuations can be investigated by looking at associated changes in gene expression, especially in the brain which controls behaviour (Whitfield *et al.*, 2003). In the case of social insects, social interactions are of special importance as labour is divided among colony members and social behaviours are directed towards nestmates in an altruistic manner (Hölldobler and Wilson, 1990). How social insects react to changes in their social environment, which additional factors influence these responses and how the presence of social parasites inside the interaction networks changes responses will be discussed in the following section.

One of the most detrimental events in social insect colonies is the loss of the queen. This is especially true for colonies that only hold a single queen representing the sole reproductive individual in the colony (Oster and Wilson, 1978). As queen loss can occur in natural settings, workers should possess reaction norms that allow the propagation of their genes, for example by producing males. Indeed, in many ant species workers lost the spermatheca but retained their ovaries, allowing them to reproduce in queenless conditions. In concordance with this, experimental queen removal was shown to have multivariate effects on the remaining workers including worker fertility and increased survival (Kronauer *et al.*, 2010; Majoe *et al.*, 2021; Negroni *et al.*, 2021a; Choppin *et al.*, 2021a; Kohlmeier *et al.*, 2017). These changes were also shown to be reflected in the level of gene expression (Manfredini *et al.*, 2014) and moreover, even

in the absence of phenotypic changes, these transcriptomic changes are already present (Taylor *et al.*, 2021). Not only the presence or absence of a queen but moreover the number of queens can have many effects on colony traits, including the lifespan and size of both queens and workers (Goodisman *et al.*, 1999; Schrempf *et al.*, 2011; Nonacs, 1988; Keller and Genoud, 1997; Calabi and Porter, 1989; Rüppell and Heinze, 1999). Species that show polymorphism in the number of queens are thus interesting study objects to investigate how these changes are regulated and whether they represent either plastic responses (**Chapter 1**) or have underlying genetic determinants (Libbrecht and Kronauer, 2014). While evidence from other systems such as *Solenopsis invicta* and *Formica selysi* suggests the latter mechanism to determine queen number polymorphism (Wang *et al.*, 2013; Purcell *et al.*, 2014; Brelsford *et al.*, 2020; Yan *et al.*, 2020b), my analysis on the ant *Temnothorax rugatulus* could not find strong evidence for a genetic determination of queen morphology and the associated queen number (**Chapter 1**). Instead, queens from the two morphs reacted differently to changes in their social environment, namely the number of queens inside the colony, by differentially expressing the same genes but in different directions (Figure 0-3). As some of these genes were related to stress response, this suggests that the two queen morphs experience different social environments as stress and that this is reflected in the level of brain gene expression.

Thus, not only does the social environment influence brain gene expression in queens of this species but this change is additionally influenced by queen morphology and the social environment these morphs are adapted to. That changes in the social environment interact with other factors is also evident from a study in the fire ant *S. invicta*: While in queenright colonies, global gene expression differed between workers performing tasks inside or outside the nest, these differences diminished when the

queen was removed (Manfredini *et al.*, 2014). Similarly, in the brain data from workers of colonies headed by macrogynous or microgynous queens, I found the interaction of queen morph and behavioural task to explain most gene expression differences (**Chapter 1**). Another system in which the social environment influences division of labour is represented by ants that lack a caste system, such as the clonal raider ant *Oocerea biroi* (Ravary and Jaisson, 2004). Here, ants switch from a reproductive to a non-reproductive phase depending on the presence of larvae (Ravary *et al.*, 2006). Thus, the social environment in which ants live largely determines other traits, including division of labour, and these can change plastically as the social environment is altered. My results add to the growing number of studies examining the transcriptomic signature of the division of labour and the factors that influence it by showing that it is not only the presence of the queen or brood that is important but also the queen's traits.

