The role of epigenetic mechanisms in the regulation of division of labor in the ant *Temnothorax longispinosus*

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Tag der mündlichen Prüfung:

“Still, if history and science have taught us anything, it is that passion and desire are not the same as truth. The human mind evolved to believe in the gods. It did not evolve to believe in biology. Acceptance of the supernatural conveyed a great advantage throughout prehistory when the brain was evolving. Thus, it is in sharp contrast to biology, which was developed as a product of the modern age and is not underwritten by genetic algorithms. The uncomfortable truth is that the two beliefs are not factually compatible. As a result, those who hunger for both intellectual and religious truth will never acquire both in full measure”

– Edward O. Wilson

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Note: Supplementary material can be found online for published chapter 1 or following the discussion for no-published chapters 2 and 3.

Zusammenfassung

Arbeitsteilung (DOL) ist ein wichtiges Organisationsprinzip komplexer biologischer Systeme, welches sich während der großen Übergänge in der Evolution wiederholt entwickelte und wesentlich zum ökologischen Erfolg von Insektengesellschaften beiträgt. Die reproduktive Arbeitsteilung führt dazu, dass sich ein oder wenige Individuen in einer Kolonie auf die Fortpflanzung konzentrieren (Königinnen/Könige), während die übrigen Gruppenmitglieder als sterile, nicht reproduktive Arbeiterinnen fungieren. In den meisten Insektengesellschaften gibt es auch unter den Arbeitern eine DOL für Aufgaben, die für das Wachstum und die Produktivität der Kolonie wichtig sind. Alle Mitglieder haben eine spezifische Rolle, wobei sie sich auf Aufgaben innerhalb der Kolonie, wie die Brutpflege oder die Pflege der Nestgenossen, und auf Aufgaben außerhalb der Kolonie, wie die Nahrungssuche oder die Verteidigung der Kolonie, konzentrieren. Obwohl das Alter eine wichtige Rolle bei der DOL spielt, bleibt die Aufteilung der Aufgaben dynamisch, und die Arbeiterinnen können je nach den Bedürfnissen der Kolonie hin- und herwechseln. Im Feld wird allgemein angenommen, dass DOL darauf zurückzuführen ist, dass die Individuen unterschiedlich auf aufgabenbezogene chemische Signale reagieren, was zu Verhaltensvariationen und Aufgabenspezialisierung führt. Daher besteht eine wichtige Frage darin, die regulatorischen Prozesse zu verstehen, die das Verhalten der Arbeiterinnen und ihre Spezialisierung auf bestimmte Aufgaben durch unterschiedliche Genexpression steuern.

In dieser Studie untersuchten wir die transkriptionellen Unterschiede zwischen verschiedenen Verhaltensphänotypen im Gehirn (d. h. im zentralen Nervensystem) und in den Fühlern (d. h. in den peripheren Sinnesorganen) der kleinen Eichelameise *Temnothorax longispinosus*. Wir fanden heraus, dass i) Verhaltensvariationen mit signifikanten transkriptomischen Veränderungen in den Fühlern einhergingen, ii) diese Veränderungen einen großen Teil des Repertoires an Geruchsrezeptor (OR)-Genen umfassten und iii) Arbeiterinnen, die sich auf die Brutpflege spezialisiert hatten, ORs der Exon-9-Genfamilie überexprimierten, von denen man annimmt, dass sie an der Erkennung sozialer Signale beteiligt sind. Diese Ergebnisse stützen unsere Hypothese, dass das periphere Nervensystem eine wichtige Rolle als sensorischer Filter bei der

Regulierung von Verhaltensunterschieden zwischen Arbeiterinnen und damit bei der DOL sozialer Insekten spielt. Bislang war man davon ausgegangen, dass alle Arbeiterinnen aufgabenbezogene Hinweise in gleicher Weise wahrnehmen, aber nur dann eine Aufgabe übernehmen, wenn das zugehörige Signal die Reaktionsschwelle des Individuums überschreitet. Unsere Ergebnisse deuten jedoch darauf hin, dass nicht alle Arbeiterinnen die Signale in gleicher Weise wahrnehmen, weil ein sensorischer Filter in den Fühlern den Zugang des zentralen Nervensystems zu chemischen Informationen, die für die Aufgabenerfüllung wichtig sind, einschränkt.

Die epigenetische Regulierung dieses sensorischen Filters ist noch unbekannt, aber frühere Experimente deuten auf eine Rolle von Kandidatengenen wie *Vg-like-A* und epigenetischen Faktoren wie Histon-Acetylierung oder miRNAs hin. Daher haben wir *Vg-like-A* durch RNAi-Injektion in die Gaster von Ammenameisen ausgeschaltet, um die Funktion dieses Signalwegs bei der Beeinflussung des Verhaltens der Arbeiterinnen innerhalb von 4-10 Tagen zu untersuchen. Wir dokumentierten eine Herunterregulierung über 10 Tage, die auch zu einigen Verhaltensänderungen führte, aber im letzten Experiment, in dem wir Veränderungen der Genexpression in verschiedenen Geweben, einschließlich der Antenne, untersuchen wollten, schlug die Herunterregulierung von *Vg-like-A* fehl. Schließlich haben wir ein ChIP-seq-Protokoll für Gehirnproben und eine bioinformatische Analysepipeline entwickelt, um die Unterschiede in der Histonacetylierung (H3K27ac) zwischen Brutpflegerinnen und Futtersammlerinnen zu untersuchen, und wir erörtern Fragen der Versuchsplanung, die unsere Ergebnisse beeinflusst haben könnten. Zusammenfassend lässt sich sagen, dass diese Arbeit einen wichtigen Beitrag zum Verständnis der molekularen Regulierung der Arbeitsteilung in Ameisenkolonien geleistet hat. Sie hat nicht nur Beweise für die Funktion eines sensorischen Filters geliefert, sondern auch Ansätze für die weitere Analyse von regulatorischen Elementen, einschließlich neuer Kandidatengene, Histonmodifikationen und miRNAs, die weitere Untersuchungen verdienen.

Summary

Division of labor (DOL) is a key organizing principle of complex biological systems that arose repeatedly during the major transitions in evolution and contributes significantly to the ecological success of insect societies. Reproductive DOL results in one or a few individuals in a colony focusing on reproduction (queens/kings), while the remaining group members function as sterile, non-reproductive workers. In most insect societies, there is also a DOL among the workers for tasks important for colony growth and productivity. All members have a specific role, concentrating on intranidal tasks such as brood or nestmate care, and outside tasks such as foraging or colony defense. Although age plays an important role in DOL, the allocation of tasks remains dynamic, and workers can switch back and forth according to the needs of the colony. It is generally accepted in the field that DOL stems from individuals differing in their response to task-related chemical signals, leading to behavioral variation and task specialization. Therefore, a major question is to understand the regulatory processes that control the behavior of workers and their specialization on specific tasks via differential gene expression.

In this study, we investigated transcriptional differences between distinct behavioral phenotypes in the brain (i.e., the central nervous system) and in the antennae (i.e., the peripheral sensory organs) of the small acorn ant *Temnothorax longispinosus.* We found that i) behavioral variation was associated with significant transcriptomic changes in antennae, ii) these changes encompassed a large portion of the repertoire of odorant receptor (OR) genes, and iii) workers specialized on brood care overexpressed ORs of exon-9 gene family thought to be involved in social cue recognition. These results support our hypothesis that the peripheral nervous system plays an important role as a sensory filter in regulating behavioral differences between workers and thus in the DOL of social insects. Until now, it had been assumed that all workers perceive task-related cues in the same way, but only take on a task if the associated signal crosses the response threshold of the individual. However, our results suggest that not all workers perceive the signals similarly because a sensory filter in the antennae restricts the central nervous system's access to chemical information that is important for task performance.

The epigenetic regulation of this sensory filter is still unknown, but previous experiments suggest a role of candidate genes such as *Vg-like-A* and epigenetic factors such as histone acetylation or miRNAs. Therefore, we proceeded to knocked down *Vg-like-A* via RNAi injection in the gaster of nurse ants to assess the function of this pathway in influencing worker behavior within 4-10 days. We documented a downregulation over 10 days, which also led to some behavioral changes, but in the final experiment, in which we aimed to investigate changes in gene expression in different tissues including the antenna, the downregulation of *Vg-like-A* failed. Finally, we developed a ChIP-seq protocol for brain samples and bioinformatic analytical pipeline to investigate differences in histone acetylation (H3K27ac) between nurses and foragers and discuss experimental design issues that may have influenced our results. In summary, this work has made an important contribution to the understanding of the molecular regulation of the division of labor in ant colonies. It has not only provided evidence for the function of a sensory filter, but also approaches for further analysis of regulatory elements, including new candidate genes, histone modifications and miRNAs, which deserve further investigation.

General Introduction

Marcel A. Caminer

Caste determination and division of labor in social insects

The evolution of life on Earth has been driven by a few major evolutionary transitions involving significant changes in the organization of living systems. These transitions involved a change in how information is transmitted between generations, often resulting in the emergence of new, more complex levels of biological organization that have played a crucial role in shaping the diversity of life today (Maynard Smith & Szathmáry, 1995). Major evolutionary transitions in individuality, a subset of these transitions, are defined as a group of individuals joining to form a new, more complex life form that can only replicate as a whole, and where there is negligible within-group conflict (Queller, 2000). The formation of a cooperative group could be transformed into a more cohesive and integrated entity that can be considered a new level of individual (organism). This step usually involves individuals specialized in performing different tasks, which makes them dependent on each other. This needs to a need to develop or improve communication to coordinate cooperation at the new group level (Cooper & West, 2018).

Many species of insects, such as ants, bees, wasps, and termites, live in highly organized societies, where different individuals within the colony perform specific tasks. They have reached an impressive level of social organization, including sophisticated communication skills, learning abilities, cooperation, including altruism and sometimes self-sacrifice, and division of labor (DOL). In most cases, they can resolve conflicts that arise within the group. Social insects are organized in colonies ranging in size from a few to a million or more non-reproductive workers and one or a few reproductive individuals, the queens (Oster & Wilson, 1978). The workers carry out various specialized tasks that are necessary for the growth and maintenance of the colony, e.g. brood care, foraging and defense of the colony (Corona et al., 2016; Gordon, 2016; Leitner & Dornhaus, 2019). This non-reproductive DOL among workers results in an increased efficiency in task performance (Amado et al. 2018; Goldsby et al. 2012). In some social insects, workers might exhibit almost little task specialization, while in others they might differentiate into developmentally-determined morphological castes that focus on only few tasks (Oster & Wilson, 1978). Therefore, caste determination stems mostly from a developmental switch controlled either by environmental factors or strong genetic effects (Schwander et al., 2010).

The distribution of tasks can change in the course of the colony's life cycle. Most colonies start with a founding queen and the number of workers increases as the colony ages. The newly emerged workers typically start as nurses and then become foragers or builders as they grow older (Gordon, 2010). The regulation of workers activity depends on the age (polyethism) and size of the colony. This relative age polyethism has a built-in flexibility that allows workers being able to respond adaptively to unexpected changes in demography with accelerated, retarded, or reversed behavioral development (Huang & Robinson 1996; McDonald &Topoff 1985; Robinson 1992). Colony size affects the probability that workers will be available to switch task in response to changing conditions (Gordon, 1989), while polyethism ensures that tasks are performed by individuals with the appropriate skills and experience. The colony as a whole might become responsive to changes in environmental conditions, such as food availability, variable task demands or worker losses, by adjusting the ratios of individual workers engaged in different tasks (Gordon, 1991).

Social communication and response thresholds model

Communication between and within the different castes in social insects are fundamental for the efficient functioning of a colony. Communication is largely based on chemical signals (or pheromones) that are produced by exocrine glands, to a lesser extent, on visual and tactile communication (Ayasse et al. 2001; Heinze & D'Ettorre 2009; Richard and Hunt 2013). The major pheromones of eusocial insects include queen pheromones, sex pheromones, alarm pheromones, and trail pheromones and as their names such they take over different roles (Hölldobler & Wilson 1990; Leonhardt et al. 2016; Wyatt 2014). In addition, cuticular hydrocarbons (CHC) are synthesized in oenocytes (a group of cells often located in the abdomen close to the cuticle) and secreted through the cuticle to the surface of the insect body (Lockey, 1988). CHCs are broadly used as cues for recognition of colony members, which is based on heritable variation in CHC production between colonies (Martin & Drijfhout 2009; Walsh et al. 2019; van Zweden & D'Ettorre 2010). Indeed, pheromones function as negative and positive feedbacks playing a crucial role in balancing the number of workers performing different tasks (Huang & Robinson 1996; Robinson 1992).

Recently studies started to elucidate caste-specific differences in the olfactory system that underlie caste-specific responses to pheromones. A chemosensory system is essential to discriminate among a large variety of chemicals in the environment. The pheromone signals are perceived with the chemosensory neurons localized in hair-like structures, called sensilla, on the antennae before being further processed by the olfactory system (Sharma et al., 2015; Villar et al., 2015; Wanner et al., 2007). There are three major classes of chemoreceptors: odorant receptors (ORs), gustatory receptors

(GRs) and variant ionotropic receptors (IRs) (Suh et al. 2014). The odorant receptor neurons (ORNs) that express ORs are specialized in detecting most volatile chemicals, including low volatility CHCs (Kwon et al., 2007). In fact, the insect OR gene family is thought to have evolved from another family of chemosensory receptors, GR genes (Brand et al., 2018; Robertson, 2019). A recent study suggests that a large and novel clade of OR genes evolved in the ancestor of all ants, potentially enhancing pheromone communication (McKenzie et al., 2016). In addition, ants and at least one social wasp have between 300–500 OR genes, which cluster into 27 subfamilies, with the 9-exon subfamily being the largest, containing between one-third and one-half of the known OR genes (Legan et al. 2021; McKenzie & Kronauer 2018; Zhou et al. 2012, 2015). Based on this, the expansion of the OR family in the Hymenoptera is associated with positive selection (Roux et al. 2014; Saad et al. 2018; Zhou et al. 2015) in both solitary as well as social species, suggesting that it can be driven by factors associated with both eusocial organization and solitary life.

High levels of cooperation and division of labor among workers in the non-reproductive caste of social insects are often considered to be determinant of their ecological success (Hölldobler & Wilson, 1990). The main model for the self-organized division of labor in social insect colonies is based on the idea that workers at different behavioral states exhibit different behavioral response thresholds to task-specific stimuli (Gordon 2016; Beshers & Fewell 2001; Duarte et al. 2012a; Page & Sandra 1998; Bonabeau et al. 1996). This response threshold model assumes that individuals have inherent thresholds for responding to stimuli associated with specific tasks, where individuals with the lowest threshold for a task will perform this task more often. For example, in ant colonies, individuals with lower thresholds may be more likely to become foragers, while those with higher thresholds may specialize in chores like nursing the brood or defending the nest (Fig. 1). Consequently, although workers are exposed to many or all task-stimuli in the colony, the probability that an individual worker will respond to the stimulus for a particular task varies (Bonabeau et al. 1998; Duarte et al. 2012b; Leitner & Dornhaus 2019). Finally, the response thresholds in social insects can change based on internal factors (such as age, colony size, spatial distribution or hunger) and external factors (such as environmental conditions) (Gove et al. 2009; Jeanson et al. 2007; Merkle & Middendorf 2004; Richardson et al. 2011; Theraulaz et al. 1998). This flexibility allows the colony to adapt to changing circumstances.

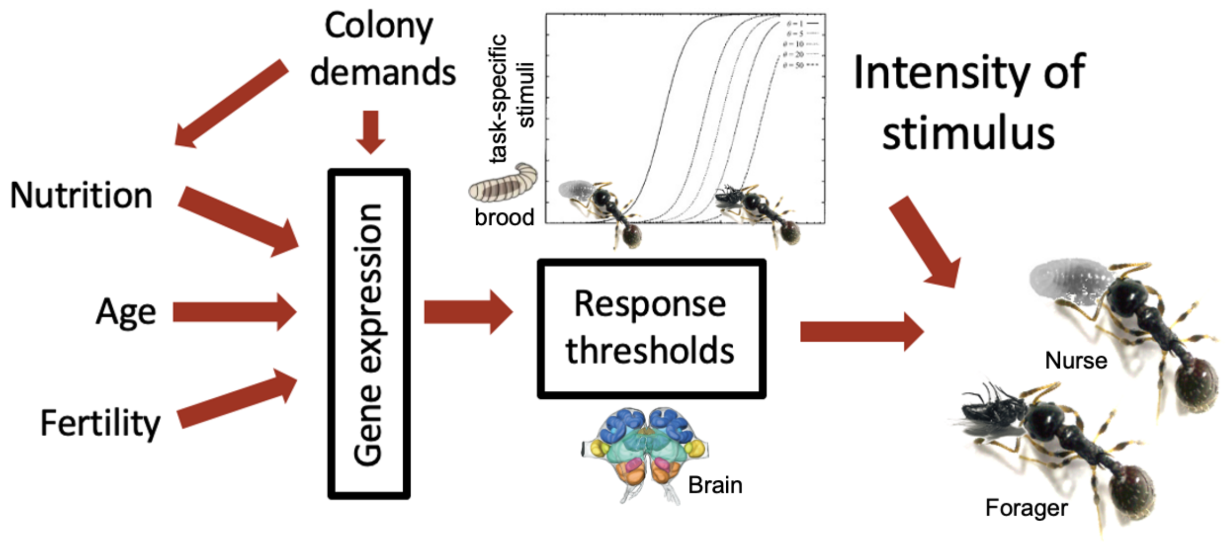

Figure 1. Division of labor in insect societies is based on the concept of response thresholds influenced by age, nutrition, fertility, experience and specially genes. (Arechavaleta-Velasco & Hunt 2003; Detrain and Pasteels 1991; Page et al. 1998; Pankiw & Page 1999; Ravary et al. 2007). Individuals with different thresholds respond differently to task-specifict stimuli. Low-threshold individuals become involved at a lower level of stimulus than high-threshold individuals. Response to the thresholds is presumably set in the central nervous system, possibly regulated via molecular pathways in the brain that correlate with behavioral variation (Beshers & Fewell 2001; Bonabeau et al.1998; Thamm & Scheiner 2014).

Gene expression and epigenetic regulation

Behavior of social insects and their caste system are controlled by an interaction between genetic and environmental effects. Gene expression and regulation can mediate plastic responses to environmental cues. Caste determination can be attributed to groups of genes whose functions are linked to developmental, hormonal, and metabolic pathways (Page & Amdam 2007; Corona et al. 2016; Weitekamp et al. 2017). The expression of these critical pathways remains highly sensitive to external cues, such as pheromone signaling and nutritional intake, and they are considered as highly responsive to factors within the social environment (Ament et al. 2008; Fischer & O’Connell 2017; Grozinger et al. 2003). One of the first behavioral genes to be identified was the gene *foraging*, which affects adult foraging behaviors and plays a role in regulating behavioral maturation in social insects (Ben-Shahar et al. 2002, 2003; Ingram et al. 2005; Lucas & Sokolowski 2009). Insulin/insulin-like growth factor signaling (IIS) and target of rapamycin (TOR) pathways then were found to play an important role in social behavior, linking changes in physiology to shifts in behavior (Ament et al. 2008, 2010). In addition, the feeding-related genes *neuropeptide F* (NPF) and gene *malvolio* (involved in the manganese transporter) are also associated with the transition from nursing to foraging behavior in honeybees (Ament et al. 2011; Ben-Shahar et al. 2004).

The conserved genetic and endocrinological networks that regulate fitness-relevant behavior, such as foraging and brood care, in their solitary ancestors have been decoupled from actual reproduction in the worker caste and now generate behavioral phenotypes (Pamminger & Hughes 2017). In eusocial insects, juvenile hormone (JH) signaling responds to nutrition and is important in various instances of caste determination, subcaste determination, and age polyethism (Bloch et al. 2009; Libbrecht et al. 2013; Nijhout & Wheeler 1982; Penick et al. 2012; Robinson 1987). Similarly, ecdysteroids, a class of steroid hormones produced in the gonads, have broad effects on physiology and social behavior (Pandey & Bloch, 2015). Levels of ecdysteroids are typically high in reproductive individuals and low in workers of species with reproductive DOL (Dolezal et al., 2012). Finally, JH affects the production of vitellogenin (Vg) which is typically involved in reproduction (Barchuk et al., 2002; Corona et al., 2016; Pinto et al., 2000). Nurses and foragers differ in ovary activity, Vg expression, and JH levels in bees (Amdam et al. 2004; Sullivan et al. 2000) and ants (Corona et al., 2007; Dolezal et al., 2012; Penick et al., 2011). Phylogenetic analyses revealed the existence of multiple genes encoding Vg in all of the ant genomes sequenced (Corona et al., 2013), suggesting the Vg genes regulate not only reproduction but also worker behavior (Corona et al., 2013). On the other side, biogenic amines have been well studied for their role regulating physiology and behavior in social insects. Biogenic amines function as neurotransmitters or neuromodulators in the nervous systems and can influence DOL, reproductive behaviors, learning and memory, stress response, aggression and defense (Cook et al. 2001; Kamhi & Traniello 2013; Raza et al. 2022; Schulz & Robinson 1999). Biogenic amines can also affect the ability of social insects to communicate with each other through chemical signals. They can influence the release and perception of pheromones, which are crucial for colony organization and social cohesion (reviewed in Zhukovskaya & Polyanovsky 2017).

DNA methylation may be one of the gene regulatory mechanisms that mediates behavior-specific changes in gene expression and affect caste differentiation in ants and honeybees (Alvarado et al. 2015; Foret et al. 2012; Kucharski et al. 2008; Lyko et al. 2010; Sweatt et al. 2016; but see Libbrecht et al. 2016). Changes in gene transcription are also associated with alteration of chromatin structure through histone post-translational modifications (hPTMs) between castes and developmental stages. For example, the level of acetylation of lysine 27 of histone H3 (H3K27ac) has been shown to be a strong predictor for caste identity (Kohlmeier et al. 2023; Bonasio et al. 2012; Simola et al. 2013a; 2016; Wojciechowski et al. 2018; Spannhoff et al. 2011). Both genome methylation and hPTMs might therefore influence caste determination through transcriptional control and alternative splicing, contributing to behavioral plasticity.

Non-coding RNAs (ncRNA), including micro-RNAs, could be another gene regulatory mechanism mediating gene expression changes associated with age and caste differentiation in ants (Seistrup et al., 2023). Micro-RNAs involved in pathways regulating caste differentiation (e.g., IIS, TOR) are differentially expressed between honeybee queen- and worker-destined larvae (Shi et al., 2015; Weaver et al., 2007). The transcriptional regulatory networks are the highly complex and interconnected molecular signaling pathways (between TFs and their target genes) underlying both gene expression and environmentally responsive regulation (Luscombe et al., 2004). Comparative analysis suggested that the abundance and diversity of TFs binding sites (TFBSs) might also play a role in the evolution of caste-specific patterns of gene expression (Simola et al. 2013b). TFBSs were found to be more divergent within ants than between social and solitary insects. Genes exhibiting important changes in the abundance of TFs between social and solitary insects showed higher levels of gene expression plasticity between castes (Simola et al. 2013b). In conclusion, the combination of *cis* epigenetic process (which involve DNA methylation, hPTMs and ncRNAs) and *trans* epigenetic process (TFs and ncRNAs) underline behavioral plasticity in social insects by their ability to maintain transcriptional patters over time.

Study system

Temnothorax longispinosus is a small monomorphic ant species that has a relatively uniform worker caste with unimodal size variation (Fig. 2). Ant colonies of this species can be found in temperate forests of Eastern North America, inside plant cavities such as hollow stems, twigs, and in acorns, as well as between clefts in rocks. Colonies contain from around 50 workers, although occasionally colonies can be found with a few hundred workers. They are relatively easy to collect and maintain under laboratory conditions. In this species, young workers usually takeover brood care inside the nest, whereas older workers focus on risky external tasks such as foraging, but workers also remain flexible (Kohlmeier et al. 2018). For example, when brood carers or foragers are removed, workers can either fast forward their behavioral development or reverse it, by taking up brood care again. In addition, task performance and underlying gene expression are largely independent of age or fecundity and are rather related to behavior (Kohlmeier et al., 2019). Recent experiments carried out on *T. longispinosus* in our lab, in which the histone acetyltransferase inhibitor C646 (Bowers et al., 2010) was administered to workers, impeded the switch of foragers back to brood care, but promoted the reversed change from brood care to foraging (Kohlmeier et al. 2023), and alter their circadian rhythms (Libbrecht et al., 2020). These behavioral results lead us to hypothesize that histone acetylation controls transcriptional changes associated with DOL. On the other hand, RNAi downregulation of the gene *Vg-like A* (an orthologous gene of vitellogenin) typically overexpressed in the fat bodies of *T. longispinosus* nurses reduced sensitivity to brood-related cues and increased the response to nestmate odors, suggesting a change in odor perception and olfaction-driven decision making in the ant brain (Kohlmeier et al. 2018). However, whether modulation occurs in at the periphery of the sensory system such as in the antennae or in higher brain regions remains unexplored.