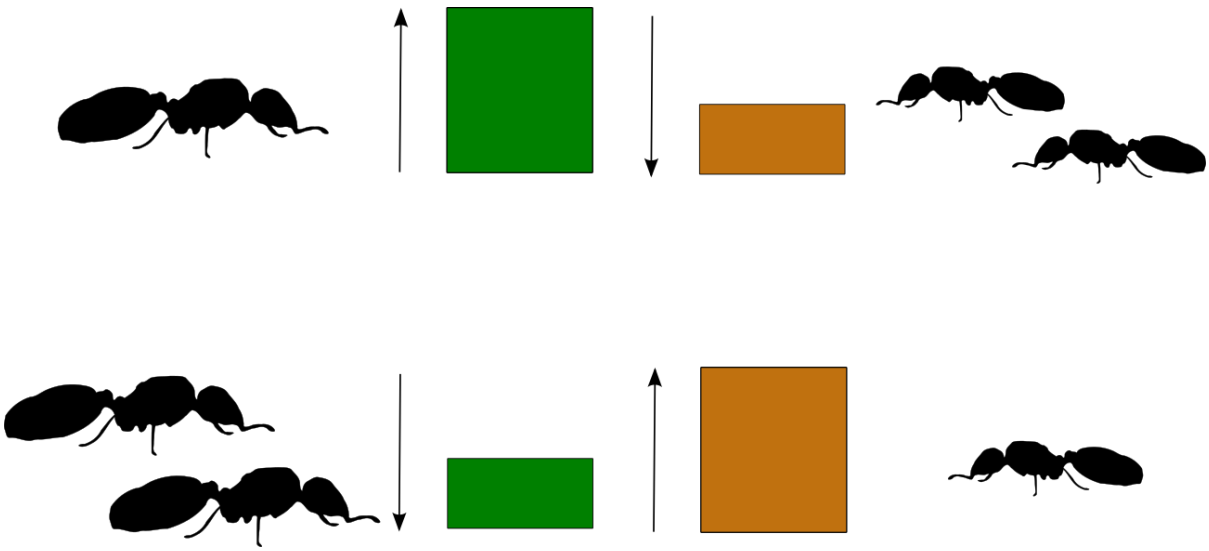

Figure 0-3 Graphical representation of genes influenced by an interaction of queen morph and queen number in *T. rugatulus*. On the left: Macrogyne queens. On the right: Microgyne queens. Top: Colonies with one queen. Bottom: Colonies with two queens. Arrows indicate the direction of changes in the expression of genes which are coloured to indicate different sets of genes.

Even more extreme than the loss of the queen for a social insect is isolation from the entire colony. In general, social isolation was shown to impact multiple traits in social insects including lifespan, immunity and behaviour as well as interest in nestmate contact (Koto *et al.*, 2015; Traniello *et al.*, 2002; Hughes *et al.*, 2002; Hewlett *et al.*, 2018; Lihoreau *et al.*, 2009). But besides experimental manipulations, social isolation of ants from their colony can also occur in the form of an altruistic act for example when infected ants leave the nest to not infect others (Heinze and Walter, 2010). Another natural scenario in which social isolation is a part of an ants' lifecycle is the founding phase of most ant species. Queens which found colonies independently, do so completely on their own without the help of workers, who will emerge from their first brood. Whether queens found the new colony all on their own or together with other queens influenced the brain gene expression of *Solenopsis* queens (Manfredini *et al.*, 2021). Similarly, the isolation of workers from their natal colony influences brain gene expression (**Chapter 2**). Thus, studies on brain gene expression are suited to investigate how social isolation shapes the neurological pathways influencing ant behaviour. But since social isolation alters many external and internal stimuli which might shape ant behaviour and gene expression, more behavioural and physiological studies are needed to unravel the exact mechanisms behind the observed changes in gene expression (Figure 0-4).