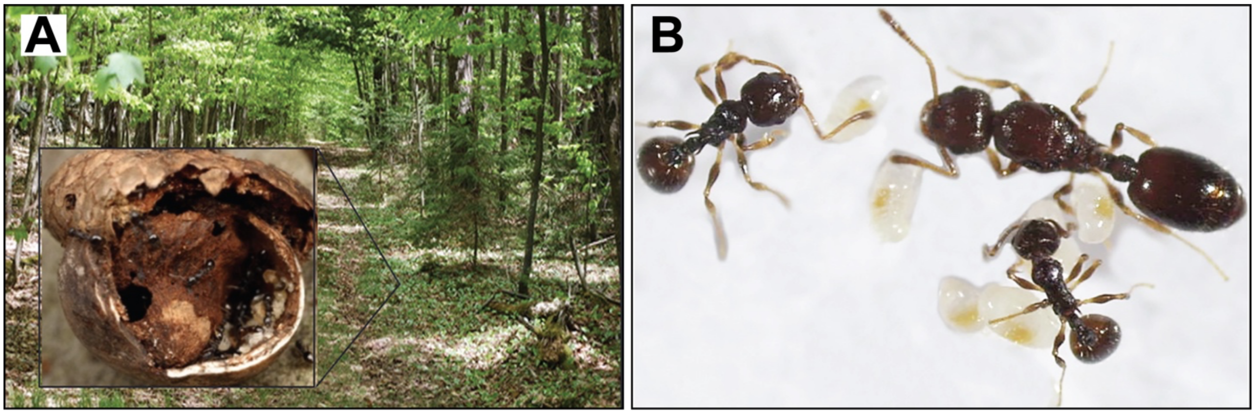

Figure 2. (**A**) Colony of *Temnothorax longispinosus*, South Bristol, New York, USA. (**B**) Queen with two workers and larvae. Pictures were taken by (**A**) Aiden Wasserman and (**B**) Marcel A. Caminer.

Objectives

In this dissertation, we investigated the molecular basis of DOL of *T. longispinosus* by analyzing the expression and functions of the genes controlling task specialization (**Chapter 1**) and the importance of *Vg-like A* (**Chapter 2**) and histone H3K27 acetylation (**Chapter 3**) for controlling expression of these genes. Here we also test the novel hypothesis that social insect workers perform different tasks because they differ in their ability to detect task-related stimuli (**Chapter 1**). For this, in a first step we performed RNA-seq to investigate gene expression differences between distinct behavioral phenotypes in the central nervous system and in peripheral sensory organs. Whereas previous studies on our model species investigated gene expression in the context of tandem-running and scouting behavior (Alleman et al., 2019) and during defense against slavemakers (Kaur et al., 2019) using a single worker brain, we focus here in task depended gene expression using two important tissues: brain and antennae. We report seven times more genes are differentially expressed in the antennae than in the brain, and these included nearly half of all OR genes. Our results demonstrate that workers performing different tasks also live in a different sensory environment, and suggest that a sensory filter may play an important role in regulating division of labor in insect societies (**Chapter 1**). However, the epigenetic regulation of this filter is yet unknown, but previously evidence suggest a role of candidate genes such as *Vg-like-A* and histone acetylation (Kohlmeier et al. 2018, 2023; Libbrecht et al. 2020). According to this, in the second step we knockdown the expression of the behavior candidate gene *Vg-like-A* using a series of RNAi for a short and a long period of time via injection, followed by behavioral assays. We achieved stable downregulation over 10 days, which also led to behavioral changes and identified only a handful of differently expression genes (**Chapter 2**). In a last step, we established a ChIP-seq protocol and developing a ChIP-seq bioinformatics pipeline to investigate the differences in histone acetylation profiles and identify H3K27ac-enriched regulatory regions between nurses and foragers (**Chapter 3**). Altogether, these results add to the existing knowledge about the molecular basis of individual variation in task allocation and DOL plasticity in *T. longispinosus*.

Chapter 1

Task-specific odorant receptor expression in worker antennae indicates that sensory filters regulate division of labor in ants

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Note: Supplementary tables and datasets are not numerically ordered because their numbers were kept from the published version where the methods section was after the discussion.

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Abstract

Division of labor (DOL) is a characteristic trait of insect societies, where tasks are generally performed by specialized individuals. Inside workers focus on brood or nest care, while others take risks by foraging outside. Theory proposes that workers have different thresholds to perform certain tasks when confronted with task-related stimuli, leading to specialization and consequently DOL. Workers are presumed to vary in their response to task-related cues rather than in how they perceive such information. Here, we test the hypothesis that DOL instead stems from workers varying in their efficiency to detect stimuli of specific tasks. We use transcriptomics to measure mRNA expression levels in the antennae and brain of nurses and foragers of the ant *Temnothorax longispinosus*. We find seven times as many genes to be differentially expressed between behavioral phenotypes in the antennae compared to the brain. Moreover, half of all odorant receptors are differentially expressed, with an overrepresentation of the 9-exon gene family upregulated in the antennae of nurses. Nurses and foragers thus apparently differ in the perception of their olfactory environment and task-related signals. Our study supports the hypothesis that antennal sensory filters predispose workers to specialize in specific tasks.

Introduction

Division of labor (DOL) is an important organizing principle of complex biological systems that arose independently during three of the major evolutionary transitions (Maynard Smith & Szathmáry, 1995). DOL was originally formulated in the context of the production process in human societies (Smith 1776), but specialization to specific tasks is also found within cells and across many organisms (Maynard Smith & Szathmáry, 1995). Examples range from bacteria, where clonal populations are divided into subpopulations focusing on different activities (van Gestel et al. 2015; Marlow et al. 2014; Veening et al. 2008; West & Cooper 2016) to multicellular organisms with differentiation of cells into different tissues and organs, and individuals performing specific roles in animal societies (Oster and Wilson 1978; Wilson 1968). To understand the evolution of complex life it is therefore essential to investigate the mechanisms that underlie DOL.

DOL in insect societies results from individuals specializing in the performance of specific tasks. In addition to the reproductive DOL between fertile queens and functionally sterile workers, there is a behavioral DOL among workers that specialize in tasks such as brood care, foraging, nest building, and defense (Herbers 1983; Pinter-Wollman et al. 2012; Wilson 1976). Several factors can affect task specialization, including age (Seeley, 1982; Tripet & Nonacs, 2004), nutrition (Blanchard et al., 2000; Toth & Robinson, 2005), morphology (Wetterer, 1999), genotype (Fewell & Page, 1993; Gove et al., 2009; Graham et al., 2006), experience (Ravary et al., 2007), and colony size (Gautrais et al., 2002; Jeanson et al., 2007; Merkle & Middendorf, 2004). In leafcutter ant species, among others, behavioral specialization of morphologically distinct groups of workers contributes to the DOL (Wilson 1980). In most social insect species, younger workers tend to perform intranidal tasks, while older individuals perform risky activities such as nest defense and foraging outside the nest (Iwasa & Yamaguchi, 2020; Wakano et al., 1998). Yet, such specialization among workers remains flexible, as foragers can revert to perform brood care when needed (Robinson et al. 2009; Shimoji et al. 2020).

Several molecular mechanisms have been implicated in the regulation of DOL. Task specialization is associated with transcriptional changes in the worker brain (Ben-Shahar et al. 2002, 2003; Ingram et al. 2005; Lucas & Sokolowski 2009; Whitfield et al. 2003; Zayed & Robinson 2012). Molecular pathways such as the insulin/insulin-like signaling (IIS), vitellogenin (Vg) and juvenile hormone (JH) pathways are involved in the regulation of worker behavior (Ament et al. 2008; Corona et al. 2013; Dolezal et al. 2012; Kohlmeier et al. 2019; Libbrecht et al. 2013; Sullivan et al. 2000). Functional manipulations have confirmed that the expression of key genes in the worker brain controls task specialization (Gospocic et al., 2017; Kohlmeier et al., 2018; Marco Antonio et al., 2008). Behavioral variation among workers is also associated with signaling of biogenic amines (e.g., dopamine, octopamine, tyramine, serotonin), which act as neurotransmitters or neuromodulators involved in the modulation of the responsiveness to task-associated stimuli (Kamhi & Traniello 2013; Mercer & Menzel 1982; Scheiner et al. 2017; Schulz & Robinson 1999).

Self-organization and collective behavior in insect societies are maintained via the exchange of chemical information (Ferguson et al., 2021). Social insects communicate primarily through glandular pheromones and complex mixtures of long-chain hydrocarbons on their cuticle. These cuticular hydrocarbons (CHC) facilitate recognition of nestmates, developmental stages, castes, sexes, and species (Martin & Drijfhout 2009;

van Wilgenburg et al. 2011). Social insects perceive chemical information via different types of sensilla in the antennae, small receptor organs embedded in the integument that are connected to sensory neurons (Altner & Prillinger, 1980; Hallberg & Hansson, 1999; Slifer, 1970). Decoding the identities of chemical compounds relies on odorant receptors (OR) located within each sensillum (Leal, 2013; Wicher & Miazzi, 2021). OR are transmembrane proteins expressed in the dendrites of olfactory receptor neurons (ORN). The largely conserved OR coreceptor (*Orco*) of insects (Sánchez-Gracia et al., 2011) is required for odorant recognition in the dendritic membrane: it forms an ion channel with specific OR, which determines the sensitivity and specificity of the ORN (Breer et al., 2019). Odorant molecules penetrate through the antenna cuticular pores and are transported by odorant-binding proteins to the ORN membrane, where they interact with receptors, leading to the generation of action potentials (Jacquin-Joly & Lucas 2005; Wilson & Mainen 2006). ORN axons relay signals from the sensilla to the glomeruli of the antennal lobes in the insect brain, which are the first processing unit for olfactory information. Then, ORN make synaptic contact with the projection neurons and local neurons, which transfer information to the central brain (Anton & Homberg 1999; de Belle & Kanzaki 1999; Hansson & Anton 2000).

Several lines of evidence indicate that OR genes play central roles in the regulation of social life of insects. First, social insect species typically harbor large numbers of OR genes (Engsontia et al. 2015; Oxley et al. 2014; Saad et al. 2018; Zhou et al. 2012, 2015). Second, some OR gene families have specifically expanded during social evolution, such as the 9-exon subfamily in ants, which appears to serve an important function in the perception of CHC (McKenzie et al. 2016; Pask et al. 2017; Smith et al. 2011; Zhou et al.

2012, 2015). Third, species that evolved socially parasitic strategies resulting in reduced behavioral repertoires show a strong and convergent reduction in the number of OR genes (Jongepier et al., 2022). Finally, experimentally produced mutant ants that lack the *Orco* gene (coding for the co-receptor necessary for OR to properly function) show impaired social behavior (Trible et al., 2017; Yan et al., 2017).

Current DOL models bring together the chemical nature of social insect communication and variation among workers in their response to chemical cues. They posit that flexible response thresholds to task-related chemical stimuli serve as regulators of worker specialization (Beshers & Fewell 2001). Workers take on a particular task when the stimulus intensity exceeds their individual threshold for this task. Therefore, individuals with lower thresholds for a given task are more likely to perform it than those with higher thresholds (Lichocki et al., 2012). Individual response decisions and task performance are modulated via numerous parameters on different time scales (Jeanson & Weidenmüller, 2014; Naug, 2016; Oster & Wilson, 1978; Ulrich et al., 2021), and despite extensive research on DOL and task allocation, these mechanisms are not fully understood.

As the name suggests, response threshold models are based on an individual's response to certain stimuli and the variation in their responsiveness over time. However, these studies do not include in their models how these cues are processed (Bonabeau et al. 1996, 1997, 1998; Oxley et al. 2014; Shiraishi et al. 2022; Theraulaz et al. 1998; Ulrich et al. 2021; Yamanaka et al. 2019). The fact that the processing of signal information has been primarily described in the insect brain (Thiagarajan & Sachse, 2022) may suggest that thresholds and associated responses are set in the central nervous system, possibly regulated via molecular pathways in the brain that correlate with behavioral variation. We propose that DOL models would benefit from considering odor sensitivity as a potential upstream sensory filter that may affect task specialization. Along these lines, we hypothesize that behavioral variation among workers may also stem from their ability and/or efficiency in detecting different signals. For example, we propose that individuals that specialize in brood care do so because they are more sensitive to brood cues, rather than (or in addition to) being more likely to respond to similar levels of brood cues. To test the hypothesis that a sensory filter regulates inter-individual behavioral variation, and thus the DOL in social insects, we investigated transcriptional signals in the brain and antennae of workers that specialize in either brood care or foraging behavior in the ant *Temnothorax longispinosus*. We found that i) behavioral variation was associated with more extensive transcriptomic differences in the antennae than in the brain, ii) these differences included a large proportion of the OR gene repertoire, and iii) individuals specializing in brood care overexpressed an OR gene family putatively involved in detecting social cues. These findings support our hypothesis that the peripheral nervous system, acting as a sensory filter, plays an important role in regulating behavioral differences between workers and thus in the DOL of social insects.

Materials and Methods

Sample collection and behavioral determination

A total of seven colonies of the ant *T. longispinosus* were selected with an average colony size of 110 ± 31.5 workers (mean ± SD, Dataset S4) at the moment when the workers were sampled. The ants were collected in the forests of the Edmund Niles Huyck Preserve, Renssellearville, NY, USA (42°31′41.0″N 74°09′38.8″W), in June of 2018 with permission. Upon collection, we housed each colony in a plaster-floored nesting box (43 cm × 28 cm × 10 cm) divided into three chambers containing a single slide nest, in 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 established at the Johannes Gutenberg University in Mainz, Germany, under a 14 h:10 h light:dark photoperiod at 18°C to a 22°C temperature. We provided honey and water ad libitum and fed crickets to the colony twice a week. To allow for visible behavioral DOL between workers of the two behavioral phenotypes, we marked, observed and recaptured ants from inside and outside the nest. We defined foragers as workers that perform outside-nest tasks, including gathering and searching for food and water and exploring the environment surrounding the nest, while nurses remained inside the dark nest and cared for the ant brood. A total of 69 workers inside (from the brood pile) and 76 workers outside the nest were marked with fine colored metal wires (0.02 mm Elektrisola, Eckenhagen, Germany). To immobilize the workers, they were placed with their heads and part of the thorax in a notch of a soft sponge without prior anesthesia. Then we marked the ants with a very thin loop around the petiole. It was then checked that the wires did not interfere with the ants' movement. We performed behavioral observations every two hours, four times a day for five days (total = 20 scans), in which we noted down how many times an individual performed brood care and foraging behavior and the position in the nest (Table S3). Based on these behavioral observations, the marked individuals found outside the nest, exploring the surroundings for food or water, were identified as foragers. These workers usually do not care for the brood and do not frequently reside on brood piles, as ant colonies organize themselves spatially in a way that reduces contact between foragers and brood (Pamminger et al. 2014). We identified nurses as workers that remain inside the nest in direct contact with brood and were unlikely to leave the nest. Foragers were found in 42% ± SD 26% of the observations outside of the nest, whereas nurses spend only 1% ± SD 3% outside. In contrast, nurses were interacting with the brood in 54% ± SD 22% of the observations, whereas we found that foragers only did this only in 2% ± SD 4% of the observations. We scanned the behavior of workers over 20 observations, albeit earlier studies have shown that a single observation makes it possible to group *T. longispinosus* and other ants reliably into nurses and foragers that differ in behavior (Kohlmeier et al. 2017; Pamminger et al. 2014), gene expression (Kohlmeier et al., 2019) and CHC composition (Kohlmeier et al. 2018). Furthermore, spatial location can alone can predict behavior in *Temnothorax* workers (Sendova-Franks & Franks, 1993). We focused in this study on individuals highly specialized on either foraging or brood care. Workers that performed both tasks regularly were not included in this study. After all observations were completed, the marked nurses and foragers were collected, directly frozen in liquid nitrogen and stored at -80 °C until further processing for dissection and pooling according to behavioral state and colony.

RNA extraction and sequencing

For RNA extraction, we removed both antennae and stored them in a 1.5 ml Eppendorf tube containing 50 µl TRIzol (Invitrogen), cut the head off and fixed it on a slide with melted dental wax. We then made an incision around the head with a surgical scalpel and removed the head capsule with forceps to expose the intact brain. Finally, we carefully pulled the brain out of the head capsule and removed the remains of other tissues that were connected to it. The dissected brain was transferred to a 1.5 ml Eppendorf tube containing 20µl PBS. Each dissection was completed in less than 5 minutes to prevent RNA degradation. We dissected brain and antennae tissues from 48 nurses and 49 foragers. We pooled the brains and antennae from seven workers from each behavioral state and colony. The only exception was the “GO” colony (NY18 E110), for which we pooled only six brains and twelve antennae from six nurses (Dataset S4) due to the loss of one sample during the dissection process. Immediately after dissection of each brain and antennae, the Eppendorf tubes were kept on dry ice while we dissected the remaining individuals. Brain and antennae tissues were homogenized with a pestle. Sample brains were transferred separately to a 1.5 ml Eppendorf tube containing 50µl of TRIzol. We added 50µl chloroform to each brain and antenna samples, gently inverted for 30 s and then centrifuged samples at 12,000 g for 15 min at 4°C. We collected the resulting supernatant and precipitated RNA with 25µl 70% ethanol. We conducted the subsequent RNA extraction with the RNeasy Mini Kit (Qiagen), following the manufacturer’s instruction. The resulting 28 samples (14 brains and 14 antennae) were stored at -80°C until library preparation.

RNA-seq libraries were prepared by Novogene Company Limited, Cambridge, UK, using the NEBNext Ultra RNA Library Prep Kit for Illumina according to the manufacturer's protocol. After amplification and purification, 28 libraries were sequenced on an Illumina NovaSeq 6000 S4 flow cell platform using a paired-end 150 bp. Approximately 43 million raw reads were generated from each library (Dataset S4).

Gene expression analyses

Raw data obtained from Novogene were checked using FastQC v.0.11.9 (Andrews, 2016), and Illumina adapters were removed using Trimmomatic v.0.36 (Bolger et al., 2014). The protein-coding genes of *T. longispinosus* together with the manual OR annotations (GCA_004794745.1; Kaur et al. 2019) and the congener *T. curvispinosus* (GCA_003070985.1) were retrieved from the NCBI database and we used Liftoff v.1.6.1 tool (Shumate & Salzberg, 2021) to assign these annotations to the recently published *T. longispinosus* genome (Jongepier et al., 2022). In total, 10,029 of 13,061 (~77%) annotated protein-coding genes were assigned from the original *T. longispinosus* assembly (genes identified as "DBV15") and 4,808 were assigned from *T. curvispinosus* (genes identified as "LOC"), for a total of 14,837. For gene expression analysis, reads were mapped to our *T. longispinosus* genome assembly, and the read counts table was generated using STAR 2.7.0 (Dobin et al., 2013) with default settings. Detailed mapping statistics for each sample is available in Dataset S4. We used the deseq2 v1.16.1 package for R to identify differentially expressed genes (Love et al. 2014). To avoid biased results due to low read counts, we removed from the counting matrix those genes for which less than 10 of the reads mapped to at least 6 of our 14 samples (n - 1 of the smallest sample size). Then, we conducted a differential gene expression analysis with DESeq2 (Love et al. 2014). We began with comparisons between nurses and foragers using the ~Colony+Task model, followed by a likelihood ratio test (LRT) approach, with colony ID as a fixed factor. Genes were considered differentially expressed if the false discovery rate (FDR), using Benjamini-Hochberg procedure, had an adjusted p-value of ≤ 0.05. The resulting lists of differentially expressed genes (DEGs) refer to genes that are overexpressed and underexpressed in foragers compared to nurses. We used the online tool Venny v.2.1 (https://bioinfogp.cnb.csic.es/tools/venny) to generate a Venn diagram containing the DEGs associated with task and tissues. Separation of DEGs by task was visualized by performing principal component analysis (PCA) with a 95% confidence ellipse using the ggplot2 v3.4.2 package for R (Wickham, 2016). For PCA, we used the transformed reads of filtered transcriptomes from all contigs using the plotPCA function provided by DESeq2. Samples “GO”, “BG” and “YY” showed a divergent expression pattern. To ensure that our results were not influenced by these deviating samples, we re-run the DEseq2 analyses repeatedly removing sample after sample. This resulted show slight shifts in the number of DEGs in the antennae (2,267 with all samples vs. 1,960 without “GO”; 1,861 without “BG”; and 1,862 without “YY”) and in the brain (339 with all samples vs. 227 without “GO”; 272 without “BG”; and 346 without “YY”). However, the main findings remained similar and a large number of differentially expressed ORs were always found in all analyses (209 with all samples vs. 195 without “GO”; 209 without “BG”; and 179 without “YY”). Finally, OR genes that were upregulated in each behavioral phenotype were visualized in a volcano plot using ggplot2. All statistical tests and graphical visualizations were performed in RStudio v.1.4.1106 (RStudio Team, 2020).

Identification of behavior candidate genes and odorant receptors

We used gene annotations based on a BlastX search of the *T. longispinosus* transcriptome compared to a list of different invertebrate proteomes (i.e., *Acromyrmex echinatior, Apis mellifera, Camponotus floridanus, Drosophila melanogaster, Harpegnathos saltator, Odontomachus brunneus, Temnothorax curvispinosus*) downloaded from the NCBI database with an E-value of 1e-5 and below. Clusters containing more than one sequence match per species were reduced to a single specimen based on the highest blast score. We constructed orthogroups across all of the above species using OrthoFinder (Emms & Kelly, 2015), including amino acid sequences from the *T. longispinosus* proteome (Kaur et al., 2019), and retained orthogroups containing caste DEG (Dataset S5) to again compare potential behavioral candidate genes previously identified as involved in regulating the division of labor in social insects (Alleman et al. 2019; Das & de Bekker 2022; Gospocic et al. 2017, 2021; Guo et al. 2020; Kohlmeier et al. 2019; Korb et al. 2021; Opachaloemphan et al. 2021; Qiu et al. 2022). GO enrichment analysis was performed with TopGO v.2.44.0 for R using a Fisher's exact test for the different gene sets compared to the whole genome with the weight01 algorithm (Alexa & Rahnenfuhrer, 2018). Only annotated GO terms with a p-value of ≤ 0.05 were considered significantly enriched.

Odorant receptor (OR) protein sets were clustered across multiple ant species using OrthoFinder to derive orthologous groups and identify subfamilies for each OR in *T. longispinosus*. To associate orthogroups with previously identified OR subfamilies, we used OR annotation in *Atta cephalotes, Acromyrmex echinatior* from Engsontia et al.

(2015), and *Camponotus floridanus*, *Harpegnathos saltator*, and *Solenopsis invicta* from Zhou et al. (2012, 2015). Missing subfamily information was labeled as "unassigned" (Dataset S3).

Results

Identification of *Temnothorax longispinosus* behavioral phenotypes

Task specialization in the ant *T. longispinosus* is neither genetically fixed nor rigid, but can change with age and in response to colony needs (Kohlmeier et al., 2019). We conducted behavioral observations of seven *T. longispinosus* laboratory colonies to identify individuals that specialize in brood care behavior (hereafter referred to as nurses), and others that specialize in foraging (hereafter referred to as foragers) (Fig 1*A*; see Methods section for more details). The grouping of workers into the two behavioral categories was based on the frequency of their location inside or outside the colony and on their behavior, especially whether they showed brood care or foraging behavior (Dataset S1). Individuals identified as nurses interacted with the brood in 54% ± 22% (mean ± sd) of the observations, and were recorded outside the nest in 1% ± 3% of the observations. On the contrary, foragers were found outside the nest in 42% ± 26% of the observations but interacted with the brood in only 2% ± 4% of the observations.

Larger task-associated transcriptomic changes in the antennae than in the brain

To investigate transcriptomic variation between nurses and foragers, we used RNA-seq to generate seven nurse and seven forager brain and antenna samples, each consisting of pooled tissue from seven workers of a single colony. Of the 14,837 genes annotated in the *T. longispinosus* genome, we found 91% (13,494) and 92% (13,683) to be expressed (FPKM > 0) in the brain and the antennae, respectively. To investigate gene expression differences between nurses and foragers, we compared full models that included task as an explanatory variable to reduced models that did not using the likelihood ratio test (LRT) method implemented in DEseq2. The influence of colony identity was controlled for by including it as an explanatory variable in both full and reduced models. A Benjamini-Hochberg adjusted p-value of 0.05 was set as a threshold to obtain genes whose variation was significantly explained by behavioral specialization in each tissue. We detected 339 differentially expressed genes (DEG) in the brain (223 upregulated in nurses, 116 in foragers), and 2,267 in the antennae (1,241 upregulated in nurses, 1,026 in foragers; Dataset S2; Fig 1*B*). We found an overlap of 162 DEG between the two tissues, including 143 DEG that showed differences in the same direction across tissues. Principal component analyses (PCA) for both brain and antenna data reveal that most samples clustered by behavioral phenotype rather than colony of origin (Fig. 1 *C*-*D*).

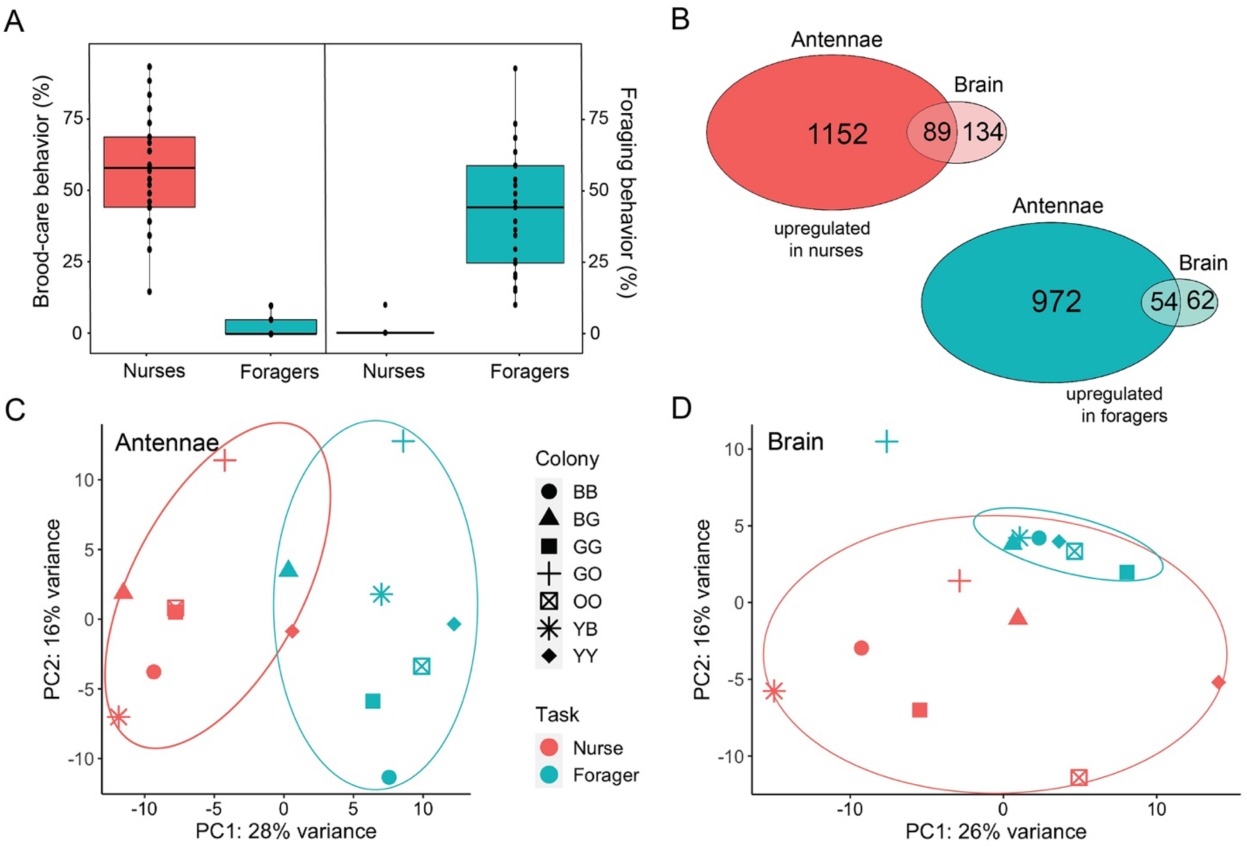

Figure 1. Variation in behavior, brain and antenna gene expression between nurses and foragers. (**A**) Boxplot showing behavioral differences between ants selected for transcriptomic analysis. Each black dots represent individual ants. For differential expression analysis, each sample contains the pooled RNA of seven ants of the respective behavioral phenotype. (**B**) Venn diagrams showing the number of DEG that were upregulated in nurses (red) and foragers (turquoise) for both antenna and brain tissues. Principal component analysis (PCA) plots based on all expressed genes for (**C**) antenna and (**D**) brain samples with 95% confidence level presented as ellipses. The color of each sample represents task (red = nurse, turquoise = forager), and the shape the colony of origin.

OR gene expression differs between antennae of nurses and foragers

To investigate whether odorant perception differs between nurses and foragers, we focused our attention on the expression of OR genes in the antennae. We found that all 419 previously annotated OR genes in the genome of *T. longispinosus* (Jongepier et al., 2022) were expressed in the antennae, and that 50% (209/419) of them were differentially expressed between nurses and foragers. Specifically, 64 OR genes were upregulated in nurses (15% of all OR genes), and 145 in foragers (35% of all ORs) (Fig 2). Then, we studied which OR subfamilies were preferentially expressed in nurses and foragers. The 64 OR genes overexpressed in nurses belonged to three OR subfamilies, while the 145 OR genes upregulated in foragers were distributed among 19 OR subfamilies. Foragers overexpressed 27, 19, 8, and 8 OR genes from the L, V, P, and H subfamilies, respectively, while no OR genes from these subfamilies were upregulated in nurses (Dataset S3). We found that 63% (27/43) of the genes from the L subfamily and 73% (8/11) from the P subfamily were overexpressed in foragers, which represent significant overrepresentations (L subfamily: Fisher's test, odds ratio = 3.67, p-value < 0.001; P subfamily: Fisher's test, odds ratio = 5.25, p-value = 0.02). For the V and H subfamilies we did not find such an overrepresentation, likely due to lower gene numbers (V subfamily: Fisher's test, odds ratio = 1.64, p-value = 0.17; H subfamily: Fisher's test, odds ratio = 1.71, p-value = 0.30). On the other hand, we found that 80% (51/64) of the OR genes overexpressed in nurses belong to the 9-exon subfamily. This results in an overrepresentation of the 9-exon subfamily in genes that were overexpressed in nurses (Fisher's test, odds ratio = 22.18, p-value < 0.001), with 45% (51/114) of this subfamily being overexpressed in nurses. In contrast, only 6% (8/137) of the 9-exon subfamily was overexpressed in foragers, which is less than expected by chance (Fisher's test, odds ratio = 0.09, p-value < 0.001). We also found that nurses overexpressed *Orco* compared to foragers (FDR p-value = 0.001).

Genes in biogenic amine pathways vary in expression between nurses and foragers

Biogenic amines have been implicated in the regulation of behavior, and they may affect sensory perception (Cook et al. 2001; Mercer & Menzel 1982; Schulz et al. 2003; Zhukovskaya & Polyanovsky 2017). We therefore screened our lists of brain and antennal DEGs for biogenic amine pathway genes. We detected five differentially expressed genes in the antennae that are associated with biogenic amine signaling. Genes in the serotonin (*5-hydroxytryptamine*, DBV15_11483), tyramine (*tyramine beta-hydroxylase*, DBV15_00422) and octopamine (*octopamine receptor*, LOC112465659) pathways were upregulated in the antennae of foragers, while nurses showed a higher expression of genes in dopamine (*dopamine 1-like receptor 2*, DBV15_07611) and octopamine (*octopamine beta2 receptor*, DBV15_10418) pathways (Fig 3 *A*-*E*). These five genes were also expressed in the brain, but not differentially expressed between nurses and foragers. Also, no other biogenic amine pathway genes were found to be differentially expressed in the brain.

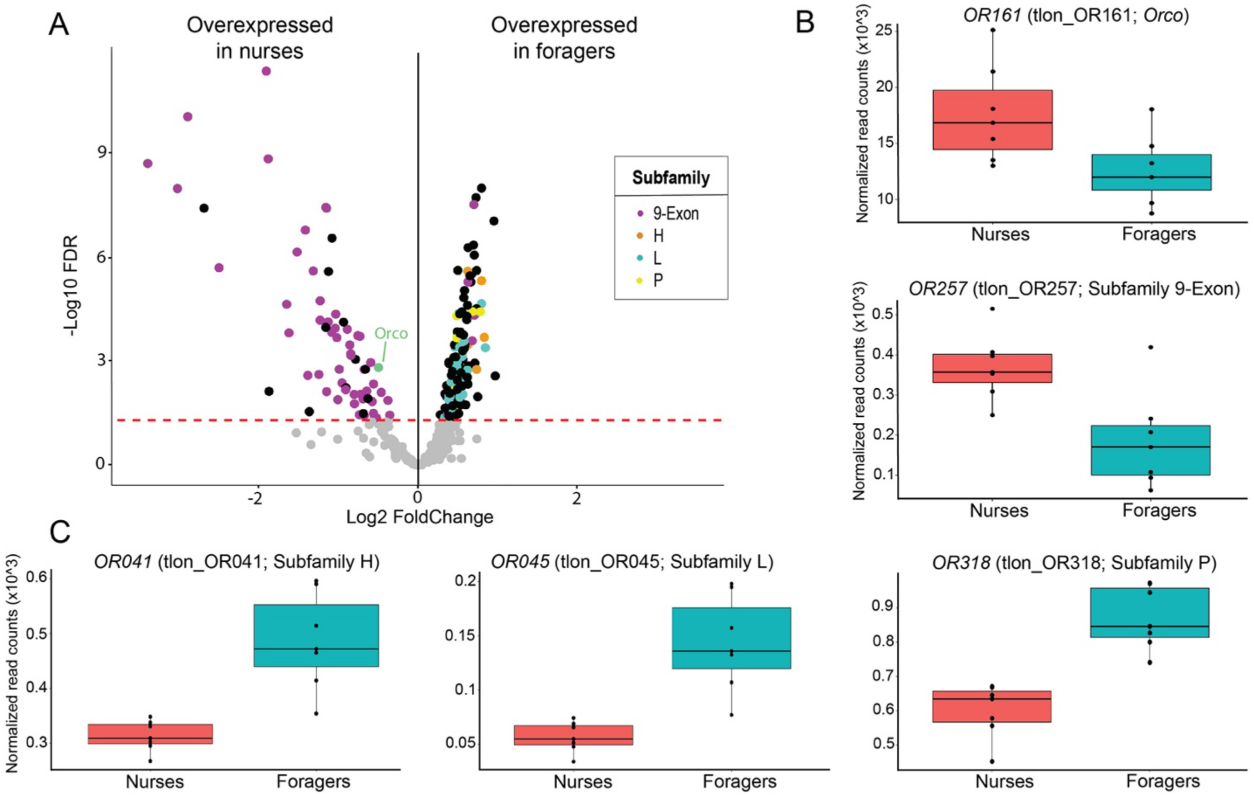

Figure 2. OR expression in the antennae differs between nurses and foragers. (**A**) Among the DEG overexpressed in nurses and foragers, the most represented OR subfamilies are indicated by different colors. The red dotted line represents the significance threshold of our differential expression analysis. (**B,C**). Boxplots representing the expression of (**B**) *Orco* and *OR257*, the gene with the highest expression difference in terms of $\log_2$ FoldChange in the 9-exon subfamily, and (**C**) *OR041*, *OR045* and *OR318*, the genes with the highest expression difference in terms of $\log_2$ FoldChange in the H, L and P subfamilies, respectively.

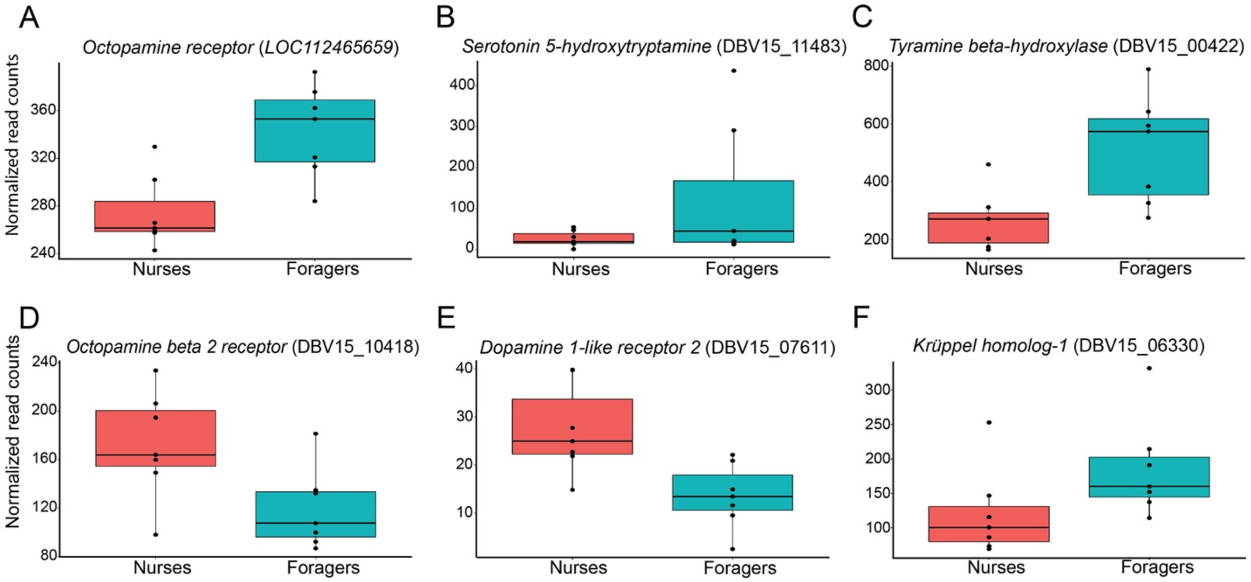

Figure 3. Expression patterns of candidate DEGs of nurse and forager antennae known to regulate division of labor in social insects (Fussnecker & Grozinger 2008; Gospocic et al. 2021; Grozinger & Robinson 2007; Jedlicka et al. 2016; Kamhi & Traniello 2013; Mercer & Menzel 1982; Scheiner et al. 2017; Schulz & Robinson 1999; Shpigler et al. 2010). Boxplots showing the expression of (**A-E**) some genes from different biogenic amines pathways, while (**F**) one behavioral candidate gene (Table S1). Gene identity is shown in parentheses.

Association between behavioral variation and molecular pathways in brain and antennae

Our analysis revealed expression differences of genes expressed in the brains of nurses and foragers involved in the regulation of task specialization in social insects. Previous studies have found that *Vg* genes and the associated JH and IIS/TOR pathways are important endocrine networks that play central roles in the regulation of lifespan, fertility and behavior in bees and ants (Ament et al. 2008; Azevedo et al. 2016; Jedlicka et al. 2016; Norman & Hughes 2016; Opachaloemphan et al. 2021; Robinson 1987; Rodrigues & Flatt 2016). Therefore, we searched and found evidence in our RNA-seq data for task-associated expression of genes involved in the metabolism, biosynthesis, and regulation of these pathways in the brain and antennae (Table S1). These genes included *conventional Vg* (LOC112466671), *Vg-like A* (named/classified as per et al. 2018; DBV15_03138), *venom carboxylesterase-6-like* (DBV15_11528), and *protein takeout* (DBV15_08771) overexpressed in the brain of nurses. Meanwhile, *allatostatin A-like* (LOC112454443) and *insulin-like growth factor I* (*IGF1*) (LOC112454447) were found overexpressed in the brain of foragers. Remarkably, *venom carboxylesterase-6-like* and *IGF1* were overexpressed in the antennae of nurses and foragers respectively. Additionally, high expression levels of the zinc-finger transcription factor *Krüppel homolog-1* (*Kr-h1*) were detected in the antennae of foragers (DBV15_06330) (Fig 3*F*). The expression of this gene has been correlated with caste and behavioral differences in the brain of social insects (Fussnecker & Grozinger 2008; Gospocic et al. 2021; Grozinger & Robinson 2007; Jedlicka et al. 2016; Shpigler et al. 2010), but little is known about its function and gene targets in the antennae.

We performed a GO enrichment analysis to gain a deeper understanding of the biological processes represented in the lists of DEG. This analysis detected many enrichments based on single one or few genes, and we mention below a few interesting processes with the number of genes driving the enrichment in parentheses (see Table S2 for complete list). Genes that were upregulated in the brain of nurses were enriched for biological processes such as *translation* (19 genes), *cellular iron ion homeostasis* (2 genes), and *catabolic process* (6 genes); while *translation* (22 genes), *endocytosis* (6 genes), *glucose metabolic process* (4 genes), and *regulation of cell cycle* (3 genes) were enriched in the antennae. In the list of genes that were overexpressed in the brain of foragers, we found that enriched processes included *peptide metabolic process* (4 genes), *methionyl-tRNA aminoacylation* (1 gene), *positive regulation of type I interferon production* (1 gene), and *cell redox homeostasis* (1 gene); while *phosphatidylinositol phosphorylation* (4 genes), *inositol phosphate dephosphorylation* (3 genes), *carbohydrate metabolic process* (17 genes), *innate immune response* (2 genes), and *nucleotide catabolic process* (3 genes) were enriched in the antennae.

Discussion

In this study, we found evidence supporting the hypothesis that variation among social insect workers in their ability to perceive different chemical signals could contribute to the regulation of task allocation, and thus to DOL in insect societies. To do so, we analyzed brain and antenna transcriptomes of nurses and foragers of the ant *T. longispinosus.* We report several lines of evidence that support our hypothesis. First, we found almost seven times as many genes to be differentially expressed between nurses and foragers in the antennae as in the brain, indicating that peripheral sensory organs may have an important function in the task specialization process of social insect workers. Second, we found that half of all OR genes of the *T. longispinosus* genome are differentially expressed between the antennae of nurses and foragers, suggesting that behavioral specialization is associated with different sensory filters that result in specific perceptions of the chemical environment. Third, our analyses revealed that nurses and foragers upregulated distinct families of OR genes, indicating that their sensory filters may target different types of chemical cues, possibly adjusted to the tasks they perform. Finally, we detected several genes in multiple biogenic pathways to be differentially expressed between the antennae of nurses and foragers, potentially involved in fine-tuning the sensitivity of the odorant filters.

Many organisms have evolved sensory filters to focus on only a subset of all environmental cues (Hempel De Ibarra et al., 2022; Patullo & Macmillan, 2010; Ponnath et al., 2022; Warrant, 2016). In insects, the peripheral olfactory filtering system enables individuals to detect and discriminate odors that convey ecologically relevant information used to mediate important behaviors such as courtship, locomotion and navigation to avoid predators and locate food or nesting sites (von der Emde & Warrant 2016). For example, the mosquito *Anopheles gambiae* strongly responds to odorant components of its vertebrate hosts that provide meals (Carey et al., 2010). Sensory filters have been selected for because they limit the amount of information perceived by the organism, and thus the energy and time required by the brain to process it (von der Emde & Warrant 2016). Within insect colonies, individuals typically exhibit many morphological and physiological traits associated with increased efficiency in task specialization. These can be understood as adaptations that allow individuals to become more proficient at their task due to learning, training, or the perception of valuable task-related information, allowing the colony to avoid the cost of task switching (Dornhaus, 2008). In this context, it is interesting to find that *T. longispinosus* workers may have different sensory filters that would serve as a basis for their task specialization, as workers would mostly perceive chemical cues that pertain to their tasks. Our findings indicate that the sensory filter of ant workers is dynamic, and its changes may underlie their behavioral maturation.

In this study, we report a higher number of DEGs between nurses and foragers in the antennae than in the brain. The brain is a heterogeneous tissue in which different cell types perform very specific functions, which differ greatly in their gene expression (Ito et al., 2013; Koch & Laurent, 1999; McCaffrey, 2015). Therefore, using the whole brain for transcriptome analyses could make it more difficult to identify genes that are differentially expressed only in specialized parts of the brains of nurses or foragers. In comparison, the antennae might have a more uniform cell composition, which could facilitate the identification of DEGs. In contrast to this prediction, a transcriptome comparison of two different behavioral phenotypes of worker honey bees revealed that transcriptional differences are much more pronounced in the antenna than in the separately studied brain parts including the mushroom body, antennal lobe and central brain (Kennedy et al., 2021). Furthermore, Chandra et al. (2018) used whole-brain RNA-seq in several ant species to detect differential expression of the gene *Ilp2* between castes, a gene that was later found to be expressed in only about 15 cells of the pars intercerebralis. This led us to conclude that it may be slightly more difficult to identify DEGs in the brain than in the antennae, but this unlikely explains the nearly seven-fold difference in the number of DEGs.

Age, genetic background, social environment, individual experiences and hormones influence behavioral differences between workers leading to DOL (Geva et al. 2005; Jeanson et al. 2007; Libbrecht & Keller 2013; Ravary et al. 2007; Schulz et al. 2002; Tripet & Nonacs 2004). In our study, we expect that age differed between individuals identified as nurses and those identified as foragers, although we did not measure it directly. However, we know from previous studies on *T. longispinosus* that workers live between one to three years and switch from brood carer to forager about one year after eclosion, when new generation of workers emerged and taken over the care of the brood (Kohlmeier et al. 2017, 2018). Previous experiments designed to disentangle gene expression associated with behavioral specialization, age and fertility showed that behavioral specialization is much more strongly associated with gene expression than age and fertility in *T. longispinosus* (Kohlmeier et al., 2019). We propose that the molecular and physiological regulators such as JH, Vg, biogenic amines, and nutritional status known to regulate task specialization could drive different OR expression patterns, which in turn would produce behavioral variation via contrasting abilities to detect different sets of odors. This hypothesis is supported by several studies (Gadenne et al., 2016) showing that the physiological condition of an animal can influence the level of receptor expression, including mating status, oviposition, feeding, circadian rhythm, experience, and aging. Alternatively, we cannot exclude that the exposure to different odors that is associated with performing different tasks may at least in part have affected OR gene expression in the antennae. However, such an effect is unlikely to explain the large-scale variation in gene expression, as there is very limited evidence that the mere exposure to odors influences the expression of the gene coding for the OR that binds this odor (von der Weid et al. 2015; Zhou et al. 2009).

According to our sensory filter hypothesis, ant workers would be expected to primarily detect chemical cues that correspond to their tasks. We found that the 9-exon subfamily of OR was overrepresented in genes that were upregulated in the antennae of nurses. Interestingly, recent studies have shown a rapid expansion of the 9-exon subfamily in ants, and several lines of evidence indicate that OR genes from this subfamily mediate complex social interactions in ant colonies (Engsontia et al. 2015; McKenzie et al. 2016; Smith et al. 2011). First, comparisons of antennal transcriptomes revealed that 9-exon OR genes are expressed more frequently in workers than in males, suggesting a role in social communication among workers (McKenzie et al. 2016; Zhou et al. 2012). Second, representative OR from the 9-exon subfamily can detect CHC extracts from several castes (Pask et al., 2017). According to McKenzie et al. (McKenzie et al., 2016), 9-exon OR genes were first expressed in solitary ancestors of aculeate wasps and facilitated CHC discrimination, likely for prey or mate recognition, with a lineage giving rise to the ancestors of ants. Moreover, OR genes of the 9-exon subfamily were convergently lost in socially parasitic ant species that lost the ability to perform brood care or foraging (Jongepier et al., 2022), suggesting that they are essential for the performance of these worker tasks. Our finding of an overexpression of 9-exon OR genes in the antennae of nurses is in line with these previous studies, and suggests that many of these receptors have important functions within the nest, such as sensing chemical cues from the larvae, queen, or other workers, and/or that they are less important for sensing task-related stimuli or other signals outside the nest. Among the OR genes overexpressed in nurses was also the co-receptor *Orco*, which is widely expressed in olfactory sensory neurons and nearly unchanged in sequence in distant insect taxa (Benton et al., 2006; Krieger et al., 2003; Larsson et al., 2004). ORs form a unique class of heteromeric cation channels composed of two related heptahelical subunits: a divergent OR subunit that confers odor specificity, and the co-receptor *Orco* subunit (del Mármol et al., 2021; Sato et al., 2008). Since those functional receptors would increase the sensitivity of the workers to odors, we propose that the overexpression of *Orco* may indicate higher olfactory sensitivity to odors in the antennae of nurses compared to foragers. This would be supported by previous studies that showed that changes in *Orco* expression can be indicative of physiological conditions and sensory receptivity (Castillo et al. 2022; Guo et al. 2017).