One change often observed when ants are isolated from their colony is reduced immunity (Hughes *et al.*, 2002; Traniello *et al.*, 2002). As social isolation usually occurs in workers which are deemed to die either through old age or disease, investment into immunity under these circumstances seems to be redundant. Another reason for a reduction in immunity following social isolation could be the lack of social immunity (Cremer *et al.*, 2007). In ants next to innate immunity, social immunity, where group

members employ specific defence strategies to reduce the spread of infections within the group for example by grooming pathogens from nestmates, determines how pathogens are spread and respectively how high the individual parasite load is (Hughes *et al.*, 2002). Ants which lack contact to their nestmates are thus more likely to be exposed to a detrimental dose of parasites such as fungi, which could trigger an immune response. An additional factor that could influence the response of workers to social isolation is the loss of the queen isolated individuals experience, which impacts fecundity and also gene expression in workers (Manfredini *et al.*, 2014; Negroni *et al.*, 2021a). Both immunity and reproduction represent costly processes that were shown to be traded-off in insects (Schwenke *et al.*, 2016). Thus, changes in fecundity are likely to result in changes in immunity. Following these suggested consequences of social isolation, isolated ants showed differential expression of genes related to immunity in the ant *Temnothorax nylanderi* (**Chapter 2**).

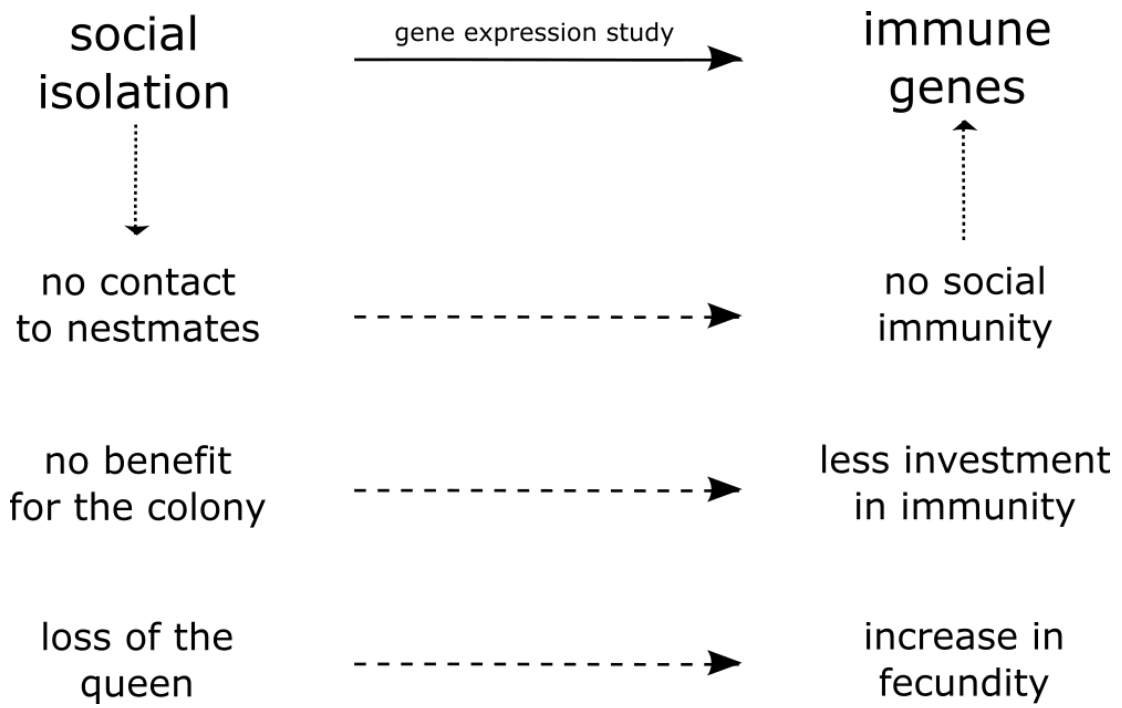

Figure 0-4 Possible causalities inducing the differential expression of immune genes in isolated workers.