The behavioral transition from nursing to foraging may be triggered by a lower efficiency in detecting brood cues via the downregulation of specific OR genes (e.g., from the 9-exon subfamily). This would result in ants moving farther away from the brood, and this change in spatial location may trigger the behavioral transition to outside tasks (Mersch et al. 2013; Richardson et al. 2021). In addition to being less efficient at detecting brood cues, the sensory filter of foragers may also become fine-tuned to detect a more diverse set of odors. Foragers overexpressed a greater number of OR gene subfamilies compared to nurses (19 and 3 for foragers and nurses, respectively), which may indicate that the olfactory system of foragers could perceive the more diverse chemical environment outside the nest. Similar to the 9-exon subfamily, the L subfamily has also been expanded in social insects (Engsontia et al. 2015; Saad et al. 2018; Zhou et al. 2015), and along with the P and H subfamilies, it has been lost in socially parasitic ants (Jongepier et al., 2022; Schrader et al., 2021). Interestingly, the OR genes from the L, P, V, and H subfamilies have been upregulated in foragers, and thus may have a task-specific function, such as recognition of chemical cues related to environmental perception or recruitment cues outside the nest. OR genes belonging to subfamilies L, H and V have been shown to be highly responsive to long-chain hydrocarbons and are overexpressed in the antennae of males and workers of the ant *Harpegnathos saltator* (Slone et al., 2017). In addition, several ORs of the H subfamily have been proposed to act as putative floral odorant detectors in the antennae of honey bees (Claudianos et al., 2014).

Finding task-specific variation in OR gene expression raises the question as to which molecular mechanisms regulate those changes. Variation in biogenic amines levels have been identified as one of the leading causes of behavioral plasticity and specialization of social insects to different tasks (Barbero et al., 2023). Functional manipulation of biogenic amines has led to changes in behavior, dominance status and reproductive activity, as well as shifts in worker task performance (Fussnecker et al. 2006; Penick et al. 2014; Yaguchi et al. 2016; Yakovlev 2018). Previous studies on insects revealed that olfaction-guided behavior is mediated by biogenic amine receptors in the antenna, and their expression is involved in fine-tuning the sensitivity of the olfactory system (Gadenne et al. 2016; Zhukovskaya & Polyanovsky 2017). Signal transduction of biogenic amine receptors is mediated by G protein-coupled receptors (GPCRs) located on the cell membrane, which trigger different signaling cascades that lead to increased or decreased cAMP level and Ca^{2+} release (Beggs et al. 2011; Blenau & Baumann 2001; Ohta & Ozoe 2014; Vleugels et al. 2015). For example, modified concentration cAMP and intracellular Ca^{2+} levels due to octopamine-induced signal transduction in the moth *Manduca sexta* (Dacks et al., 2006) activate *Orco*, leading to changes in ORN sensitivity (Getahun et al., 2013; Stengl & Funk, 2013). Our results suggest that the biogenic amine signaling pathway may modulate the sensory filtering function of insect antennae and alter sensitivity to various signals. We found that genes encoding tyramine and its precursor, octopamine, are upregulated in forager antennae, similar to genes involved in serotonin signaling. Tyramine and dopamine (which was upregulated in nurses) have been implicated in modulating taste and olfactory receptor neurons, while serotonin may serve as a neurotransmitter and neurohormone in antennal vessels and mechanosensory organs (Zhukovskaya & Polyanovsky, 2017). Serotonin influences foraging activity (Schulz et al. 2003) and regulates food intake in many animals (Blundell & Halford, 1998; French et al., 2014; Voigt & Fink, 2015). Dopamine signaling also plays an important role in controlling the insect circadian clock and mediating clock-controlled behavioral phenotypes such as locomotion (Beninger, 1983; Liang et al., 2019). In our focal species *T. longispinosus*, inside workers were found to exhibit a stronger circadian rhythmicity than foragers, which may be regulated via differences in the acetylation of histone proteins (Libbrecht et al., 2020).

Changes in behavior and olfactory sensitivity in insects could be related to the expression of genes involved in IIS, target of rapamycin (TOR), JH and Vg pathways, according to age, circadian rhythm, mating and feeding status (Gadenne et al. 2016). For example, appetite state in *D. melanogaster* is signaled by insulin, which upregulates a peptide receptor on the olfactory receptor cells that innervate the DM1 glomerulus. Activation of the DM1 glomerulus is enough to drive the fly to reach for food (Root et al., 2011). Recent studies have shown that pheromone release and odor sensitivity appear to be under JH control in *Schistocerca gregaria* and *Locusta migratoria*, which could lead to behavioral changes (Anton et al. 2007; Guo et al. 2020; Tawfik et al. 1997, 2000; Wiesel et al. 1996). Finally, an experimental downregulation of *Vg-like A* in *T. longispinosus* workers resulted in decreased brood care behavior and a lower sensitivity to brood-related chemical cues, suggesting changes in odor perception and olfactory-driven decision making (Kohlmeier et al. 2018). Our results reveal that genes associated with all these pathways were differentially expressed in the brain and antennae between nurses and foragers, predicting a link between their role in task-associated behavioral changes and the regulation of odor perception. Given the central role of IIS, Vg, JH, and TOR pathways in regulating division of labor in social insects, and our finding of task-associated patterns of the antennal expression of genes from multiple biogenic amines, we hypothesize that these modulators and hormones could be involved in the regulation of the olfactory filter. How the detailed molecular mechanisms of these pathways in the brain are causally linked to the complex changes in olfactory perception in the antennae should therefore be investigated next.

Conclusion

Our transcriptomic analyses of the brain and antennae of *T. longispinosus* nurses and foragers provide support to our hypothesis that behavioral variation and task specialization in ant workers are regulated via differences in olfactory perception. We predict that antennal physiology acts as sensory filters that limit the type and amount of chemical information passed to the brain. This would allow workers to target relevant chemical information from the environment and discriminate signal from noise without using energetically costly processing by the central nervous system. We argue that this sensory filter is flexible and regulated through changes in physiological conditions such as age, nutrition, and hormones. Variation among workers in their efficiency to detect specific chemical cues would result in task specialization and division of labor. The information perceived by the peripheral ORs is transmitted to the primary brain center of the olfactory pathway, the glomeruli of the antennal lobes. The question now arises whether there are differences between nurses and foragers in the morphology or physiology of the antennal lobes. A limited subset of active ORs and glomeruli might be easier to process and less energy consuming. Our study opens novel avenues of research to better understand the role of sensory filters in controlling DOL in insect societies.

Data availability

Numerical source data found in Supplementary Data S1 was used to create Figure 1*A*, and Data S2 for Figures 2*B-C* and 3. Raw sequencing reads generated for this study have been deposited in NCBI under BioProject PRJNA926589 (http://www.ncbi.nlm.nih.gov/bioproject/926589).

Code availability

Data analysis (Dataset S1–5) and visualization for this study was done using code written in R and Bash, which can be found on Mendeley (https://data.mendeley.com/datasets/yyg46xmph6.3).

Author Contributions

M.A.C., R.L., and S.F. conceived the study; M.A.C. conducted the research; M.A.C., M.M., R.L., and D.H. contributed to the development of the data analytical protocol; M.A.C. analyzed the data; and M.A.C., R.L., M.M., P.B., and S.F. wrote the manuscript.

Supplementary material

Table S1. List of candidate genes related to behavior phenotypes.

Gene Annotation	Locus (*T. longispinosus*)	Organism reference	Locus reference	Orthogroup	Task	Tissue	Pathways and genes	References
protein takeout isoform	DBV15_08771	*Camponotus floridanus*	LOC105257386	OG0007297	Nurse	Brain	JH: signalling	(Das & de Bekker, 2022)
vitellogenin-1 (Vg conventional)	LOC112466671	*Harpegnathos saltator*	LOC105181726	OG0003549	Nurse	Brain	Vitellogenin	(Das & de Bekker, 2022; Gospocic et al., 2021; Kohlmeier et al., 2018; Opachaloemphan et al., 2021)
vitellogenin-2-like (Vg like-A)	DBV15_03138	*H. saltator*	LOC105191299	OG0009030	Nurse	Brain	Vitellogenin	(Gospocic et al. 2021; Kohlmeier et al. 2018)
farnesyl pyrophosphate synthase	LOC112466955	*Drosophila melanogaster*	Fpps	OG0000401	Nurse	Brain	JH: biosynthesis	(Korb et al., 2021)
venom carboxylesterase-6 (Jhe1)	DBV15_11528	*H. saltator*	LOC112590013	OG0002262	Nurse	Brain/Antenna	JH: regulation of JH biosynthesis	(Das & de Bekker, 2022; Gospocic et al., 2021)
ribosomal protein S6	DBV15_00490	*D. melanogaster*	RpS6	OG0007546	Nurse	Brain/Antenna	IIS / TOR	(Korb et al., 2021)
dopamine 1-like receptor 2	DBV15_07611	*D. melanogaster*	Dop1R2	OG0001377	Nurse	Antenna	biogenic amines	(Gramates et al., 2022)

octopamine beta2 receptor	DBV15_10418	*D. melanogaster*	Octbeta2R	OG0000466	Nurse	Antenna	biogenic amines	(Gramates et al., 2022)
aldehyde dehydrogenase	DBV15_01763	*D. melanogaster*	Aldh-III	OG0000175	Nurse	Antenna	JH: biosynthesis	(Korb et al., 2021)
cyclin G	DBV15_03888	*D. melanogaster*	CycG	OG0003395	Nurse	Antenna	IIS / TOR	(Korb et al., 2021)
happyhour	DBV15_11653	*D. melanogaster*	hppy	OG0000352	Nurse	Antenna	IIS / TOR	(Korb et al., 2021)
insulin receptor isoform (InRL)	DBV15_10224	*H. saltator*	LOC105183944	OG0001487	Nurse	Antenna	IIS	(Gospocic et al., 2021)
Pi3K21B	DBV15_03081	*D. melanogaster*	Pi3K21B	OG0002238	Nurse	Antenna	IIS	(Korb et al., 2021)
Ras oncogene at 64B	DBV15_02802	*D. melanogaster*	Ras64B	OG0007075	Nurse	Antenna	IIS / TOR	(Korb et al., 2021)
sarcoplasmic calcium-binding protein 2	DBV15_05072	*D. melanogaster*	Scp2	OG0005773	Nurse	Antenna	JH: metabolism	(Korb et al., 2021)
shaggy	DBV15_09601	*D. melanogaster*	sgg	OG0000404	Nurse	Antenna	IIS / TOR	(Gospocic et al., 2021; Korb et al., 2021)
allatostatin A	LOC112454443	*D. melanogaster*	AstA	OG0007104	Forager	Brain	JH: regulation of JH biosynthesis	(Korb et al., 2021)
insulin-like growth factor I (IGF1)	LOC112454447	*H. saltator*	LOC105186969	OG0004678	Forager	Brain/Antenna	IIS	(Gospocic et al., 2021; Opachaloemphan et al., 2021)
5-hydroxytryptamine (serotonin) receptor 2A	DBV15_11483	*D. melanogaster*	5-HT2A	OG0000533	Forager	Antenna	biogenic amines	(Gramates et al., 2022)

octopamine receptor in mushroom bodies	LOC112465659	*D. melanogaster*	Oamb	OG0001501	Forager	Antenna	biogenic amines	(Gramates et al., 2022)
tyramine beta hydroxylase	DBV15_00422	*D. melanogaster*	Tbh	OG0006646	Forager	Antenna	biogenic amines	(Gramates et al., 2022)
density regulated protein	DBV15_10283	*D. melanogaster*	DENR	OG0004331	Forager	Antenna	IIS / TOR	(Korb et al., 2021)
dreadlocks	DBV15_03530	*D. melanogaster*	dock	OG0001746	Forager	Antenna	IIS / TOR	(Korb et al., 2021)
juvenile hormone-inducible protein 26	DBV15_02220	*D. melanogaster*	JhI-26	OG0000832	Forager	Antenna	JH: regulation of JH biosynthesis	(Korb et al., 2021)
Krueppel homolog 1-like	DBV15_06330	*H. saltator*	LOC112589664	OG0008125	Forager	Antenna	JH: signalling	(Gospocic et al., 2021; Opachaloemphan et al., 2021)
rapamycin-insensitive companion of Tor	DBV15_07973	*D. melanogaster*	rictor	OG0003860	Forager	Antenna	IIS / TOR	(Korb et al., 2021)

Table S2. List of enriched GO biological process terms of the DEG in the brain and the antenna.

Gene Set (DEG)	Tissue	GO ID	Term	Annotated	Significant	Expected	Fisher (*p*-value)
Nurse	Brain	GO:0006412	translation	73	19	1.79	0.000
Nurse	Brain	GO:0006879	cellular iron ion homeostasis	5	2	0.12	0.006
Nurse	Brain	GO:0009056	catabolic process	155	6	3.79	0.024
Nurse	Brain	GO:0030155	regulation of cell adhesion	1	1	0.02	0.025
Nurse	Brain	GO:0010040	response to iron (II) ion	1	1	0.02	0.025
Nurse	Brain	GO:0030245	cellulose catabolic process	1	1	0.02	0.025
Nurse	Brain	GO:0009204	deoxyribonucleoside triphosphate catabolic process	1	1	0.02	0.025
Nurse	Brain	GO:0006559	L-phenylalanine catabolic process	2	1	0.05	0.048
Nurse	Brain	GO:0006572	tyrosine catabolic process	2	1	0.05	0.048
Nurse	Brain	GO:0007160	cell-matrix adhesion	2	1	0.05	0.048
Nurse	Brain	GO:0006546	glycine catabolic process	2	1	0.05	0.048
Nurse	Brain	GO:0019236	response to pheromone	2	1	0.05	0.048
Nurse	Brain	GO:0030334	regulation of cell migration	2	1	0.05	0.048
Forager	Brain	GO:0006518	peptide metabolic process	89	4	0.96	0.001
Forager	Brain	GO:0006431	methionyl-tRNA aminoacylation	1	1	0.01	0.011
Forager	Brain	GO:0032481	positive regulation of type I interferon production	1	1	0.01	0.011
Forager	Brain	GO:0045454	cell redox homeostasis	2	1	0.02	0.022
Forager	Brain	GO:0046855	inositol phosphate dephosphorylation	4	1	0.04	0.043
Nurse	Antenna	GO:0006412	translation	73	22	8.93	0.000
Nurse	Antenna	GO:0006897	endocytosis	15	6	1.83	0.006

Nurse	Antenna	GO:0006006	glucose metabolic process	10	4	1.22	0.006
Nurse	Antenna	GO:0051726	regulation of cell cycle	9	3	1.1	0.015
Nurse	Antenna	GO:0015012	heparan sulfate proteoglycan biosynthetic process	3	2	0.37	0.040
Forager	Antenna	GO:0046854	phosphatidylinositol phosphorylation	6	4	0.57	0.001
Forager	Antenna	GO:0046855	inositol phosphate dephosphorylation	4	3	0.38	0.003
Forager	Antenna	GO:0005975	carbohydrate metabolic process	81	17	7.74	0.004
Forager	Antenna	GO:0045087	innate immune response	2	2	0.19	0.009
Forager	Antenna	GO:0009166	nucleotide catabolic process	4	3	0.38	0.025
Forager	Antenna	GO:0007229	integrin-mediated signaling pathway	9	3	0.86	0.045
Forager	Antenna	GO:0006166	purine ribonucleoside salvage	4	2	0.38	0.047

Table S3. Behaviors and positions annotations during nest scans. Nursing behavior highlighted in red was defined as the number of observations a worker antennating, grooming, feeding or carrying a brood item, and its position inside the nest (on the brood or near to the brood pile). Foraging behavior highlighted in blue was defined as the number of observations an individual was found outside the nest, collecting food or water.

Position	
Inside the nest	On the brood pile
	Near the brood pile
	Surroundings of the nest
	Near the entrance
Outside the nest	Chamber 1
	Chamber 2
	Chamber 3
Behavior	
Brood care	Antennation, grooming, feeding or carrying the brood
Nestmate care	Antennation, grooming, feeding or carrying a nestmate
Forager	Collecting food/drinking water outside the nest
Others	Resting
	Walking
	Being groomed or fed
	Grooming itself

Chapter 2

Vitellogenin-like-A gene expression patterns in relation to task differentiation in the ant *Temnothorax longispinosus*

Marcel A. Caminer, [redacted]

Abstract

Social insect colonies are characterized by division of labor. The dynamic regulation of behavioral genes plays an important role in this. One gene that controls the brood care behavior of the workers of the ant *Temnothorax longispinosus* is *Vitellogenin-like-A* (Vg-like-A). This gene is mainly expressed in the fat body, not in the peripheral or central nervous system as one might assume for a behavioral gene. In this study, we address the question of how exactly the expression of this gene affects the behavior of ant workers. A *Vg-like-A* titre could influence the expression of downstream genes in other tissues, such as the brain or the antenna. We question which other genes are involved in this network, how the communication between the different tissues takes place and how fast these molecular processes occur. To investigate the effects of downregulation of *Vg-like-A* on behavior and temporal changes in gene expression in sister ants, we used RNA interference (RNAi) by injecting dsRNA of *Vg-like A* directly into the gaster of the ants, followed by behavioral observations and transcriptome analyses of brain, antennae and fat body of *T. longispinosus*. We succeeded in a stable downregulation over 10 days, which also led to behavioral changes. However, these were only weakly pronounced, presumably because the injection lowered the motivation of all workers, including those in the control treatment. We also identified only a handful of genes whose expression depended on the treatment and the time of injection. We discuss why only such weak transcriptional shifts occurred in our study.

Introduction

Division of labor (DOL) is a characteristic trait of complex biological entities, from biofilms and multi-cellular organisms to complex animal societies such as those of the social insects (Beshers & Fewell 2001; van Gestel et al. 2014). Social insects live in highly organized societies where individuals can be grouped into castes taking over different roles. These roles include reproduction, which is typically carried out by the queen, and all other work in the colony such as foraging and raising the offspring, which is carried out by the workers. In addition to reproductive DOL between queens and workers, workers also divide the labor specializing on specific tasks (Wilson 1976; Herbers 1983; Pinter-Wollman et al. 2012). Task specialization can be related to morphological differences (Wilson 1971; Oster & Wilson 1979; Hölldobler & Wilson 1990), may be in part genetic (Fraser et al., 2000; Hughes et al., 2003; Schwander et al., 2005) and typically varies with age (Wilson 1971). In most social insects, young workers take care of the brood and switch to risky tasks outside the nest later in life. This transition from intranidal to extranidal tasks (e.g., foraging) may be delayed, accelerated and even reversed in response to modifications of the social environment (Robinson 2009; Shimoji et al. 2020; Robinson et al. 1997; Amdam 2011).

Workers vary in their response to task-associated stimuli such as chemical signals, either because they have different response thresholds or because they perceive the cues themselves differently (Bonabeau et al. 1998; Caminer et al. 2023). In both cases, gene expression could play an important role by influencing the molecular physiology of the central or peripheral nervous system or the fat body. In some social insects, an influence of transcriptional activity on the division of labor has already been demonstrated. For example, brood care and foraging behavior are affected by hormones involved in the regulation of the vitellogenin gene (Vg), including juvenile hormone (JH), ecdysone and several neuropeptides (Engelmann 1983; Engelmann & Friedel 1974; Guidugli et al. 2005; Hagedorn 1985; Wyatt & Davey 1996). In honeybees, high expression of the single Vg gene is positively linked to brood care behavior and negatively to the onset of foraging. When Vg is downregulated, JH titres increase and result in a reduction of brood care, precocious foraging, and a forager-like transcriptional profile (Marco Antonio et al. 2008; Nelson et al. 2007; Nunes et al. 2013; Azevedo et al. 2016). However, this Vg and JH association may be different in other social insects such as ants (Libbrecht et al. 2013; Pamminger et al. 2016), suggesting fundamental variation in gene networks that regulate behavior.

All egg-laying species, including insects, supply their eggs with vitellin as the main yolk protein. The precursor of vitellin is Vg, which is synthesized extra-ovarially by the fat body - a tissue of insects comparable to the liver- , secreted into the haemolymph and then sequestered by competent oocytes via receptor-mediated endocytosis (Raikhel & Dhadialla, 1992; Sappington & Raikhel, 1998; Snigirevskaya & Raikhel, 2005). In addition to the primary function of egg yolk formation, Vg is a protein associated with other functions, including task specialization, pleiotropic effects in female castes, regulation of hormonal dynamics, longevity, gustatory responsiveness, and reproductive physiology of many social insects (Excels 1974; Amdam & Omholt 2003; Guidugli et al. 2005; Nelson et al. 2007; Münch & Amdam 2010). Vg underwent several duplication events, followed by diversification and subfunctionalization in the social insects, which consequently exhibit a variable number of *Vg* genes. For example, *Vg-like-A*, *Vg-like-B*, and *Vg-like-C*, which are orthologous to the *conventional Vg*, have emerged in the Hymenoptera (Morandin et al., 2014). The different Vg copies can be assigned to specific clusters, are expressed in a caste- and behavior-specific manner and are associated with specific reproductive and non-reproductive functions (Corona et al., 2013; Morandin et al., 2014).

Recent studies have shown that *Vg-like-A* responded more strongly than the *conventional Vg* to inflammatory and oxidative stress conditions in honeybees (Salmela et al., 2016) and play an important role in over-wintering workers longevity (Ricigliano et al., 2018). Additionally, *Vg-like-A* overexpressed in nurses compared to foragers is linked to the regulation of brood care behavior in the ponerine and *Temnothorax longispinosus* ants (Kohlmeier et al., 2018; Miyazaki et al., 2021). A downregulation of *Vg-like-A* by constantly feeding Dicer-substrate small interfering RNA (dsiRNA) to the entire *T. longispinosus* colony, showed shifted the interest of brood-care workers from brood-related cues to odors of adult nestmates. This result indicates a change in odorant perception or assessment and olfaction driven decision-making in the ant brain (Kohlmeier et al., 2018).

Social insects communicate mainly through exchange of chemical information including pheromones and cuticular hydrocarbons (Wilson 1965). Odorant perception occurs in the antennae, a peripheral sensory organ equipped with numerous sensilla (sensory receptors) that houses a large and diverse subfamily of odorant receptors (OR) capable of detecting a wide range of chemical compounds (McKenzie et al., 2016). Comparative transcriptomic analysis of *T. longispinosus* worker antennae revealed that half of all OR receptors are differentially expressed between nurses and foragers (Caminer et al., 2023). These workers, specialized in different tasks, show transcriptional activity in their antenna from ORs of different subfamilies, suggesting that they perceive their olfactory environment and task-related cues differently. Therefore, the molecular physiology of the antennae could act as a sensory filter, limiting the type and amount of chemical information transmitted to the brain and promoting worker specialization to specific tasks (Caminer et al., 2023). In the same study, *Vg-like-A* was found to be overexpressed in brain of nurses, suggesting that it may be involved in regulating this sensory filter.

All these findings support the idea that *Vg-like-A* plays an important role in regulation behavior and thus division of labor in ant societies. However, it remains unclear how exactly *Vg-like A*, which is mainly expressed in the fat body (Kohlmeier et al. 2018), influences the behavior of ant workers. A *Vg-like A* titre could modulate the expression of downstream genes in the peripheral or central sensory system such as in the antennae, the antennal lobes or in higher brain regions. It also remains unexplored which other genes are involved in this network, how the communication between the different tissues occurs and how fast these molecular processes are. To investigate the effects of a downregulation of *Vg-like-A* on behavior and changes in gene expression in ant nurses over time, we used RNA interference (RNAi) via injection of dsRNA of *Vg-like A* directly into the gaster of ants, followed by behavioral observations and transcriptome analyses of the brain, antennae, and fat body of *T. longispinosus*. In this species, the activities of the workers are not regulated by the queen herself or by other dominant nest inhabitants. Moreover, there are no morphological worker castes, but a clear age-polyethism. The emergence of new workers during the summer months pushes older workers to the nests' periphery, away from the brood pile, and so they take on other tasks around or outside the nest (Mackay, 1983). Since all workers in a *T. longispinosus* colony are highly related (Foitzik & Herbers, 2001), the differences in behavior are probably caused by shifts in gene expression, although genetic influences cannot be completely ruled out. In this study, we predict that nurses of *T. longispinosus* reduce their brood care behavior after downregulation of *Vg-like-A* and want to test how fast these behavioral changes happen. Tissue-specific transcriptional analyses will reveal which other genes change their activity as a consequence of the downregulation of *Vg-like A* and in which tissues these changes occur. We are analyzing several time points in order to be able to better break down the sequence of events and possibly identify a *Vg-like-A* regulated gene network. In particular, we are interested in whether the activity of *Vg-like A* can directly or indirectly alter the expression of odorant receptors in the antenna, which is so different between nurses and foragers (Caminer et al. 2023).