My findings as well as the described possible mechanisms behind them represent examples of how the social environment can shape different life-history traits in ants and how this is reflected on the level of gene expression. However, they also highlight the importance of multi-factorial designs in such transcriptomic studies to disentangle different factors influencing individual gene expression as well as the need for studying behavioural and physiological changes in parallel to transcriptomic changes to detect possible causalities.

In the two chapters discussed above, I considered the social environment as a community of individuals of the same species living together. In addition, the interactions between ant hosts and their social parasites can also be considered social since the hosts direct certain social behaviours towards the parasites and both live in the same colony (D'Ettorre and Heinze, 2001). Thus, the parasite colony also represents a form of the social environment not only for the parasite but also for the inhabiting host workers. In parasitized populations, host workers occur naturally in both parasitic and non-parasitic nests and should therefore possess respective reaction norms adapted to both situations. But how do both social environments differ? In parasitic nests, multiple species coexist together even though in parasitized host populations aggression against parasites as well as conspecifics from other colonies is usually high (D'Ettorre *et al.*, 2004; Pamminger *et al.*, 2011; Kleeberg *et al.*, 2015). Additionally, in parasitic nests no host queen is present, so host workers experience a situation similar to queen loss. Despite these differences, host workers living within the parasitic nest perform all the necessary worker chores such as nursing the parasitic brood and feeding the parasitic workers (Hölldobler and Wilson, 1990), even though records in *T. longispinosus* parasitized by *T. americanus* suggest that hosts can rebel against the parasites by destroying their brood (Achenbach and Foitzik, 2009). These differences in

the social environment of exploited host workers are likely reflected at the level of gene expression, particularly in the brain, the centre for behaviour, but also in the antennae, which are primarily involved in recognition. When *L. acervorum* host pupae were placed in parasitic colonies of *H. sublaevis*, the emerging host workers exhibited transcriptomes of the brain and antennae that differed from those of their sisters living in the mother or conspecific colony (**Chapter 4**). Similarly, in another host-parasite system, the parasitic wasp *Polistes sulcifer* and its host *Polistes dominula*, transcriptomes of parasitized hosts changed even before physiological changes were apparent, in this case, induction of fertility (Taylor *et al.*, 2021). My results provide the first line of evidence that in *H. sublaevis* colonies parasitic manipulation of hosts, be it via avoiding detection or by chemical manipulation, is incomplete since host transcriptomes diverge from their free-living sisters. The question remains whether the social behaviours performed by hosts inside parasitic nests such as nursing the parasitic brood are a) due to a **lack of conspecific recognition** because the profile of the parasite is either too similar or not differentiable (Kleeberg and Foitzik, 2016; Jongepier and Foitzik, 2016a; Kleeberg *et al.*, 2017) b) because they **learned to accept the odour of the parasitic nest** and the inhabiting parasites as their own or (Carlin and Hölldobler, 1983; Le Moli and Mori, 1987) c) because **parasites behaviorally or chemically manipulate** host workers (Jongepier *et al.*, 2015; Foitzik *et al.*, 2003). It is, however, likely that not only one of the above-mentioned mechanisms is true, but that multiple act together. Future studies should also investigate which genes are differentially expressed in exploited host workers depending on parasite prevalence, as these may be subject to selection in hosts and responsible for defence strategies such as parasitic brood destruction (Achenbach and Foitzik, 2009). In general, dulotic social parasites exert strong selection pressure on their hosts (Foitzik *et al.*, 2009). Thus, hosts are under selection to adapt to them by implementing respective countermeasures as

parasite attacks are often detrimental for the colony since many adults including the queen can be killed and brood is stolen (Foitzik and Herbers, 2001b). Such strategies include elevated aggression of hosts as well as a diversification of CHC profiles (Jongepier and Foitzik, 2016a; Kleeberg *et al.*, 2015). But since in a population only a part of the colonies are actually parasitized, and expressing such defences is costly for hosts, many of these defence responses are expected to be plastic and only induced when parasites are present. This plasticity was extensively studied on the population level showing that in the presence of parasites host colonies adapted by employing respective defences (Jongepier *et al.*, 2014; Bauer *et al.*, 2009; Kleeberg *et al.*, 2015; Pamminger *et al.*, 2013). Therefore, after I studied the transcriptomic influence of the social environment on the individual (**Chapter 2**) and the colony level (**Chapter 1, Chapter 4**), I next investigated how the presence of parasites in the population influences gene expression and other behavioural and physiological traits in *T. longispinosus* hosts (**Chapter 5**). This revealed that the parasite prevalence of the host population affected brain gene expression of host defenders while encountering a parasite less than the parasite prevalence of the population the parasite originated from. Similarly, parasites from populations with higher parasite prevalence were less often attacked by hosts, probably because they carry fewer recognition cues (**Chapter 5**). Thus, the plastic response hosts show when facing a parasite are in this case determined by traits of the parasite and not by the social environment the hosts originated from. This suggests that parasites lead the coevolutionary arms race, and have evolved infection traits that circumvent successful defences by hosts. Studies investigating the effect of the parasite prevalence are thus ideal to study the coevolutionary consequences of host-parasite interactions and how these are reflected on the transcriptomic level (**Chapter 3**). More population-genomic studies are needed to exactly determine which traits shaped by coevolution in both hosts and parasites

represent genetic variants and which are plastically expressed depending on the social environment they act in.

How phenotypic plasticity can aid the (co)evolution of parasites

Obligate parasitism where a parasitic species completely relies on its host for survival is very often related to losses. These losses can occur on the morphological, physiological or behavioural level and very often can be traced back to the level of the genome. For example, parasitic plants lost the ability to perform photosynthesis through relaxed selection on photosynthesis-related genes as they exploit their host plant's resources instead (Krause, 2008). Similarly, tapeworms show a loss of homeobox genes, which are important transcription factors, determining the adult morphology, possibly because their lifestyle allows for a simplified body plan (Tsai *et al.*, 2013; Poulin and Randhawa, 2015). In insect social parasites the transition to the parasitic lifestyle and the exploitation of host social behaviour renders genes related to social behaviour in the parasites redundant. This is, for example, evident in workers of the dulotic ant *T. americanus* which in contrast to their hosts do not overexpress a gene related to social behaviour in ants, *Gp-9* (**Chapter 7**). Additionally, in this and other systems, the transition to social parasitism is accompanied by the loss of certain odorant receptors (Schrader *et al.*, 2021; Jongepier *et al.*, 2022; Sun *et al.*, 2021; Chapter 3). These 9-exon receptors are especially important in the detection of nestmate cues and thus are not extensively required by social parasites which show a reduced repertoire of social behaviours (Zhou *et al.*, 2015; Engsontia *et al.*, 2015; McKenzie and Kronauer, 2018). But there are also gains related to the parasitic lifestyle, which often relate to the infection and exploitation of hosts. For example, parasitic helminths often gained the ability to suppress their hosts' immune defences or even manipulate their host's behaviour (Maizels *et al.*, 2004; Hughes and Libersat, 2019;

Libersat *et al.*, 2008). In the case of social parasites, these gains are morphological adaptations designed for efficient host infiltration as well as behavioural strategies such as raiding (Wilson, 1971). In both the endo- and social parasites the question remains whether these gains in function are facilitated by exploiting phenotypic plasticity already present in the host or non-parasitic ancestor.