Materials and Methods

Tissue-specific expression of *Vg-like-A* via RT– qPCR

Colonies of the ant species *T. longispinosus* were collected with permission in June 2018 in the woods of the Edmund Niles Huyck Preserve, Rensselearville, NY, USA (42°31′41.0″N 74°09″38.8′W). In the laboratory, we placed each colony in a plaster-bottomed nest box (43 cm × 28 cm × 10 cm) divided into three chambers and containing a single slide nest into which the colony moved. A slide nest is an artificial nesting site consisting of a small Plexiglas cavity nestled between two glass slides and covered with red foil. Colonies were maintained at Johannes Gutenberg University in Mainz, Germany, at a temperature of 18 °C to 22 °C and a light-dark schedule of 14 h:10 h, provided with water ad libitum, and fed with honey and crickets twice weekly.

To determine the expression levels of the brood-care gene *Vg-like-A* (Kohlmeier et al. 2018) between behavioral phenotypes and in different tissues, 34 workers inside (from the brood pile) and 30 workers outside the nest, belonging to seven colonies, were marked with fine colored metal wires (0.02 mm Elektrisola, Eckenhagen, Germany) between the petiole and the post-petiole. We conducted hourly behavioral observations (total = 24 scans) eight times daily for three days, noting how often an individual engaged in brood care and foraging and where it was in the nest. Behavioral variables such as antennation, grooming, feeding, or carrying brood, and collecting and searching for food and water were noted. Location within the nest (i.e., on the brood, near the brood, or around the nest) and outside the nest were also recorded (Table 1). Based on these behavioral and location observations, we selected two marked individuals per colony inside (nurses) and two outside the nest (foragers) that exhibited the higher percentage of brood care or foraging behavior.

We dissected brain, fat body, and antenna tissues from 14 nurses and 14 foragers. Dissected tissues were transferred separately to a 1.5 ml Eppendorf tube containing 100 µl TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Two workers from each behavioral phenotype and colony were pooled per sample. Subsequent RNA extraction was performed using Direct-zol RNA MicroPrep kits (Zymo Research, USA) according to the manufacturer's instructions. We quantified the extracted RNA concentrations using the Qubit RNA HS Assay Kit and the Qubit 2.0 Fluorometer (Invitrogen). Total RNA from 42 samples (14 brains, 14 fat bodies, and 14 antennae) was reverse transcribed using the QuantiTect Reverse Transcription cDNA Synthesis Kit (Qiagen). According to the manufacturer's instructions, the extracted RNA was treated with RNase-free DNase to remove any contamination with genomic DNA, and 1µg (per reaction) was used to synthesize complementary DNA (cDNA). The resulting cDNA was used as a template for PCR amplification with *Vg-like-A* specific forward and reverse primers (Table 2). After initial denaturation at 95 °C for 1 min, PCR was performed for 35 cycles: Denaturation at 95 °C for 1 min, annealing at 57 °C, and extension at 72 °C for 1 min. A final extension phase of 10 min at 72 °C completed the PCR reaction. PCR products were separated by electrophoresis on a 1% agarose gel. Quantitative PCR (qPCR) was performed using a Mic qPCR Cycler (Bio Molecular Systems) and the Biozyme Blue S'Green qPCR Kit Separate ROX (Biozyme). The reaction mixture (20 µL) contained 10 pmol of the RT-PCR primers (Table 2) and 3 ng/µL of cDNA template quantified with Qubit. After activation of hotstart Taq DNA polymerase for 2 minutes at 95°C, the reaction was amplified with 40 cycles of denaturation at 95°C for 5 seconds and annealing at 57°C for 20 seconds. Mic qPCR Cycler software generated amplification plots and assessed fluorescence in real time. Melting curves of the PCR products were analyzed to ensure that they were a single product. Three technical replicates were used for each of the 42 samples. The expression levels of *Vg-like-A* were normalized by quantifying the expression levels of alpha-tubulin (housekeeping gene) in each sample. Relative quantification of mRNA levels was determined by the comparative cycle threshold (CT) method (Livak & Schmittgen, 2001). Each biologically independent sample was measured three times and averaged.

To determine whether there was a correlation between behavior and expression of *Vg-like-A* in each tissue, the mean behavioral values (% brood care and % foraging) of the two individuals used to produce that qPCR sample were taken. All statistical analyses were performed (unless specifically noted) in RStudio, version 1.4.1106 (RStudio Team, 2020).

Table 1. Behaviors and location annotations during nest scans. Nursing behavior was defined as the number of observations a worker antennating, grooming, feeding or carrying a brood item, and its location inside the nest. Foraging behavior was defined as the number of observations an individual was found outside the nest, collecting food or water.

Location	
Inside the nest	On the brood pile
	Near the brood pile
	Surroundings of the nest
	Near the entrance
Outside the nest	Chamber 1
	Chamber 2
	Chamber 3
Behavior	
Brood care	Antennation, grooming, feeding or carrying the brood
Nestmate care	Antennation, grooming, feeding or carrying a nestmate
Forager	Collecting food/drinking water outside the nest

Table 2. Primers used in this study.

Gene	Name	Sequence (5’ to 3’)	Amplicon length	Usage
Vg-like-A template	FP-Vg174.1-Tn	CAGGATTCGAAGACGCCTAAG	309	PCR
	RP-Vg174.1-Tn	GTCGCTCTGCTGATGTATGAA		
Vg-like-A	FQ-Vg174.1-Tn	CCGTCAGGATTGTCGCTTAT	89	qPCR
	RQ-Vg174.1-Tn	CTCGTGTACCATGAACCAATAGA		
Alpha tubulin	FQ-Tubulin-Tn	TCCGTTGACTATGGCAAGAAG	107	qPCR
	RQ-Tubulin-Tn	GTGTGCGTCGTCAAGATAGAA		
EGFP template	FP-EGFP	GCTGACCCTGAAGTTCATCTG	349	PCR
	RP-EGFP	CTGCTTGTCGGCCATGATATAG		
dsRNA *EGFP*	T7-FP-EGFP	TAATACGACTCACTATAGGGAGAGCTGACCCTGAAGTTCATCTG	395	RNAi
	T7-RP-EGFP	TAATACGACTCACTATAGGGAGACTGCTTGTCGGCCATGATATAG		
dsRNA *Vg-like-A*	T7-FP-Vg174.1-Tn	TAATACGACTCACTATAGGGAGACAGGATTCGAAGACGCCTAAG	355	RNAi
	T7-RP-Vg174.1-Tn	TAATACGACTCACTATAGGGAGAGTCGCTCTGCTGATGTATGAA		

Silencing expression of *Vg-like-A* by RNA interference (RNAi)

Previous studies already showed a control of *Vg-like-A* expression on the division of labor in the ant *T. longispinosus*. An RNAi knockdown of *Vg-like-A* in the fat body of workers via feeding dsiRNA resulted in a decrease in brood care behavior within a week and an increase in nest mate care over several weeks (Kohlmeier et al. 2018). However, how *Vg-like-A* affects the expression of other genes in the brain, fat body, and antennae of nurses, and how rapidly this occurs, is still completely unknown. To address this question, we first attempted to suppress the expression of *Vg-like-A* using an interference double-stranded RNA (dsRNA) injected directly into the gaster. RNAi was performed with dsRNA synthesized according to the manufacturer's instructions using the Megascript RNAi Kit (Ambion). A partial sequence of the *Vg-like-A* target gene (355 bp) was amplified with gene-specific primers containing the T7 promoter region at the 5′-end (Table 2). T7 RNA polymerase was inactivated after 4 hours of synthesis at 37°C. A 395 bp long fragment of enhanced green fluorescent protein (EGFP) was synthesized as a control dsRNA (Table 2). The synthesized dsRNAs were quantified using a spectrophotometer (NanoDrop; Thermo Scientific) and a 1% agarose gel.

We selected eight colonies and divided each of them into two (treatment and control) to create artificial colonies (total = 14) with equal numbers of nurses (n = 18), foragers (n = 2), larvae (n = 20), and queens (n = 1). Nests were transferred to plaster-bottomed Petri dishes (50 mm x 10 mm) with half of the lid covered with a red plastic filter to simulate dark conditions. The ants were fed ad libitum with crickets, honey and water. Five nurses per artificial colony were marked with colored metal wires for subsequent injection with *Vg-like-A* RNAi treatment or EGFP RNAi control. Ants were removed from nests and anesthetized with CO2 before being fixed to an injection plate with double-sided adhesive tapes. For injection, glass capillaries with an outer diameter of 1.14 mm and a length of 3.5 inches (Drummond) were pulled using a Narishige PC-128 pulling machine, and the tips were opened and beveled using a grinder (Bachofer). A Nanoject II auto-nanoliter injector (Drummond) was used to inject 128 ng of dsRNA (128 nl) at a concentration of 1µg/µl between the first and second segments of the ant's abdomen. Control individuals were injected with 100 ng of dsEGFP (Vatanparast et al. 2021, 2022; Vatanparast & Kim 2017). To determine the specific time period during which RNAi affects the expression of the target gene after injection, we decided to test the downregulation of *Vg-like-A* over various time periods (1, 2, 3, 5, and 10 days) with the respective controls. Eight ants died during the injection, reducing the number of replicates from 5 to 7 for some treatments (i.e., 3, 5, and 10 days) and controls (i.e., 5 and 10 days), while the rest of the replicates for each treatment and control remained at 8 samples. RNAi efficiency was determined by qPCR as described above.

Behavior observations, RNA extraction and sequencing

Our preliminary results showed strong evidence that the expression of *Vg-like-A* is reduced by the RNAi treatment compared to the control across at days 1, 2, 3 and 5 post-injections ($P < 0.02$, by the chi-square test controlled for colony ID). Based on these results, and in order to demonstrate the effects of reduction-of-function of *Vg-like-A* in nurse’s behavior and on tissue-specific gene expression, we proceed to knockdown this gene followed by behavioral observations and RNA-seq analysis for transcriptional signatures at days 3 and 10 post-injections. Twenty-eight artificial colonies with 37 ± SD

2.4 workers, 29 ± SD 6.1 larvae, and 1 queen, were transferred in small slide nests. Four nurses per colony (total = 112 individuals) were marked with colored metal wires and injected with *Vg-like-A* RNAi treatment or control (dsEGFP) following the methodology described above. Ants that did not survive the injection were excluded. We conducted behavioral observations every hour, eight times a day in the two days (total = 16 scans) prior to sacrificing the ants at days 3 and 10, in which we noted how many times an individual spent performing brood care and foraging behavior as well as their location in the nest (Table 1).

We selected one focal ant per colony to dissect the brain, fat body and antennae, following by RNA extraction with 100µl of TRIzol and purification with the Direct-zol RNA MicroPrep Kits (Zymo Research). The resulting 84 samples (28 brains, 28 fat body, and 28 antennae) were stored at -80°C until library preparation. RNA-seq libraries were prepared by Novogene Company Limited, Cambridge, UK, using the NEBNext Ultra RNA Library Prep Kit for Illumina according to the manufacturer's protocol. Samples “F_577_B” and “F_791_B” for fat body tissue fail during the library preparation. After amplification and purification, libraries were sequenced on an Illumina NovaSeq 6000 using a paired-end 150 bp. We sequenced a minimum of 8 Gb per sample, resulting in around 30 million raw reads were generated from each library (Dataset S1).

Differential gene expression analysis

Illumina adapters of the raw data obtained from Novogene were removed using Fastp v. 0.22.0 (Chen et al. 2018). Subsequently, the quality of the reads was assessed using fastQC v. 0.11.9 (Andrews, 2016) and sequences flagged as poor quality were removed

(Andrews, 2010). Filtered reads were mapped against the *T. longispinosus* genome assembly (Jongepier et al., 2022), and the read counts table was generated using STAR 2.7.0 (Dobin et al., 2013) with default settings. Detailed mapping statistics for each sample is available in Dataset S1. The gene count matrix was analyzed using DESeq2 v.1.32.0 package for R to identify differentially expressed genes per tissue (Love et al. 2014). To avoid biased results due to low read counts, we removed from the counting matrix those genes for which less than 10 of the reads mapped to at least 7 of our 28 samples (n - 1 of the smallest sample size). We used a likelihood ratio test (LRT) to compare models that included ~Day + Treatment as factors to test for main effects of treatment, as well as to test their interaction (~Day * Treatment). Gene expression was considered to differ between treatment and control if the false discovery rate (FDR), using Benjamini-Hochberg procedure, had an adjusted p-value of ≤ 0.05, yielding in a list of differentially expressed genes (DEGs). Finally, InterProScan v. 5.46-81.0 (Jones et al., 2014) was run on the protein sequences of the *T. longispinosus* genome to obtain functionally annotations for each contig.

Statistical analysis of ant behavior

Principal component analysis (PCA) and analysis of variance (ANOVA) were used to assess the degree of change in brood care behavior and positioning of ants between 94 *Vg-like-A* RNAi treated and control nurse ants. All annotated nest behavioral and location traits (Table 1) were included in the analysis, but only components with eigenvalues greater than 1 were considered. We split the data set into two independent PCA analyses as behavior and location are two different types of values recoded at each timepoint. PC

I and II values were extracted and compared between treatments and controls after ensuring that they conformed to a normal distribution. To examine correlations between behavior and *Vg-like-A* expression, we calculated correlation coefficients between PC behavioral values and *Vg-like-A* gene expression obtained from RNA-seq analysis. All statistics were performed using RStudio v.1.4.1106. (RStudio Team, 2020).

Results

Tissue-specific expression of *Vg-like-A* in nurses and foragers of *T. longispinosus*

We quantified the expression levels of *Vg-like-A* in three different tissues of nurses and foragers of *T. longispinosus* via qPCR. Workers identified as foragers were found outside the nest in 17% ± SD 15% of the time, while nurses spent only 3% ± SD 4% outside the nest. In contrast, focal nurses interacted with brood in 11% ± SD 6% of observations, while we found that foragers did so in only 1% ± SD 2% of observations. Each qPCR sample consists of two individuals of the same type and colony. In particular, the *Vg-like-A* gene was strongly expressed in the fat body of the nurses, whereas it had a lower expression level in the foragers (t = 2.7, d.f. = 6.3, P = 0.03). This differentiation occurred mainly in the fat body, whereas only low expression of *Vg-like-A* was detected in brain and antennal tissues (Fig. 1).

The two workers pooled per colony, belonging to the same behavioral phenotype selected for one qPCR sample, showed quite similar behavior in terms of percentage of brood care or foraging. Therefore, we decided to use the median of these behaviors to determine possible relationships between the expression of *Vg-like-A* in the fat body and behavior. While in nurse workers, the amount of time workers spent brood caring was not associated with the expression of *Vg-like-A* (Fig. 2 A), foragers that cared more for brood had higher expression of *Vg-like-A* (r = 0.76, p = 0.045; Fig. 2B).

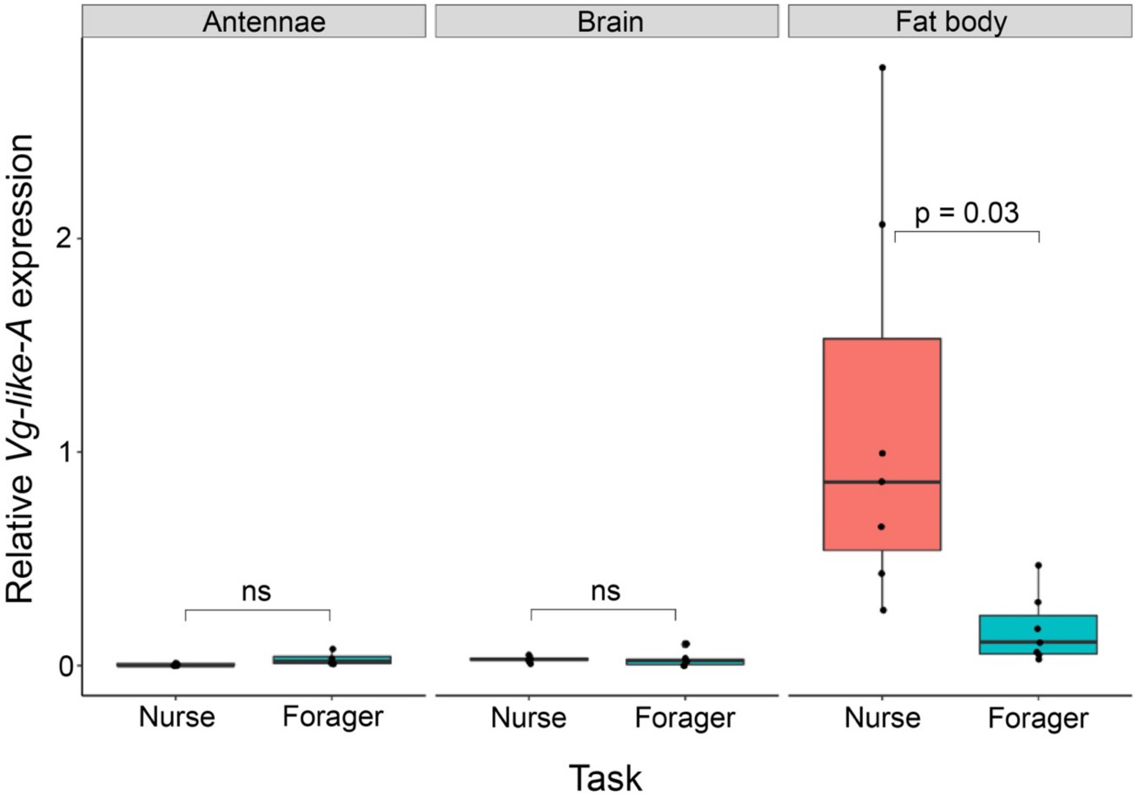

Figure 1. Tissue-specific expression pattern of *Vg-like-A* gene in nurses and foragers of *T. longispinosus*. Each point represents two pooled individuals belonging to the same colony and behavioral phenotype. Expression levels in dissected tissues were quantified via qPCR relative to *Alpha tubulin* expression. Means and standard deviations of seven biological replicates are shown.

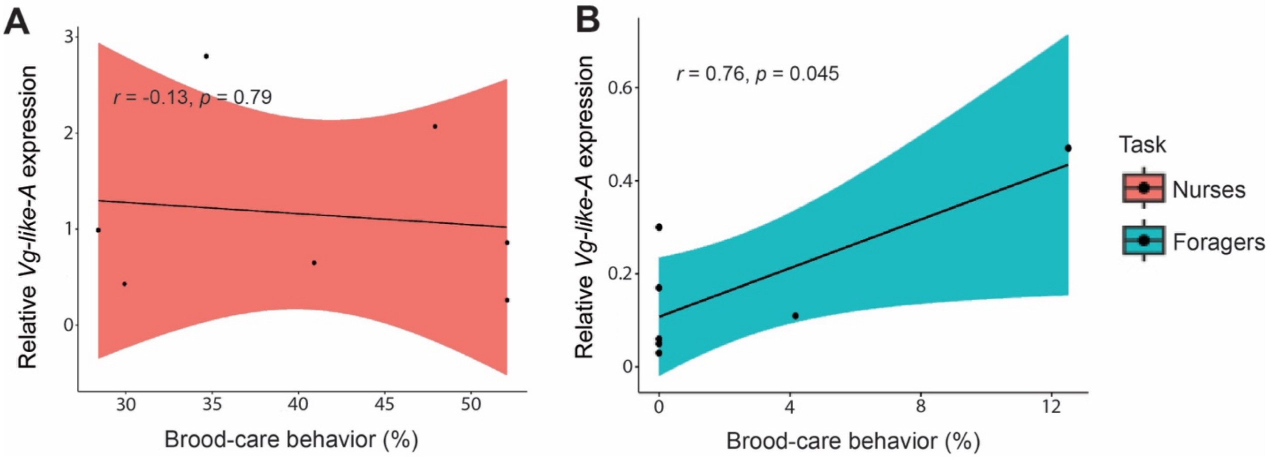

Figure 2. Correlation between brood care behavior and *Vg-like-A* gene expression in fat body of (**A**) nurses and (**B**) foragers. Each point represents two pooled individuals belonging to the same colony and behavioral phenotype. Correlation is determined by how well a line fits the plotted data, not by the slope of the line. Black lines represent best-fit linear regressions and indicate positive or negative correlations.

Vg-like-A expression, regulation and behavioral progression

We performed a series of knockdown experiments to determine the function of *Vg-like-A* and to uncover the effects of *Vg-like-A* on the signalling pathways or networks regulating task choice and thus on division of labor. First, we wanted to know how long it takes for *Vg-like-A* to be downregulated via injecting a dsRNA into the abdomen of ants. During the experimental period of between days 3 and 10 post-injections, the mortality rate was generally low at 5% (3/56) and 27% (15/56), respectively. We found evidence that the relative expression of *Vg-like-A* was reduced overall by RNAi treatment (main effect of treatment, $X^2 = 25.6$, $P < 0.001$). In general, *Vg-like-A* increased over the course of the experiment (main effect of time, $X^2 = 12.1$, $P < 0.001$), but more so in control than in RNAi treatment (interaction time:treatment, $X^2 = 4.7$, $P = 0.03$). *Vg-like-A* was significantly downregulated at days 1, 2, 3, and 10 (all P values < 0.02), with a reduction in expression between from 56% to 76%. We found only a trend of a downregulation at day 5 ($P = 0.08$, 45% reduction) (Fig. 3). Two components with the higher percentage of explained variance and eigenvalues > 1.0 were extracted from each behavioral and location PCA. The two behavioral PCs accounted for 33.6% of the total variation. PC I (17.8% of the variance) had high loadings on walking, antennae and grooming the brood; PC II (15.8% of the variance) had high loadings on carrying the brood and resting (Fig. 4A; Table S2). Overall, comparison of PC I between *Vg-like-A* RNAi-treated and control nurse ants revealed slight differences at day 3 ($X^2 = 2.89$, $P = 0.08$), but no changes at day 10 ($X^2 = 0.05$, $P = 0.82$) after the injections. Meanwhile, comparison of PC II showed a trend of reduced nursing behavior at day 10 ($X^2 = 3.76$, $P = 0.052$), but not at day 3 ($X^2 = 0.5$, $P = 0.47$) (Fig. 4C). On the other hand, two location PCs accounted for 47.7% of the total variation. PC I (28.4% of the variance) had high loadings on near to the brood, on the brood, and surrounding the nest; PC II (19.3% of the variance) had high loadings on surrounding the nest, chamber 2 and 3 (Fig. 4B; Table S3). Comparisons of PC I showed that nurse ants treated with *Vg-like-A* RNAi were significantly less likely to be near brood on day 3 than the control group ($X^2 = 3.82$, $P = 0.045$), but this change was not found on day 10 ($X^2 = 0.24$, $P = 0.62$). Meanwhile, comparison of PC II showed no change in position between control and treatment at day 3 ($X^2 = 0.76$, $P = 0.38$) and 10 ($X^2 = 01.55$, $P = 0.21$) (Fig. 4D).

To investigate transcriptomic variation in nurses with a *Vg-like-A* downregulation during a short (3 days) and long (10 days) period of time, we used RNA-seq to generate *Vg-like-A* RNAi and control brain (n = 28), antenna (n = 28), and fat body (n = 26) samples. After quality controls and mapping process, 12 antennae and 6 fat body poor quality samples with an insufficient number of aligned reads (< 50%) were excluded from the DEseq2 analysis (see Dataset S1 for samples discarded). Of the 14,646 protein-coding genes in the *T. longispinosus* genome, we found that 70% (10,352), 67% (9,938), and 62% (9,061) were expressed (FPKM > 0) in the brain, fat body and the antennae respectively. Using an adjusted p-value of 0.05 as significance threshold, we detected only a single differentially expressed genes (DEG) in the brain and three DEGs in the antennae testing the interaction between day and treatment (Table 3). Tlon_g03598 (*Importin subunit alpha*-7), Tlon_g13163 (*reverse transcriptase*), and Tlon_g17763 (*prohormone-2-like*) genes were upregulated at day 3 and downregulated at day 10 in the antennae of treatment nurses, while Tlon_g08480 (uncharacterized protein) gene was downregulated at day 3 and upregulated at day 10 in the brain. On the other hand, DEseq2 analyses testing the main effect of treatment resulted in no DEGs for all tissues. Plotting *Vg-like-A* expression in fat body (Fig. 5A) and brain (Fig. 5B) showed a slight downregulation level in nurses treated with RNAi in both tissues at days 3 and 10. However, these results were not significant as those quantified with qPCR in fat body (Fig. 3).