Insect social parasites share a common non-parasitic ancestor with their host representing unique model systems to investigate how parasitic behaviour evolved in closely-related species sharing the same environmental constraints (Borowiec *et al.*, 2021; **Chapter 3**; Feldmeyer *et al.*, 2017; Beibl *et al.*, 2005). Social insects in general display a high degree of phenotypic plasticity including the differentiation between reproductive queens and non-reproductive workers (Wilson, 1971). Such changes in reaction norms as a response to changes in the environment have been previously proposed to facilitate evolution (Crispo, 2007; Price *et al.*, 2003; West-Eberhard, 1989). Thus, it is likely that this plasticity has somehow aided the phenotypic evolution of social parasites. Cini et al. proposed two likely scenarios of how phenotypic plasticity can aid the molecular evolution of social parasitism (Cini *et al.*, 2015): In the first scenario, parasites might facilitate already existing plasticity in host phenotypes by only expressing a certain phenotype, thereby driving the genetic fixation of this specific phenotype and the deletion of others ("phenotype deletion"). For this to be the case two conditions would need to be in place: first, a conserved set of genes would need to be responsible for certain phenotypes in parasites and hosts. Second, these phenotypes would need to be expressed under different conditions in the parasite. In the case of social parasites where the reproductive division of labour seems to be less pronounced or completely missing (Calis *et al.*, 2002; Dapporto *et al.*, 2004; Aron *et al.*, 1999), one could therefore imagine that parasites express the queen phenotype across

environments, implying a deletion of the worker phenotype. Another possible scenario might be that parasites acquire novel phenotypes as a response to the environment which likely involves the acquisition of novel genomic features and therefore might be more expensive ("phenotype shift"). Evidence for the first scenario can be found for example in the Cape honeybee where queen-biased genes are also upregulated in parasitic workers possibly explaining their high reproductive potential inside host colonies (Korb *et al.*, 2021; Aumer *et al.*, 2018). Similarly, in the parasitic paper wasps, *Polistes sulcifer,* worker-biased genes were downregulated (Cini *et al.*, 2015). In one of my study systems, the dulotic ant *T. americanus,* workers show high reproductive potential, in some cases over 70% of male-destined eggs were worker-produced (Foitzik and Herbers, 2001a). By comparing transcriptomes of different developmental stages, sexes and castes between the parasite and its host *T. longispinosus*, I aimed to see whether a conserved set of genes drives these phenotypes in both species or whether the transcriptomic signature of parasitic workers resembles more the one of queens, matching the phenotype deletion scenario (**Chapter 7**). My results suggest that transcriptomes in early developmental stages are more conserved between parasites and hosts than are adult transcriptomes and that molecular functions are conserved in larvae, pupae but also workers, while transcriptomes of sexuals differed more strongly between host and parasite. That queens of both species showed different sets of caste-related genes suggests that in this case there is not a set of toolkit queen-genes responsible for the reproductive phenotype. Moreover, we found a high functional similarity between genes overexpressed in workers of both species, suggesting even parasitic workers express certain worker-related genes. Together this does not support the phenotype deletion scenario where the worker phenotype becomes obsolete and therefore a queen-like expression is fixated. But, we found a higher number of differentially expressed genes to overlap between worker pupae, adult workers and

queens in *T. americanus*. This might suggest some extent of similarity of parasitic queens and workers on the transcriptomic level, at least partially matching the phenotype deletion model. The reduced differentiation between the worker and queen phenotype might in the end lead to the evolution of gene losses due to relaxed selection on worker-biased genes including those involved in social behaviour (e.g., Jongepier *et al.*, 2022). Additionally, I investigated convergence on the behavioural level: Both parasite and host use tandem-running as a recruitment strategy to relocate their nest or to initiate raiding parties (Möglich *et al.*, 1974; Wilson, 1959). While host workers possess the ability to perform nest recruitment throughout the year, parasitic workers initiate raiding parties only during a certain period in summer. I used brain transcriptomes of recruiters and followers, to investigate whether the evolution of raiding behaviour, a characteristic of dulotic ants, was facilitated by the reuse of genes responsible for nest site relocation in the host. Again, we were not able to detect a conserved set of genes responsible for these very similar behaviours in both the parasite and its host (**Chapter 6**). Overall, these results suggest that social parasites use rather genomic innovations to express specific phenotypes than to reuse genes already present in the non-parasitic ancestor. Another possibility might be a combination of the shift and deletion model. Due to the long speciation time between our two species (~17 mya, see Prebus, 2017), deletion might have occurred first but later the more specialized behaviour involved more and more specialized gene functions and gene-regulatory networks might have been rewired.