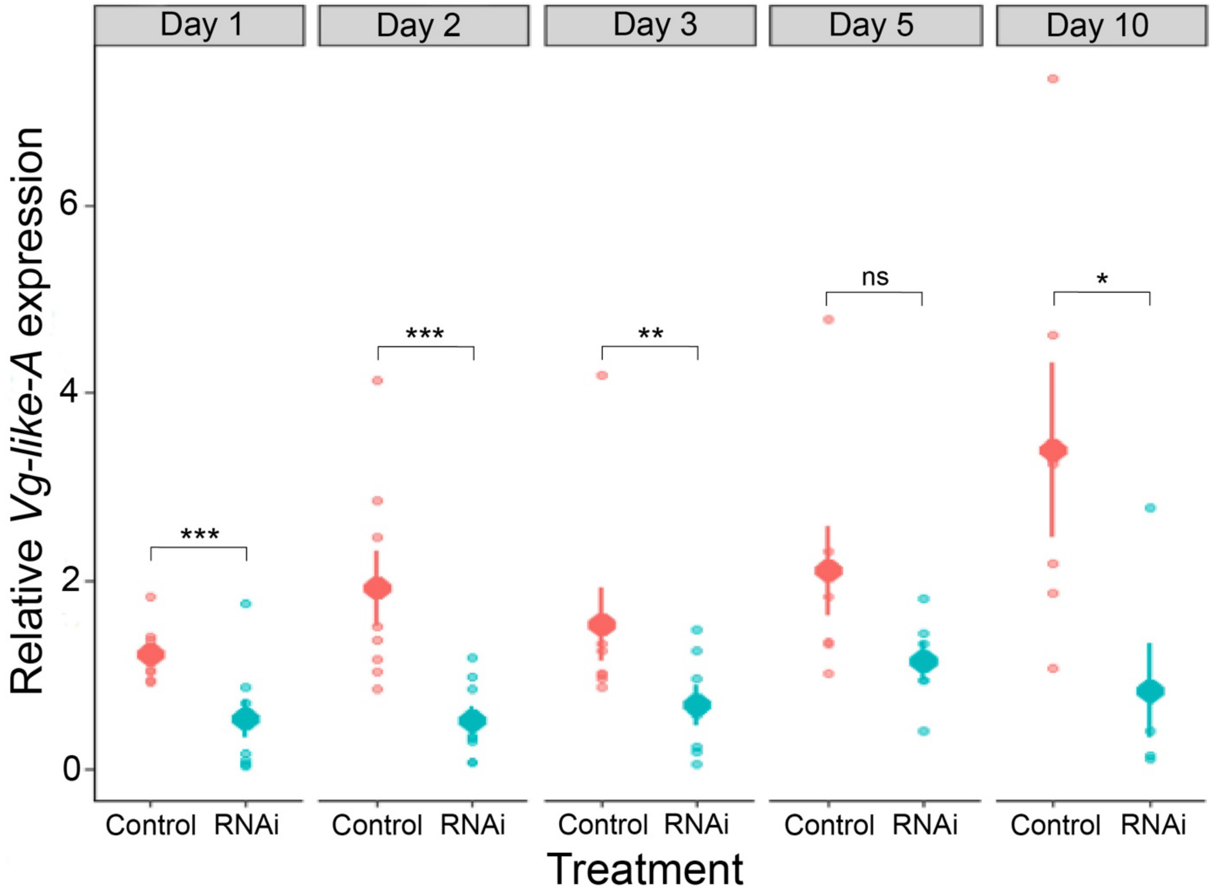

Figure 3. Downregulation expression pattern of *Vg-like-A* gene in fat body of nurses. Expression levels were quantified via qPCR relative to *Alpha tubulin* expression. Each point represents a single individual. Means are shown as large filled circles and standard deviations as lines.

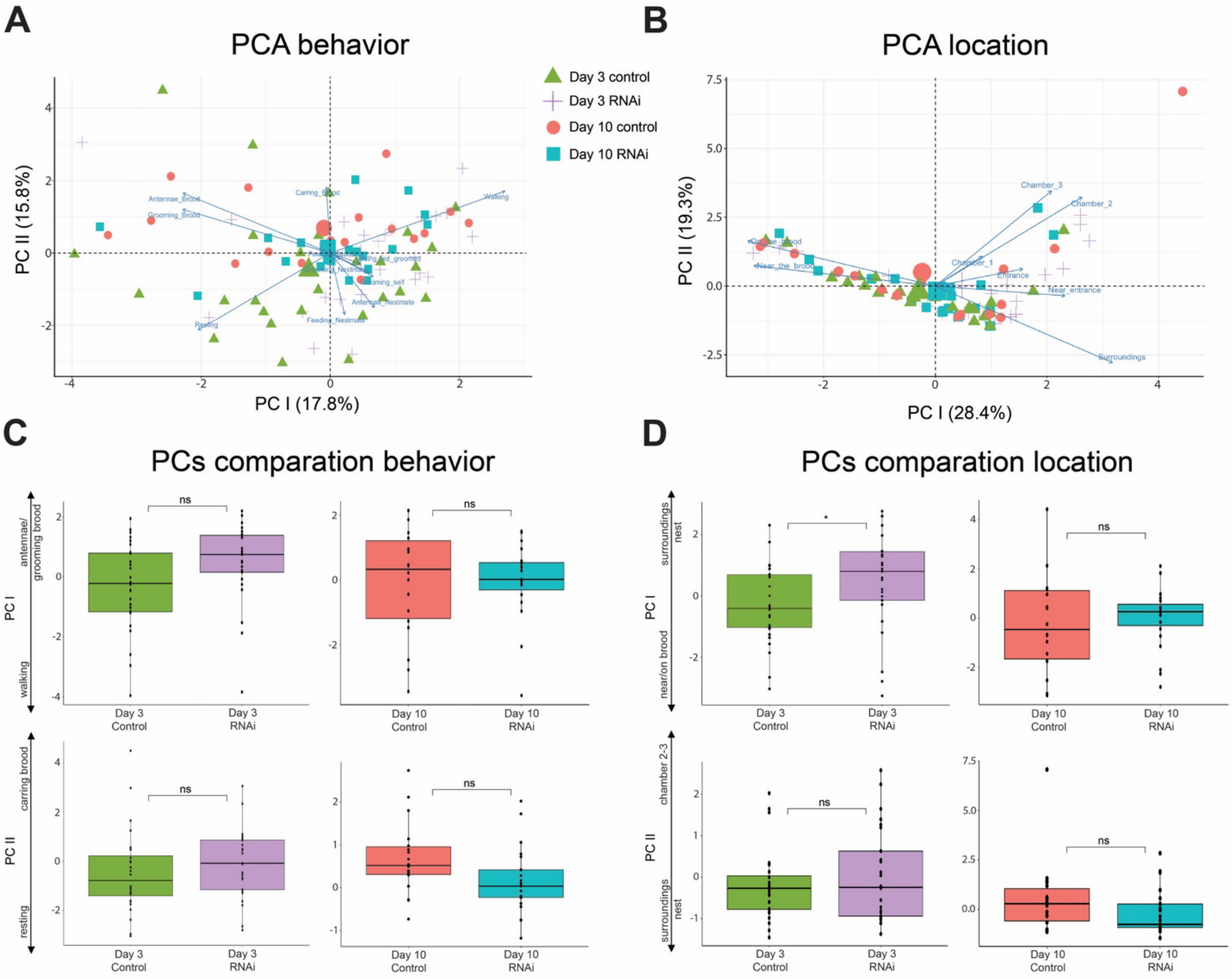

Figure 4. Axes I and II from Principal Components Analysis based on (**A**) twelve behavioral and (**B**) eight location variables. Comparations of PCs values (**I**-**II**) between 94 nurses treated with *Vg-like-A* RNAi and control at days 3 and 10 post-injections, extracted for the (**C**) behavior and (**D**) location PCA analyses.

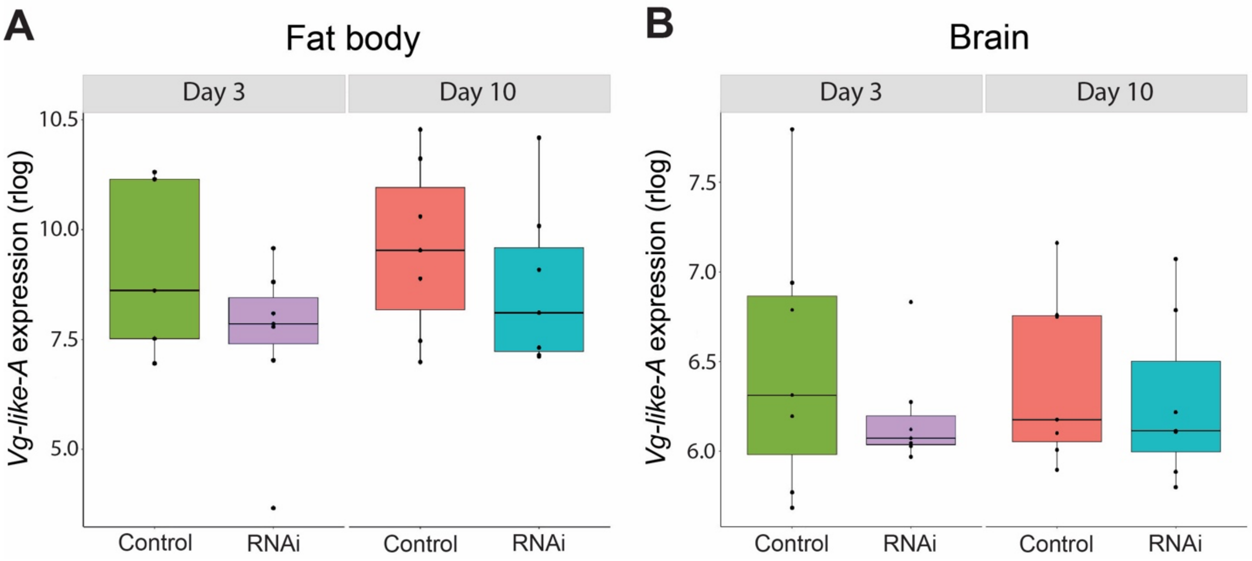

Figure 5. *Vg-like-A* gene expression differences between nurses treated with RNAi and control in (**A**) fat body and (**B**) brain tissues at days 3 and 10.

Table 3. List of DEG related to change in *Vg-like-A* expression in brain and antennae of nurses treated with RNAi vs control.

Gene ID	**Tissue**	**log2FC**	**FDR (Padj)**	**UniProt annotations**	**Blast**	**Reference locus**	**Reference organism**
Tlon_g08480	Brain	7.92	0.016	--	uncharacterized protein	LOC112462309	*Temnothorax curvispinosus*
Tlon_g03598	Antennae	-13.65	0.04	Armadillo/beta-catenin-like repeat	importin subunit alpha-7	LOC112460547	*T. curvispinosus*
Tlon_g13163	Antennae	-11.26	< 0.001	Reverse transcriptase (RNA-dependent DNA polymerase)	--	--	--
Tlon_g17763	Antennae	-19.84	< 0.001	--	prohormone-2-like	LOC112453658	*T. curvispinosus*

Discussion

DOL in insect societies is determined by a complex interaction of worker age (Huang & Robinson, 1996), morphology (Wilson 1971), fertility (Robinson 2002; Feldmeyer et al. 2014), environmental influences (Theraulaz et al., 1998), individual worker experience (Ravary et al. 2007) and gene expression (Ben-Shahar 2005; Gospocic et al. 2017; Gräff et al. 2007). In this study, we downregulated the behavioral gene *Vg-like-A* in nurses of the ant species *T. longispinosus* to investigate changes in gene expression and worker behavior. The mRNA expression level of *Vg-like-A* significantly decreased after the first day of injection of dsRNA into the nurses and the downregulation by a single injection could maintained until day 10. We found that nurses treated with *Vg-like-A* RNAi were less likely to be near the brood on the third day and showed a tendency to decrease brood care activity on the tenth day after injection. These results may confirm previous studies suggesting that high expression of *Vg-like-A* causes workers to become physiologically more focused on brood care activity, thus influencing task specialization in *T. longispinosus* (Kohlmeier et al. 2018). Despite this, we found no evidence that *Vg-like-A* downregulation causes significant changes in the transcriptional activity of brain, antennae, or fat body of treated brood care workers. Only the expression of a few genes was influenced by an interaction between time and RNAi treatment.

Although a previous study showed a significant reduction in brood care activity after 7 days using a feeding dsiRNA protocol (Kohlmeier et al., 2018), we found here only a tendency at day 10 with no behavioral changes three days after injection. However, we found that RNAi treated workers were less often close to the brood, compared to control workers. The difference between the two studies could be due to the different protocol or the fact that different types of workers were studied. Our injection protocol targeted only a few workers in the colony. It could therefore be that the injury from the injection alone, independent of treatment, reduced the likelihood that workers conducted brood care, and other workers that were not injected compensated by conducting more brood care. The feeding protocol of the previous study (Kohlmeier et al., 2018), affected all workers in the colony equally, and workers were not injured. So it could be that brood care intensities were higher in the control treatment, which could make the differences to the RNAi treatment more obvious. In fact, the nurses showed brood care behavior more than 20% of the time (Kohlmeier et al. 2018), whereas we can only show this in 11% of the observations for nurses. Furthermore, the size of the dsRNA fragment injected in our experiment was 355 bp, which cleaves into multiple duplexes of 21-23 nucleotides (nt) termed short interfering RNAs (siRNAs; Bernstein et al. 2001; Ketting et al. 2001), involved in silencing the expression of the target gene. Experiments in the red flour beetle demonstrated that long dsRNA fragments are more effective than the siRNA products of Dicer activity (Wang et al. 2013). Another difference is that in our experiment we used adult workers, whereas in the previous experiment *Vg-like-A* was also downregulated in newly developed workers (Kohlmeier et al., 2018). Younger workers often specialize more on brood care within a colony and are therefore very sensitive to brood-care stimuli. Indeed, when comparing the rate of brood care activity in our experiment and in the one. This high sensitivity to brood stimuli is based on the high expression of *Vg-like-A* in young workers, reduced during maturation (Kohlmeier et al., 2018). At the same time, a number of workers and brood were removed during the creation of our artificial experimental colonies, which may have led to a change in DOL. Typically, the emergence of new young workers displaces the older ones to the periphery and thus takes over other tasks in the nest area or outside the nest (Mackay, 1983). In these outer areas of the nest, however, the intensity of brood care stimuli is even lower (Tofts, 1993), so that older workers switch back to brood care only under changing environmental conditions in order to ensure the survival of the entire colony (Jongepier & Foitzik, 2016). Removing brood and adult group members may have resulted in more workers being responsible for brood care, and the intensity of this stimulus may have been reduced in both control and RNAi-treated nurses. Finally, the amount, duration of exposure and technique of RNAi treatment are different. Studies involving dsRNA injection in other insect species have showed direct correlations between dose and the potency of RNAi treatment (e.g., Arakane et al. 2005; Boisson et al. 2006; Whyard et al. 2009). Whereas here we injected a single dose of 128 nl of dsRNA concentrated at 1 µg/µl, in Kohlmeier et al. (2018) the entire colony was fed with 15 µl of sucrose solution that included a dsiRNA at the same concentration per day. Therefore, unlike the previous dsiRNA feeding experiment, in our study the amount and time of RNAi input per individual were controlled. In conclusion, these variations between experiments could produce differences in *Vg-like-A* downregulation and thus in brood care behavior.

The switch in workers' task from nursing to foraging seems to depend on several genes, which are related to changes in maturation (Corona et al., 2013; Guidugli et al., 2005; Shapira et al., 2001), and may include various orthologs of Vg. In particular, a long-term knockdown of *Vg-like-A* (33 days of feeding) has been shown to cause young worker ants to decrease their investment in brood care, but to increase their caring for adult nestmates, a behavior intermediate to foraging activity (Kohlmeier et al. 2018). In this context, it is interesting that our results show that RNAi-treated nurses were more likely to be away from the brood pile on day 3 (but not on day 10). Therefore, a longer and more consistent downregulation over 33 days might have led to stronger changes in the *Vg-like-A* controlled network. In our experiment, the effect of the treatment might have worn off as early as 10 days after injection, leading to a return to the proximity of the brood in the nest. Unexpectedly, we found a positive correlation between *Vg-like-A* expression and brood care behavior in foragers, but not in nurses. This could be explained by the fact that despite a downregulation of *Vg-like-A* during the maturational switch from nursing to foraging behavior, *Vg-like-A* may still remain acting at a lower level in the sensitivity to brood-care stimuli. The interplay between Vg-like genes, JH, insulin signaling (IIS), and target of rapamycin (TOR) pathways according to age could play a role in task-associated behavioral changes and the regulation of odor perception levels (Caminer et al. 2023; Gadenne et al. 2016). In contrast, no correlation was detected in nurses, perhaps due to a higher variation in *Vg-like-A* expression and brood care stimulus that workers are exposed within the nest.

Vg-like genes have been identified in many social and solitary insects (Morandin et al., 2016), and also function in oxidative stress responses (Salmela et al., 2016). This could explain not only why ant queens show an upregulation of Vg and defensin after mating (Chérasse et al., 2019), but could also be responsible for the longevity of ant queens compared to workers (Corona et al., 2013). In general, the nurses injected with dsRNA showed an increase in *Vg-like-A* expression over the course of our experiment, but more so in the control group than in the treatment group (Fig. 3). This variation may reflect the response to oxidative stress and may be related to changes in the immune system as a result of the injection injury. *Vg-like-A* has been shown to be involved in stress resistance in honeybees (Salmela et al. 2016), indicating that this genes in involved in behavioral, immunity and resistance to oxidative stress pathways in ants. These results lead us to speculate that the sensitivity to the *Vg-like-A* RNAi treatment might be linked to other immunity pathways. However, the specific mechanism behind the role of the *Vg-like-A* gene in regulation both DOL and its relationship to the immune system need further investigation.

Most eukaryotic organisms, including insects, have a common machinery for sequence-specific post-transcriptional gene silencing, which is triggered by the presence of dsRNA and leads to the degradation of the mRNA in question (Fagard et al., 2000). Several RNAi techniques have been used to analyze gene function by a reduction of function approach in plants and animals (e.g., Varsha Wesley et al. 2001; Tabara et al. 1999; Aravin et al. 2001). By applying Vg-like-A dsRNA in *T. longispinosus* nurses, we detected a few changes in gene expression in the brain (3 genes) and antennae (1 gene), but only in interaction with the time of injection. Unfortunately, these genes were not well annotated in either identity or function at the time of this study. Thus, we could not find any link with the regulation of behavior or odorant perception. It is unclear why the downregulation of the *Vg-like-A* gene led to changes in behavior, albeit a weak one, but not to change in gene expression, especially in the fat body tissue into which it was injected. However, we also need to consider that the number of replicates in some tissues such as antennae was very low, which could affect our ability to detect DEGs. On the other hand, the efficiency of RNAi-mediated knockdown seems to depend in some cases on the identity and nature of the target gene. Possible explanations are that the dsRNA reagents or the resulting siRNA molecules are degraded in a sequence-specific manner, or that silencing specificity depends on the stability of the mRNA in question. Thus, genes with efficient regulatory feedback mechanisms could prevent depletion of mRNA levels with higher transcription rates (Bellés, 2010). The fast recovery of mRNA levels in the fat body after *Vg-like-A* dsRNA would reflect this possibility (Fig. 5A). Finally, the significant differences between *Vg-like-A* expression levels between qPCR and RNA-seq methods (Figs. 3 vs 5A) could be due to differences between both technologies, in which RNA-seq has a much broader dynamic range, making it a more sensitive method, but possibly more susceptible to within-group variation (Ozsolak & Milos, 2011).

Conclusions

For several decades, Vg has been extensively studied due to its variable copy number and diverse regulatory functions in insects. The exact mechanisms of how Vg influences ant behavior is still unclear. In contrast to honeybees, it does not seem to follow a strict pattern, and quite different behavioral changes can occur. The interaction between Vg genes, together with other regulatory mechanisms of endocrine systems such as JH, may be involved in fundamental behavioral changes in the evolution of DOL ants. We expect further studies to improve the efficiency of less invasive dsRNA applications by oral ingestion or dsRNA spraying methods to increase the feasibility of RNAi techniques. In addition, future comparative work in other ant species looking at the relationships between *Vg-like-A* altered behaviors and genetic patterns will provide a better understanding of the life history conditions that have determined the function of this orthologous gene in ants.

Supplementary material

Table S1. Summary of ant antenna, brain, and fat body transcriptome data sets and mapping results. Samples marked with * were excluded of the DEseq2 analysis.

Sample ID	Category	Day	Tissue	Raw reads (total)	Raw reads one strand	Trimmed	Trimmed retained %	Uniquely mapped reads number	Uniquely mapped reads %	Multimapping reads	Unmapped many mismatches %	Unmapped too short %	Unmapped other %
A_235_R	Control	3	Antennae	32,846,068	16,423,034	16,346,708	99.54	12,881,588	78.80	625,941	7.89	2.38	7.02
A_455_G*	Control	3	Antennae	21,999,850	10,999,925	10,916,908	99.25	6,296,851	57.68	345,968	3.20	3.38	32.50
A_577_B*	Control	3	Antennae	26,616,876	13,308,438	13,221,475	99.35	5,999,276	45.38	456,222	3.11	4.04	43.84
A_658_B*	Control	3	Antennae	26,454,632	13,227,316	13,189,466	99.71	5,795,264	43.94	217,181	5.90	2.21	46.27
A_842_G	Control	3	Antennae	27,124,052	13,562,026	13,497,036	99.52	10,555,768	78.21	675,604	4.62	2.26	9.84
A_844_B*	Control	3	Antennae	43,641,564	21,820,782	21,579,872	98.90	10,756,728	49.85	656,664	3.33	4.72	38.96
A_932_G*	Control	3	Antennae	39,551,510	19,775,755	19,590,535	99.06	8,257,144	42.15	307,156	4.81	5.27	46.17
A_188_B	RNAi	3	Antennae	34,332,034	17,166,017	17,116,468	99.71	13,375,167	78.14	658,510	6.66	2.39	8.92
A_307_S*	RNAi	3	Antennae	23,924,136	11,962,068	11,902,813	99.50	5,016,850	42.15	869,461	5.05	8.16	36.91
A_691_R	RNAi	3	Antennae	29,614,278	14,807,139	14,761,267	99.69	7,545,827	51.12	1,601,529	8.16	7.14	22.47
A_707_G	RNAi	3	Antennae	15,998,902	7,999,451	7,965,762	99.58	5,659,425	71.05	298,520	6.76	2.64	15.74
A_747_S	RNAi	3	Antennae	26,597,758	13,298,879	13,237,135	99.54	9,921,969	74.96	808,852	4.92	2.61	11.30
A_894_S	RNAi	3	Antennae	83,576,856	41,788,428	41,561,029	99.46	25,253,935	60.76	1,664,734	5.68	3.72	25.72
A_906_G*	RNAi	3	Antennae	27,325,248	13,662,624	13,528,855	99.02	3,325,263	24.58	171,349	2.26	6.46	65.40
A_122_S	Control	10	Antennae	27,304,742	13,652,371	13,588,409	99.53	10,751,882	79.13	808,177	4.10	2.13	8.52
A_166_B	Control	10	Antennae	22,562,994	11,281,497	11,185,758	99.15	7,607,901	68.01	500,002	4.77	3.85	18.75

Sample ID	Category	Day	Tissue	Raw reads (total)	Raw reads one strand	Trimmed	Trimmed retained %	Uniquely mapped reads number	Uniquely mapped reads %	Multimapping reads	Unmapped many mismatches %	Unmapped too short %	Unmapped other %
A_213_G	Control	10	Antennae	27,859,518	13,929,759	13,871,056	99.58	11,114,096	80.12	585,084	7.38	2.37	5.85
A_370_S	Control	10	Antennae	20,522,910	10,261,455	10,188,975	99.29	7,143,020	70.11	396,428	4.55	3.21	18.18
A_584_S	Control	10	Antennae	21,766,942	10,883,471	10,833,381	99.54	8,382,528	77.38	618,209	8.10	2.08	6.66
A_791_B	Control	10	Antennae	29,832,766	14,916,383	14,855,258	99.59	7,909,130	53.24	771,830	5.31	22.91	13.27
A_987_G*	Control	10	Antennae	37,292,770	18,646,385	18,489,725	99.16	8,324,248	45.02	427,436	3.57	6.39	42.65
A_268_B	RNAi	10	Antennae	33,330,222	16,665,111	16,567,939	99.42	9,558,320	57.69	1,422,693	4.66	3.79	24.79
A_276_G*	RNAi	10	Antennae	51,086,914	25,543,457	25,315,003	99.11	9,494,587	37.51	502,776	2.10	4.51	53.86
A_321_S*	RNAi	10	Antennae	21,071,204	10,535,602	10,479,796	99.47	4,720,621	45.04	298,715	4.86	3.48	43.69
A_480_B	RNAi	10	Antennae	31,315,036	15,657,518	15,612,614	99.71	10,889,508	69.75	820,016	6.38	3.48	15.08
A_510_B*	RNAi	10	Antennae	24,871,902	12,435,951	12,320,179	99.07	4,972,800	40.36	507,270	3.13	5.33	46.79
A_699_R*	RNAi	10	Antennae	41,522,190	20,761,095	20,476,841	98.63	6,688,455	32.66	450,527	2.29	6.84	55.95
A_858_B	RNAi	10	Antennae	38,678,694	19,339,347	19,238,181	99.48	14,340,543	74.54	763,880	7.91	2.69	10.82
B_235_R	Control	3	Brain	29,191,300	14,595,650	14,516,309	99.46	12,145,139	83.67	497,423	6.99	1.79	4.10
B_455_G	Control	3	Brain	31,884,146	15,942,073	15,840,271	99.36	12,336,475	77.88	704,725	4.27	2.10	11.25
B_577_B	Control	3	Brain	28,978,944	14,489,472	14,423,051	99.54	11,669,013	80.91	771,792	4.21	1.68	7.82
B_658_B	Control	3	Brain	28,823,104	14,411,552	14,339,097	99.50	9,376,864	65.39	344,349	7.85	1.89	22.43
B_842_G	Control	3	Brain	29,663,354	14,831,677	14,755,081	99.48	12,812,901	86.84	486,932	4.53	1.79	3.51
B_844_B	Control	3	Brain	29,813,966	14,906,983	14,825,003	99.45	12,765,825	86.11	658,395	4.38	1.79	3.24
B_932_G	Control	3	Brain	32,407,590	16,203,795	16,106,057	99.40	13,158,837	81.70	383,136	7.75	2.09	6.04
B_188_B	RNAi	3	Brain	26,769,452	13,384,726	13,308,159	99.43	11,289,860	84.83	609,735	4.77	1.84	3.94

Sample ID	Category	Day	Tissue	Raw reads (total)	Raw reads one strand	Trimmed	Trimmed retained %	Uniquely mapped reads number	Uniquely mapped reads %	Multimapping reads	Unmapped many mismatches %	Unmapped too short %	Unmapped other %
B_307_S	RNAi	3	Brain	35,939,598	17,969,799	17,879,678	99.50	13,905,493	77.77	628,495	6.03	1.79	10.85
B_691_R	RNAi	3	Brain	26,164,874	13,082,437	13,016,600	99.50	10,425,963	80.10	508,509	6.58	1.94	7.44
B_707_G	RNAi	3	Brain	33,624,338	16,812,169	16,718,238	99.44	13,813,889	82.63	662,175	7.64	1.94	3.81
B_747_S	RNAi	3	Brain	27,627,296	13,813,648	13,739,758	99.47	11,503,251	83.72	778,663	4.20	1.75	4.61
B_894_S	RNAi	3	Brain	27,954,522	13,977,261	13,906,508	99.49	11,641,371	83.71	483,321	6.45	1.88	4.44
B_906_G	RNAi	3	Brain	27,399,268	13,699,634	13,622,728	99.44	11,046,231	81.09	636,906	7.38	1.86	4.96
B_122_S	Control	10	Brain	29,683,806	14,841,903	14,755,076	99.41	12,344,217	83.66	683,577	4.77	1.90	5.01
B_166_B	Control	10	Brain	31,164,528	15,582,264	14,755,076	94.69	12,648,974	81.62	954,032	4.88	2.01	5.26
B_213_G	Control	10	Brain	26,999,616	13,499,808	13,419,633	99.41	11,197,909	83.44	504,683	6.88	1.84	4.05
B_370_S	Control	10	Brain	27,282,176	13,641,088	13,570,810	99.48	11,525,778	84.93	461,496	4.48	1.78	5.38
B_584_S	Control	10	Brain	28,253,134	14,126,567	14,054,753	99.49	11,206,334	79.73	642,672	8.37	1.82	5.47
B_791_B	Control	10	Brain	27,003,694	13,501,847	13,440,908	99.55	11,414,483	84.92	684,783	4.49	1.86	3.59
B_987_G	Control	10	Brain	31,269,760	15,634,880	15,550,988	99.46	12,531,918	80.59	563,568	6.45	1.73	7.57
B_268_B	RNAi	10	Brain	30,888,900	15,444,450	15,358,339	99.44	13,118,274	85.41	645,372	4.91	1.95	3.47
B_276_G	RNAi	10	Brain	28,894,580	14,447,290	14,366,978	99.44	11,749,409	81.78	648,978	3.91	1.68	8.05
B_321_S	RNAi	10	Brain	36,638,032	18,319,016	18,223,019	99.48	14,604,602	80.14	708,335	6.23	2.00	7.68
B_480_B	RNAi	10	Brain	30,621,558	15,310,779	15,232,901	99.49	12,946,021	84.99	604,169	4.46	1.80	4.74
B_510_B	RNAi	10	Brain	34,268,448	17,134,224	17,049,694	99.51	14,401,509	84.47	817,349	4.61	1.82	4.27
B_699_R	RNAi	10	Brain	25,910,918	12,955,459	12,895,386	99.54	10,769,607	83.52	573,217	4.77	1.90	5.32
B_858_B	RNAi	10	Brain	28,770,370	14,385,185	14,302,005	99.42	12,042,017	84.20	419,431	6.07	1.72	5.03

Sample ID	Category	Day	Tissue	Raw reads (total)	Raw reads one strand	Trimmed	Trimmed retained %	Uniquely mapped reads number	Uniquely mapped reads %	Multimapping reads	Unmapped many mismatches %	Unmapped too short %	Unmapped other %
F_455_G	Control	3	Fat body	44,521,760	22,260,880	22,147,243	99.49	17,605,969	79.50	1,188,006	4.77	2.09	8.22
F_658_B	Control	3	Fat body	34,924,984	17,462,492	17,415,340	99.73	4,100,335	23.54	149,373	2.09	0.96	72.54
F_842_G	Control	3	Fat body	27,259,724	13,629,862	13,548,906	99.41	10,352,021	76.40	680,015	4.12	2.06	12.35
F_844_B	Control	3	Fat body	38,970,178	19,485,089	19,391,045	99.52	15,908,802	82.04	1,497,494	4.31	1.75	4.14
F_932_G	Control	3	Fat body	28,269,556	14,134,778	14,033,494	99.28	7,647,617	54.50	213,794	7.01	2.48	34.47
F_188_B	RNAi	3	Fat body	29,750,558	14,875,279	14,796,068	99.47	11,100,595	75.02	953,818	3.87	2.04	12.57
F_307_S*	RNAi	3	Fat body	30,071,042	15,035,521	14,980,863	99.64	4,856,368	32.42	309,317	3.31	1.35	60.84
F_691_R	RNAi	3	Fat body	29,875,040	14,937,520	14,899,577	99.75	1,617,393	10.86	113,766	0.95	0.83	86.60
F_707_G	RNAi	3	Fat body	33,919,024	16,959,512	16,864,741	99.44	12,765,877	75.70	692,488	7.09	2.16	10.92
F_747_S	RNAi	3	Fat body	29,927,534	14,963,767	14,886,201	99.48	11,781,028	79.14	850,850	3.91	1.59	9.60
F_894_S	RNAi	3	Fat body	32,780,660	16,390,330	16,317,744	99.56	13,578,970	83.22	612,257	7.62	1.77	3.60
F_906_G	RNAi	3	Fat body	26,269,462	13,134,731	13,012,669	99.07	3,719,661	28.58	512,555	2.45	2.59	62.41
F_122_S	Control	10	Fat body	29,317,962	14,658,981	14,597,430	99.58	10,625,985	72.79	1,098,339	3.93	1.93	13.73
F_166_B	Control	10	Fat body	23,721,638	11,860,819	11,806,707	99.54	8,813,310	74.65	1,281,926	4.27	1.99	7.94
F_213_G	Control	10	Fat body	30,318,812	15,159,406	15,080,019	99.48	11,277,121	74.78	923,104	5.58	1.95	11.48
F_370_S	Control	10	Fat body	25,812,020	12,906,010	12,840,612	99.49	9,509,485	74.06	1,249,228	3.84	1.77	10.48
F_584_S	Control	10	Fat body	28,064,462	14,032,231	13,965,218	99.52	11,280,457	80.78	735,716	7.20	1.73	4.96
F_791_B	Control	10	Fat body	32,830,372	16,415,186	16,328,816	99.47	13,532,825	82.88	891,486	4.15	1.88	5.58
F_987_G*	Control	10	Fat body	25,090,836	12,545,418	12,450,715	99.25	5,657,745	45.44	358,040	3.27	2.42	45.97
F_268_B	RNAi	10	Fat body	26,533,030	13,266,515	13,184,673	99.38	8,735,054	66.25	748,995	3.70	1.85	22.43

Sample ID	Category	Day	Tissue	Raw reads (total)	Raw reads one strand	Trimmed	Trimmed retained %	Uniquely mapped reads number	Uniquely mapped reads %	Multimapping reads	Unmapped many mismatches %	Unmapped too short %	Unmapped other %
F_276_G	RNAi	10	Fat body	27,479,138	13,739,569	13,672,820	99.51	8,495,073	62.13	672,396	2.78	1.61	28.53
F_321_S*	RNAi	10	Fat body	31,330,220	15,665,110	15,626,871	99.76	2,331,968	14.92	207,232	1.78	1.70	80.26
F_480_B	RNAi	10	Fat body	28,088,046	14,044,023	13,967,898	99.46	10,806,394	77.37	929,874	4.68	2.03	9.18
F_510_B	RNAi	10	Fat body	28,742,854	14,371,427	14,306,886	99.55	11,755,279	82.17	933,650	4.10	1.71	5.45
F_699_R	RNAi	10	Fat body	28,404,560	14,202,280	14,131,119	99.50	11,338,018	80.23	855,561	4.06	1.79	7.76
F_858_B	RNAi	10	Fat body	23,440,978	11,720,489	11,657,935	99.47	9,130,382	78.32	564,610	6.28	2.05	8.39

Table S2. Character loadings, eigenvalues, and percentage of explained variance for behavior Principal Components (PC). The analysis was based on twelve behavior variables of *Vg-like-A* RNAi-treated and controls nurses. Bold figures indicate highest loadings.

Variable	**PCA behavior**				
	PC I	**PC II**	**PC III**	**PC IV**	**PC V**
Antennae Brood	**0.66**	-0.48	0.03	0.23	-0.06
Grooming Brood	**0.66**	-0.35	0.15	0.09	0.02
Feeding Brood	-0.04	0.05	**0.74**	0.07	0.04
Carrying Brood	0.02	**-0.53**	-0.09	0.37	0.28
Antennae Nestmate	-0.2	0.43	-0.1	0.08	**0.55**
Grooming Nestmate	-0.18	0.16	-0.31	0.6	0.38
Feeding Nestmate	-0.06	0.49	0.3	0.27	-0.29
Carrying Nestmate	0.00	-0.00	0.00	0.00	-0.00
Resting	0.59	**0.61**	-0.22	-0.32	0.02
Walking	**-0.78**	-0.49	-0.06	-0.3	-0.1
Grooming self	-0.19	0.19	-0.18	**0.56**	**-0.61**
Being fed/groomed	-0.13	0.09	**0.72**	0.12	0.21
Eigenvalue	1.96	1.74	1.37	1.17	1.04
%	17.79	15.84	12.45	10.6	9.45

Table S3. Character loadings, eigenvalues, and percentage of explained variance for location Principal Components (PC). The analysis was based on eight location variables of *Vg-like-A* RNAi-treated and controls nurses. Bold figures indicate highest loadings.

Variable	**PCA location**			
	PC I	**PC II**	**PC III**	**PC IV**
On the brood	**-0.71**	0.34	0.2	-0.24
Near the brood	**-0.68**	0.16	-0.17	0.21
Surroundings the nest	**0.67**	**-0.58**	-0.43	-0.04
Near entrance of the nest	0.49	-0.07	**0.65**	-0.2
Entrance	0.33	0.13	**0.71**	0.31
Chamber 1	0.18	0.23	-0.12	**0.87**
Chamber 2	0.55	**0.68**	-0.22	-0.09
Chamber 3	0.44	**0.73**	-0.22	-0.24
Eigenvalue	2.28	1.54	1.3	2.6
%	28.44	19.31	16.26	13.26

Chapter 3

Development of a histone acetylation protocol to study the regulation of division of labor in the ant *Temnothorax longispinosus*

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Abstract

The success of social insects is based on their division of labor. A defining trait of their colonies is the presence of different castes (e.g., workers / soldiers / queens), which are usually not genetically determined, but phenotypically plastic in development. Thus, the same genotype can give rise to very different phenotypes and epigenetic regulation of gene expression plays an important role in this process. In this study, we developed a method to investigate differences in histone acetylation between nurses and foragers of *Temnothorax longispinosus* ants. We propose a number of adaptations to the ChIP-seq protocol to make this technique applicable to ants. Although our analyses were not ultimately conclusive, we discuss important experimental design issues, including sample quality, controls, antibody validation, sonication, experimental replication, depth of sequencing, number of replicates and assessment of data quality, that may have influenced our results. This may be helpful for optimizing future protocols.

Introduction

Epigenetics is defined as the inheritance of biological variation independent of changes in the DNA sequence, and its study has focused on the mechanisms by which multiple phenotypes arise from a single genotype. In recent years, epigenetic processes such as DNA methylation, different histone modifications, non-coding RNAs and their role in developmental and behavioral plasticity have been studied extensively in social insects (Ashby et al. 2016; Lyko & Maleszka 2011; Simola et al. 2013a; Yan et al. 2015). Social insects, such as ants, termites, some of the bees and wasps offer a natural experimental system to investigate the molecular bases of epigenetic processes that influence the whole organism. In ants for example, morphologically and behaviorally distinct types of individuals arise from a single genome, carry out different tasks and relate to other individuals according to their social status to benefit the colony as a whole (Hölldobler & Wilson, 1990). The mechanisms underlying the caste differences of different social insects are diverse. In some species, such as the honeybee, it is environmental factors such as diet (Shi et al., 2011; Slater et al., 2020), while in others genetic factors influence caste determination (Libbrecht & Keller, 2013). Many social insect species probably lie somewhere between these two extremes (Matsuura et al., 2018).

One of the most striking adaptations in ants is division of labor (DOL), where reproduction is monopolized by a single or a few queen(s) and all the remaining colony tasks are taken over by workers specialized on caring for the queen, eggs, larvae and pupae, digging tunnels, expanding the nest, and collecting food and materials to protect the colony from predators and parasites. Workers also show age polyethism, focusing early in life on internal chores and switching later to extrinsic tasks such as nest defense and foraging (Hölldobler & Wilson, 1990). Because of this extreme phenotypic and behavioral plasticity, ants have emerged as models for the investigation of complex social behavior and for the study of epigenetic mechanisms that control gene activity leading to distinct adaptive phenotypes (Smith et al. 2008; Yan et al. 2014).

Many lines of evidence indicate that epigenetic pathways are regulating the characteristic shifts in behavior in ants. Castes of the ant species *Harpegnathos saltator* and *Camponotus floridanus* vary in DNA methylation (Bonasio et al., 2012) and non-coding RNA (Shields et al., 2018). Pharmacological and molecular manipulation of histone acetylation induce soldiers to behave as foragers in *C. floridanus* (Simola et al. 2016) and produce a change of the circadian rhythmicity in *Temnothorax longispinosus* (Kohlmeier et al. 2023; Libbrecht et al. 2020), suggesting a direct role of histone modifications in the social behavior. While research specifically on histone modifications in ants is limited, studies on other social insects, such as honey bees, have indicated their involvement in development, lifespan, caste determination and behavioral plasticity (Jin et al. 2023; Paoli et al. 2014; Spannhoff et al. 2011; Wojciechowski et al. 2018; Zhang et al. 2023).

Histone modifications are regulatory mechanisms that influence gene expression by altering the accessibility of DNA to the cellular machinery responsible for transcription. The histone tails undergo posttranslational modifications including acetylation, phosphorylation, methylation, and ubiquitination, that play important roles in the organization of euchromatin and heterochromatin (Kouzarides, 2007). One major modification is the modulation of the positive charge density of core histones by the addition or removal of acetyl groups (Mukherjee et al. 2015). Lysine acetylation is a reversible posttranscriptional modification of proteins and plays a key role in the organization of multiprotein complexes involved in gene regulation in many cellular and developmental processes (Choudhary et al., 2009).The level of lysine-27 acetylation of histone H3 (H3K27ac) was demonstrated to be a powerful predictor for social behavior and caste differentiation in social insects (Jin et al. 2023; Simola et al. 2013a, 2016; Wang et al. 2020; Wojciechowski et al. 2018). Overall, these studies suggest that H3K27ac may be an important regulator of gene expression and behavior in ants, and further research is needed to fully understand its role in DOL.

In the ant *T. longispinosus*, variation in behavior among monomorphic workers is related to high colony productivity (Modlmeier et al., 2012). In this species, task specialization is usually neither genetically fixed nor rigid, but changes with age and in response to the needs of the colony. In fact, gene expression depends less on age than on the task in which the ant is specialized (Kohlmeier et al., 2019). A recent study shows that inhibiting p300/CBP histone acetyltransferases (HATs) and manipulating colony composition so that brood carers are needed impairs the ability of older workers to return to brood care. The reverse manipulation, in which foragers were removed, showed that inhibition of 300/CBP histone acetyltransferases increased the ability of young workers to accelerate their behavioral development and switch to foraging (Kohlmeier et al., 2023). These results suggest that HAT in combination with social signals indicating task demands, play an important role in modulating behavior. In this study, we investigated the differences in histone acetylation (H3K27ac) patterns in the brains of nurses and foragers of the ant *T. longispinosus* to understand their role in task allocation among workers. To do this, we performed a chromatin immunoprecipitation protocol followed by sequencing (ChIP-seq) analysis of nurse and forager brains. We expect to find differentially enrichment regions (DERs) between behavioral phenotypes, and whether they are correlated with the differentially expressed genes identified by previously RNA-seq analysis (Caminer et al., 2023). We describe the protocol and sample preparation for a ChIP-seq analysis and provide an overview of the most important pitfalls of experimental design, molecular work in the laboratory and bioinformatic analysis.

Materials and Methods

Chromatin preparation

To determine the importance of histone acetylation in task allocation in *T. longispinosus* ant workers, we developed a ChIP-seq protocol. We used 9 of the 20 µl of the same seven nurse and seven forager pooled brain samples used for a RNA-seq analysis (see Caminer et al. 2023), added to a final concentration of 100 µl PBS. Each sample consist of pooled tissue from seven workers of a single colony. Chromatin was cross-linked in a fixation buffer (50 mM HEPES at pH 8, 1 mM EDTA at pH 8, 0.5 mM EGTA at pH 8, 100 mM NaCl, 11% formaldehyde) for 10 min at room temperature (RT) with rotation. Reactions were quenched using 250 mM glycine for 10 min at 4°C rotation. Afterwards, fixed brains were washed with 200 µl PBS, centrifuged (1500g/ 10 min at 4°C) and supernatant was discarded. Pellets were suspended in 300 µl lysis buffer 1 (10% glycerol, 50 mM HEPES at pH 7.5, 140 mM NaCl, 0.5% NP-40, 0.25% Triton X-100, 1 mM EDTA at pH 8) and incubated for 10 min on a rotator mixer at 4°C. Lysates were then centrifuged (1500g/ 10 min at 4°C), and pellets were suspended in 300 µl lysis buffer 2 (10 mM Tris-HCl at pH 8, 200 mM NaCl, 1 mM EDTA at pH 8, 0.5 mM EGTA at pH 8) and incubated for another 10 min on a rotator mixer at RT. Lysates were centrifuged (1500g/ 10 min at 4°C), pellets were suspended in 145 µl sonication buffer 3 (10 mM Tris at pH 8, 100 mM NaCl, 1 mM EDTA at pH 8, 0.5 mM EGTA at pH 8, 0.1% sodium deoxycholate, 0.17 mM N-Lauryl sarcosine) and incubated overnight on a rotator mixer at 4°C. Buffers 1 and 3 of these steps contained complete protease inhibitor cocktail (Roche). Chromatin was sonicated into small fragments with a Covaris S220 sonicator in a 130 µL micro tube for 15 min (duty factor: 2%; intensity: 3, cycles/burst: 200). Lysates were cleared by centrifugation (20,000g/2 min at 4°C), and sonication and was checked with the Agilent 2100 Bioanalyzer.

Chromatin immunoprecipitation (IP) and ChIP-seq library preparation

Chromatin concentration was measured with dsDNA HS Assay Kit with Qubit 2.0. Ninety microliters chromatin aliquots were diluted in 510 µl ChIP dilution buffer 3 (10 mM Tris at pH 8, 100 mM NaCl, 1 mM EDTA at pH 8, 0.5 mM EGTA at pH 8, 0.1% sodium deoxycholate, 0.17 mM N-Lauryl sarcosine), the remaining sample (15 µl) was diluted in 85 µl TE buffer (10 mM Tris-HCl at pH 8, 1 mM EDTA at pH 8) and used as Input control. Four microliters of antibody H3K27ac (Abcam, ab4729) was added to the ChIP samples according to the manufacturers' instructions, and samples were incubated overnight on a rotator mixer at 4°C. We added 10 µl of magnetic protein A and G Dynabeads (Invitrogen) of each reaction, and samples were incubated for 3 h on a rotator mixer at 4°C. Beads were washed once with 200 µl buffer 3, once with 200 µl TE buffer, followed by two elutions into 300 µL of elution buffer (1% SDS, 100 mM $NaHCO_3$) on a rotator mixer for 20 min at RT. Two micrograms of RNase A were added to the chromatin immunoprecipitation (IP) and Input samples, and incubated for 30 min at 37°C. Afterward, samples were incubated for 3 h with 200 µg of proteinase K at 55°C, and then overnight at 65°C. DNA was extracted and purified with Phenol/Chloroform protocol followed by ethanol precipitation. Pelleted DNA was resuspended in 15 µL TE. Chromatin DNA concentration was measured before and after the immunoprecipitation and DNA precipitation process, obtaining a recovery of 50% in Input and 3% in IP samples. Brain DNA Input/IP samples contained ~1.3 and ~0.22 ng/µl, respectively.

ChIP-seq libraries were prepared using the Ovation ultralow system v2 DNA Library Prep Kit for Illumina following the manufacturer’s instructions. Libraries were amplified by PCR for 16 cycles, purified using Agencourt Ampure XP beads, and quantified by Qubit 2.0 and Agilent 2100 Bioanalyzer. Twenty-eight libraries were pooled and sequenced by Beijing Genomics Institute (BGI), Pekin, China on the Illumina HiSeq Xten platform with 150 bp paired-end. Three flow cell lanes were used to generate around 74 M raw reads.

ChIP-seq analysis

Raw reads obtained from the BGI were checked using FastQC v.0.11.9 (Andrews, 2016), and Illumina adapters were removed using Trimmomatic v.0.36 (Bolger et al., 2014). Sequencing reads from multiple lanes belonging to the same sample were merged and aligned to the reference *T. longispinosus* genome (Jongepier et al., 2022) with Bowtie2 v.2.4.2 (Langmead & Salzberg, 2012). SAMtools v.1.9 (Li et al., 2009) was used to convert SAM files to the binary BAM format, and then sort, remove duplicates and unmapped reads. Detailed mapping statistics for each sample is available in Table 1. PCA plots, correlation heatmap between samples, and fingerprint plots, which show ChIP enrichment compared to background, were performed using Deeptools v.3.4.1 (Ramírez et al., 2014) to investigate differences in enrichment levels between samples. After mapping reads to the genome and filtering steps, two different peak calling approaches were used with MACS2 v.2.2.7.1 (Feng et al., 2012) for identify regions of enrichment. First, Input and IP files for each sample were linked for individual peak calling (“single peaks”). Second, all Input files and IP files were pooled together to call peaks (“pooled peaks”). Overlapping peaks from the “single peaks” analysis were subsequently merged by bedtools v.2.26.0 (Quinlan & Hall, 2010) using the default intersect intervals functions with at least 1 bp overlap, to produce the final consensus peak list. Numbers of reads per peak were counted using featureCounts program from the Subread package (v.1.6.3) (Liao et al. 2014). The sample quality was determined through visual assessment using the IGV browser (Thorvaldsdóttir et al., 2013) showing enrichment signals in the IP compared with the Input. A third alternative analysis was implemented in which we defined promoter regions (as the region spanning 2 kb upstream and 5.0 kb downstream of the transcription start site of a gene) from the gff file and used individual BAM files in DiffBind instead of peak files (“enrichment promoters”). DERs between nurses and foragers were called using DiffBind (Stark & Brown, 2011) with the design model ~Colony+Task and an adjusted *p*-value <0.05 for all the analyses.