While social parasites and their hosts are very often closely related (Emery, 1909), this is not the case for endoparasites and their hosts, therefore parasite traits are not likely to originate from host traits. However, the alterations induced by the parasites in their hosts are commonly referred to as the extended phenotype of parasites (Dawkins,

1982). Again, similar to the two models proposed above, the question arises whether the parasites manipulate their host to express an already existing phenotype in a different context (similar to phenotype deletion) or express completely new phenotypes (similar to the phenotype shift model). The red-berry abdomen for example which is expressed by *Cephalotes atratus* when infected with the nematode *Myrmeconema neotropicum* represents a completely new phenotype (Poinar and Yanoviak, 2008). On the other hand, in *Polistes dominula* wasps infection with *Xenos vesparum* made workers express a transcriptomic profile resembling the one of gynes, matching at least partially a deletion of the typical worker profile in infected individuals (Geffre *et al.*, 2017). In the ant *T. nylanderi* infection with the cestode *Anomotaenia brevis* induces multiple phenotypic changes, the most obvious being the less sclerotized cuticle resulting in the yellow appearance of infected workers (Plateaux, 1972; Trabalon *et al.*, 2000). Moreover, infected ants are less active, show reduced flight response and live longer than their uninfected nestmates (Beros *et al.*, 2015, 2021). Interestingly, their survival is similar to the one observed in queens which raises the question of whether for this alteration the already existing plastic queen phenotype is highjacked by the parasite so that it is expressed in a different context, namely in infected workers. My analysis on abdominal gene expression could only find a very small number of genes to overlap between queens and infected workers, rejecting this hypothesis (**Chapter 8**). Whether the prolonged lifespan of infected workers is induced by parasitic manipulation via the release of specific substances into the host's haemolymph needs to be further investigated using proteomic analyses.

Future work

While the studies performed so far, could not detect evidence for reuse of already existing genes in the evolution of dulotic behaviour in ants (**Chapter 6 and 7**), further experiments are required to confirm the role of existing phenotypic plasticity in the evolution of dulosis. Raiding behaviour for example might have evolved from foraging instead of nest relocation behaviour (Buschinger, 1986; Darwin, 1859). Thus, a follow-up experiment should investigate whether genes between foraging hosts and raiding parasites overlap. Moreover, two problems might hinder us to detect any shared transcriptomic signature in the above-mentioned studies: a) the long speciation time between the social parasite *T. americanus* and its host *T. longispinosus* (Prebus, 2017) and b) the fact that both raiding and nest relocation are performed in different contexts. Therefore, choosing another host-parasite system with a shorter speciation time (Blaimer *et al.*, 2018; Prebus, 2017) might be more suited to detect the origin of raiding behaviour. Ants of *T. curvispinosus* for example were shown to also raid other colonies when put nearby, offering a possibility to compare transcriptomic signatures of workers of both the social parasite *T. duloticus* and its host *T. curvispinosus* during raiding (Wilson, 1975). Especially in younger parasites, the deletion of a phenotype might not be complete, allowing for a certain recovery of ancestral behaviours such as brood care and foraging. Studying the reversibility of these behaviours in different parasites of which some were already shown to partially be able to recover foraging activities (Stuart and Alloway, 1985), would allow direct comparison with hosts performing these behaviours. If indeed the same genes would be used for these behaviours in both hosts and parasites, this would provide the first evidence for the phenotype deletion model in the context of dulotic ants. Otherwise, support for the phenotype shift scenario can be found by looking closer at the respective genes in the parasite to see how they evolved. Transposable elements, for example, could promote

the adaptation of parasites to their new lifestyle as these were shown to allow another ant, *Cardiocondyla obscurior*, to adapt to novel environments (Schrader *et al.*, 2014).

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Curriculum Vitae

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