Table 1. NGS read map statistics for ChIP-seq brain samples. Comprehensive statistics for the 28 ChIP-seq read maps analyzed in our study.

Sample ID	Colony ID	Task	Input/IP	Raw reads (total)	Clean reads one strand	Mapped %	Uniquely mapped reads number	Uniquely mapped reads %	Peak calling narrow
BBN	NY18 J239	Nurse	Input	78,887,104	39,443,552	94.75	26,110,744	79.82	6,141
			IP	64,436,968	32,218,484	89.04	21,135,170	74.3	
BBF	NY18 J239	Forager	Input	69,542,240	34,771,120	90.21	21,848,397	70.58	2,205
			IP	81,586,784	40,793,392	74.46	21,627,846	55.08	
BGN	NY18 I134	Nurse	Input	75,702,960	37,851,480	89.81	23,075,867	67.66	896
			IP	83,472,302	41,736,151	44.97	13,214,539	31.47	
BGF	NY18 I134	Forager	Input	92,229,502	46,114,751	90.97	29,043,269	70.96	1,482
			IP	87,406,618	43,703,309	67.82	21,340,521	49.75	
GGN	NY18 J278	Nurse	Input	80,172,202	40,086,101	94.92	26,334,052	79.61	16,658
			IP	85,682,890	42,841,445	86.41	28,370,083	72.14	
GGF	NY18 J278	Forager	Input	74,881,892	37,440,946	94.7	24,342,007	76.36	5,498
			IP	73,316,948	36,658,474	71.88	20,164,070	56.51	
GON	NY18 E110	Nurse	Input	71,830,854	35,915,427	93.45	23,673,687	77.36	1,271
			IP	61,322,806	30,661,403	54.1	12,047,854	39.37	
GOF	NY18 E110	Forager	Input	78,960,542	35,915,427	93.45	23,673,687	77.36	2,157
			IP	66,659,760	33,329,880	92.19	22,408,629	76.17	
OON	NY18 J282	Nurse	Input	64,487,542	32,243,771	87.96	16,532,071	54.81	1,907
			IP	70,211,448	35,105,724	32.31	8,329,529	23.46	
OOF	NY18 J282	Forager	Input	71,183,048	35,591,524	93.93	23,283,993	75.76	2,318
			IP	74,969,494	37,484,747	67.65	18,643,010	50.53	
YBN	NY18 J350	Nurse	Input	74,560,672	37,280,336	93.61	24,515,207	77.21	2,252

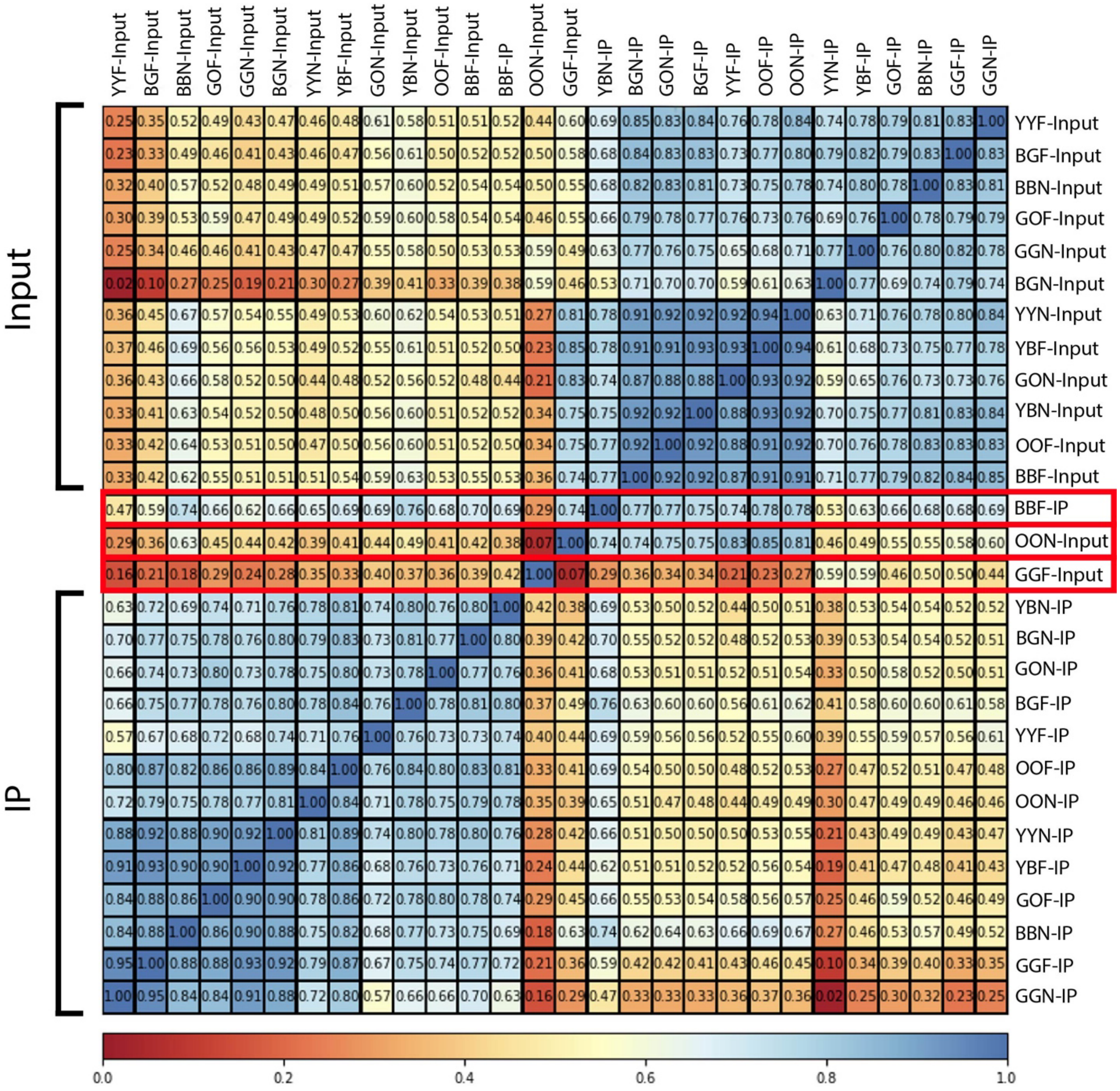
			IP	76,048,080	38,024,040	78.56	21,458,287	58.94	
YBF	NY18 J350	Forager	Input	82,769,686	41,384,843	94.93	27,793,526	81.45	2,654
			IP	68,552,982	34,276,491	89.39	22,507,941	72.93	
YYN	NY18 J350	Nurse	Input	84,007,442	42,003,721	95	28,132,252	81.07	2,346
			IP	65,327,292	32,663,646	86.19	20,807,008	68.93	
YYF	NY18 J35	Forager	Input	71,722,762	35,861,381	94.22	23,499,318	77.19	555
			IP	65,014,672	32,507,336	50.36	11,641,728	35.77	

Results

ChIP was performed for the well-characterized active H3K27ac histone mark that have been linked to task and caste determination in social insects (Kohlmeier et al. 2023; Simola et al. 2013a, 2016; Wojciechowski et al. 2018). We examined changes of H3K27ac levels in nurse and forager brains of *T. longispinosus* ants. The ChIP-seq produced an average of 74 million reads per sample (range from 61 to 92 million), which surpassed the recommended guidelines for ChIP-seq quality (Sims et al., 2014), and an average alignment rate of 82% to the genome (range from 32 to 95%) (Table 1). The read coverage similarity between 28 samples was represented as a heatmap with a hierarchical clustering. Consistently, we observed a high correlation between the IP samples and the same between the Input samples. The analysis produced two distinct clusters: samples with the active H3K27ac histone mark (IP) and controls (Input) (Fig. 1). However, three samples were observed with different patterns from those two groups ("BBF IP", "GGF Input", and "OON Input").

Overall, our "single peak" analysis produced a large variation of peak numbers across samples (from 555 to 16,658 peaks) with only one sample exhibiting more than 10,000 peaks. To determine enrichment of the H3K27ac mask, we measured the signal-to-noise ratio used to compare the level of a desired signal to the level of background noise. For this, we compared coverage inside and outside the so-called peak regions. The results showed peaks with less coverage than the background noise on average (Fig. 2), which indicate low signal and a high background noise across samples. In addition, we visualized using the IGV browser whether IP peaks were different from Input peaks and showed highly enriched signals. IGV visual exploration revealed that there was high variance within regions in scaffolds related to the level of background noise. These regions mostly showed peaks in the IP data, which points to artifacts (Fig. 3).

Due to low number of peaks obtained, we merge BAM files from all the samples prior to peak calling to improve the sensitivity of the peak calling by increasing the depth of read coverage. This “pooled peaks” analysis produced 22,374 peaks. Finally, our alternative “enrichment promoters” analysis produced 13,028 enrichment promoter regions that were defined from the gff file. Although there was no clear sign of region enrichments, we proceed running DiffBind analyses to identify differentially bound regions. We used the single calling peaks, pooled peaks and enrichment promotors regions to investigate differences in H3K27ac mark between behavior phenotypes. PCAs analyses showed high overlapping between nurses and foragers (Figs. 4A-C), while our differential biding analyses did not yield any DERs (FDR < 0.05, Diffbind).



	YYF-Input	BGF-Input	BBN-Input	GOF-Input	GGN-Input	BGN-Input	YYN-Input	YBF-Input	GON-Input	YBN-Input	OOF-Input	BBF-Input	BBF-IP	OON-Input	GGF-Input	YBN-IP	BGN-IP	GON-IP	BGF-IP	YYF-IP	OOF-IP	OON-IP	YYN-IP	YBF-IP	GOF-IP	BBN-IP	GGF-IP	GGN-IP
YYF-Input	0.25	0.35	0.52	0.49	0.43	0.47	0.46	0.48	0.61	0.58	0.51	0.51	0.52	0.44	0.60	0.69	0.85	0.83	0.84	0.76	0.78	0.84	0.74	0.78	0.79	0.81	0.83	1.00
BGF-Input	0.23	0.33	0.49	0.46	0.41	0.43	0.46	0.47	0.56	0.61	0.50	0.52	0.52	0.50	0.58	0.68	0.84	0.83	0.83	0.73	0.77	0.80	0.79	0.82	0.79	0.83	1.00	0.83
BBN-Input	0.32	0.40	0.57	0.52	0.48	0.49	0.49	0.51	0.57	0.60	0.52	0.54	0.54	0.50	0.55	0.68	0.82	0.83	0.81	0.73	0.75	0.78	0.74	0.80	0.78	1.00	0.83	0.81
GOF-Input	0.30	0.39	0.53	0.59	0.47	0.49	0.49	0.52	0.59	0.60	0.58	0.54	0.54	0.46	0.55	0.66	0.79	0.78	0.77	0.76	0.73	0.76	0.69	0.76	1.00	0.78	0.79	0.79
GGN-Input	0.25	0.34	0.46	0.46	0.41	0.43	0.47	0.47	0.55	0.58	0.50	0.53	0.53	0.59	0.49	0.63	0.77	0.76	0.75	0.65	0.68	0.71	0.77	1.00	0.76	0.80	0.82	0.78
BGN-Input	0.02	0.10	0.27	0.25	0.19	0.21	0.30	0.27	0.39	0.41	0.33	0.39	0.38	0.59	0.46	0.53	0.71	0.70	0.70	0.59	0.61	0.63	1.00	0.77	0.69	0.74	0.79	0.74
YYN-Input	0.36	0.45	0.67	0.57	0.54	0.55	0.49	0.53	0.60	0.62	0.54	0.53	0.51	0.27	0.81	0.78	0.91	0.92	0.92	0.92	0.94	1.00	0.63	0.71	0.76	0.78	0.80	0.84
YBF-Input	0.37	0.46	0.69	0.56	0.56	0.53	0.49	0.52	0.55	0.61	0.51	0.52	0.50	0.23	0.85	0.78	0.91	0.91	0.93	0.93	1.00	0.94	0.61	0.68	0.73	0.75	0.77	0.78
GON-Input	0.36	0.43	0.66	0.58	0.52	0.50	0.44	0.48	0.52	0.56	0.52	0.48	0.44	0.21	0.83	0.74	0.87	0.88	0.88	1.00	0.93	0.92	0.59	0.65	0.76	0.73	0.73	0.76
YBN-Input	0.33	0.41	0.63	0.54	0.52	0.50	0.48	0.50	0.56	0.60	0.51	0.52	0.52	0.34	0.75	0.75	0.92	0.92	1.00	0.88	0.93	0.92	0.70	0.75	0.77	0.81	0.83	0.84
OOF-Input	0.33	0.42	0.64	0.53	0.51	0.50	0.47	0.50	0.56	0.60	0.51	0.52	0.50	0.34	0.75	0.77	0.92	1.00	0.92	0.88	0.91	0.92	0.70	0.76	0.78	0.83	0.83	0.83
BBF-Input	0.33	0.42	0.62	0.55	0.51	0.51	0.51	0.54	0.59	0.63	0.53	0.55	0.53	0.36	0.74	0.77	1.00	0.92	0.92	0.87	0.91	0.91	0.71	0.77	0.79	0.82	0.84	0.85
BBF-IP	0.47	0.59	0.74	0.66	0.62	0.66	0.65	0.69	0.69	0.76	0.68	0.70	0.69	0.29	0.74	1.00	0.77	0.77	0.75	0.74	0.78	0.78	0.53	0.63	0.66	0.68	0.68	0.69
OON-Input	0.29	0.36	0.63	0.45	0.44	0.42	0.39	0.41	0.44	0.49	0.41	0.42	0.38	0.07	1.00	0.74	0.74	0.75	0.75	0.83	0.85	0.81	0.46	0.49	0.55	0.55	0.58	0.60
GGF-Input	0.16	0.21	0.18	0.29	0.24	0.28	0.35	0.33	0.40	0.37	0.36	0.39	0.42	1.00	0.07	0.29	0.36	0.34	0.34	0.21	0.23	0.27	0.59	0.59	0.46	0.50	0.50	0.44
YBN-IP	0.63	0.72	0.69	0.74	0.71	0.76	0.78	0.81	0.74	0.80	0.76	0.80	1.00	0.42	0.38	0.69	0.53	0.50	0.52	0.44	0.50	0.51	0.38	0.53	0.54	0.54	0.52	0.52
BGN-IP	0.70	0.77	0.75	0.78	0.76	0.80	0.79	0.83	0.73	0.81	0.77	1.00	0.80	0.39	0.42	0.70	0.55	0.52	0.52	0.48	0.52	0.53	0.39	0.53	0.54	0.54	0.52	0.51
GON-IP	0.66	0.74	0.73	0.80	0.73	0.78	0.75	0.80	0.73	0.78	1.00	0.77	0.76	0.36	0.41	0.68	0.53	0.51	0.51	0.52	0.51	0.54	0.33	0.50	0.58	0.52	0.50	0.51
BGF-IP	0.66	0.75	0.77	0.78	0.76	0.80	0.78	0.84	0.76	1.00	0.78	0.81	0.80	0.37	0.49	0.76	0.63	0.60	0.60	0.56	0.61	0.62	0.41	0.58	0.60	0.60	0.61	0.58
YYF-IP	0.57	0.67	0.68	0.72	0.68	0.74	0.71	0.76	1.00	0.76	0.73	0.73	0.74	0.40	0.44	0.69	0.59	0.56	0.56	0.52	0.55	0.60	0.39	0.55	0.59	0.57	0.56	0.61
OOF-IP	0.80	0.87	0.82	0.86	0.86	0.89	0.84	1.00	0.76	0.84	0.80	0.83	0.81	0.33	0.41	0.69	0.54	0.50	0.50	0.48	0.52	0.53	0.27	0.47	0.52	0.51	0.47	0.48
OON-IP	0.72	0.79	0.75	0.78	0.77	0.81	1.00	0.84	0.71	0.78	0.75	0.79	0.78	0.35	0.39	0.65	0.51	0.47	0.48	0.44	0.49	0.49	0.30	0.47	0.49	0.49	0.46	0.46
YYN-IP	0.88	0.92	0.88	0.90	0.92	1.00	0.81	0.89	0.74	0.80	0.78	0.80	0.76	0.28	0.42	0.66	0.51	0.50	0.50	0.50	0.53	0.55	0.21	0.43	0.49	0.49	0.43	0.47
YBF-IP	0.91	0.93	0.90	0.90	1.00	0.92	0.77	0.86	0.68	0.76	0.73	0.76	0.71	0.24	0.44	0.62	0.51	0.51	0.52	0.52	0.56	0.54	0.19	0.41	0.47	0.48	0.41	0.43
GOF-IP	0.84	0.88	0.86	1.00	0.90	0.90	0.78	0.86	0.72	0.78	0.80	0.78	0.74	0.29	0.45	0.66	0.55	0.53	0.54	0.58	0.56	0.57	0.25	0.46	0.59	0.52	0.46	0.49
BBN-IP	0.84	0.88	1.00	0.86	0.90	0.88	0.75	0.82	0.68	0.77	0.73	0.75	0.69	0.18	0.63	0.74	0.62	0.64	0.63	0.66	0.69	0.67	0.27	0.46	0.53	0.57	0.49	0.52
GGF-IP	0.95	1.00	0.88	0.88	0.93	0.92	0.79	0.87	0.67	0.75	0.74	0.77	0.72	0.21	0.36	0.59	0.42	0.42	0.41	0.43	0.46	0.45	0.10	0.34	0.39	0.40	0.33	0.35
GGN-IP	1.00	0.95	0.84	0.84	0.91	0.88	0.72	0.80	0.57	0.66	0.66	0.70	0.63	0.16	0.29	0.47	0.33	0.33	0.33	0.36	0.37	0.36	0.02	0.25	0.30	0.32	0.23	0.25

Figure 1. Heatmap that shows the correlation read counts between 28 ChIP-seq samples. Note that samples highlighted in red are not clustered in their respective IP and Input groups.

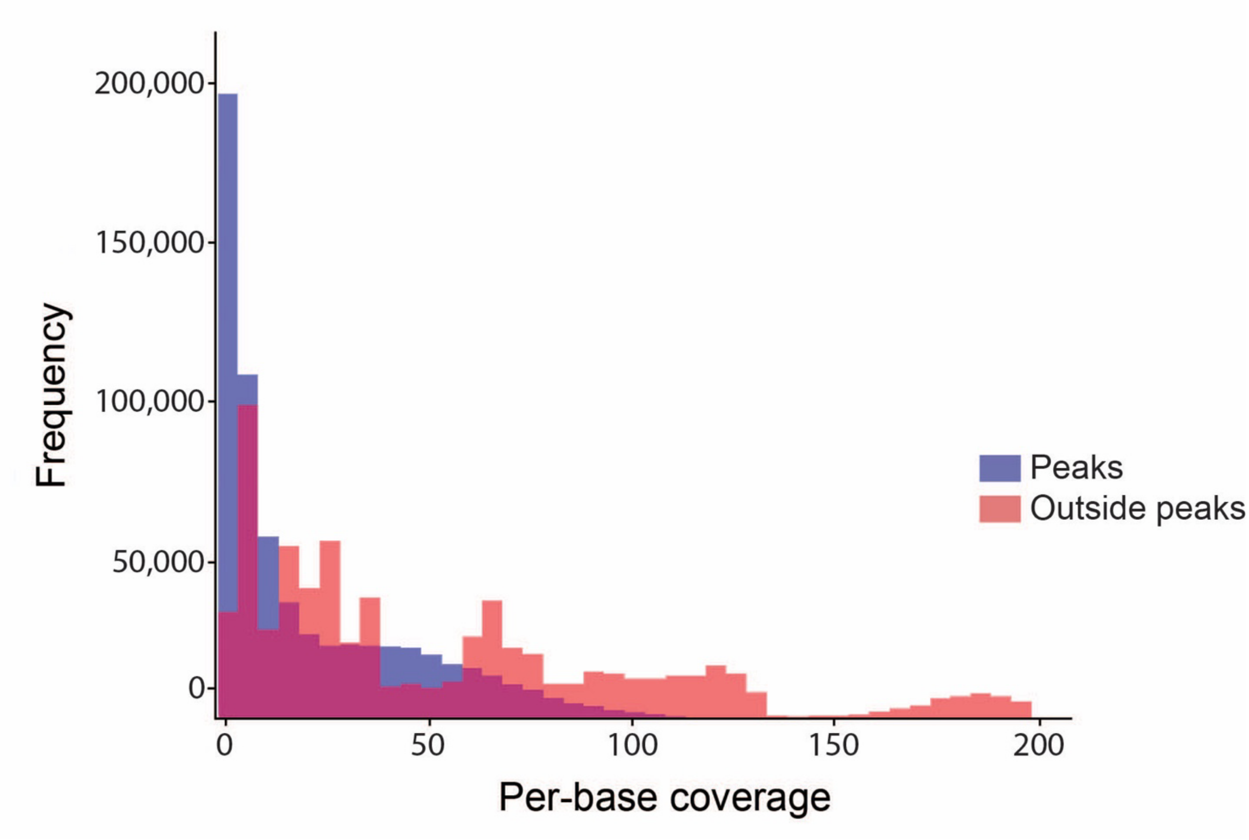


Figure 2. Distribution comparison of per-base coverage between peaks and outside of peaks. Notice that the peaks (blue) have no higher coverage than background noise (red) on average.

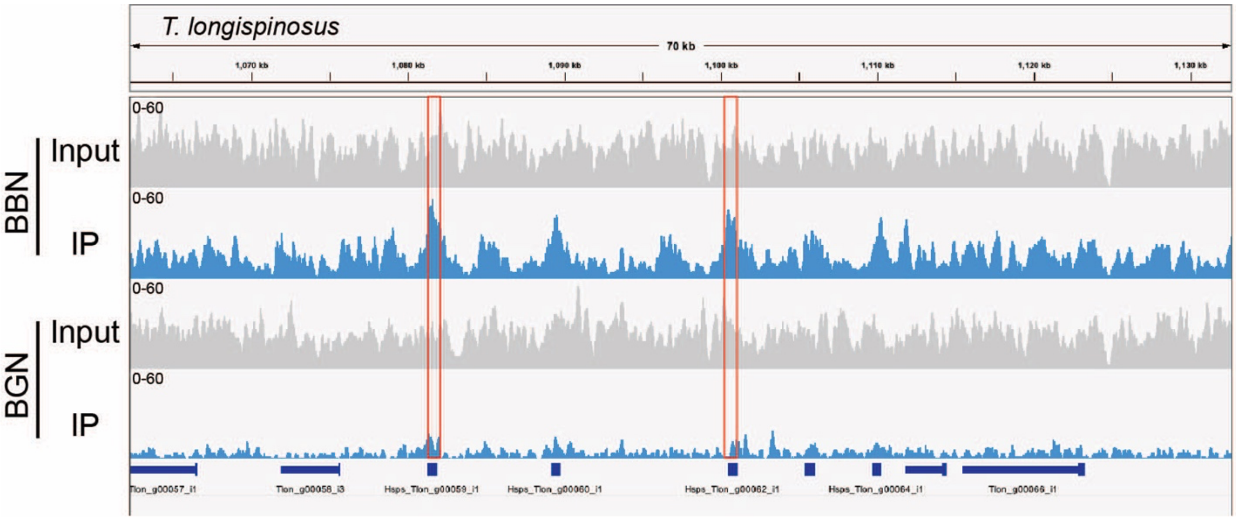


Figure 3. Genome browser plot illustrates ChIP-seq peaks of two samples. Each sample is displayed on scaffold 1 using both Input (grey) and IP (blue) data for quality control purposes. Calling peaks are highlighted with red.

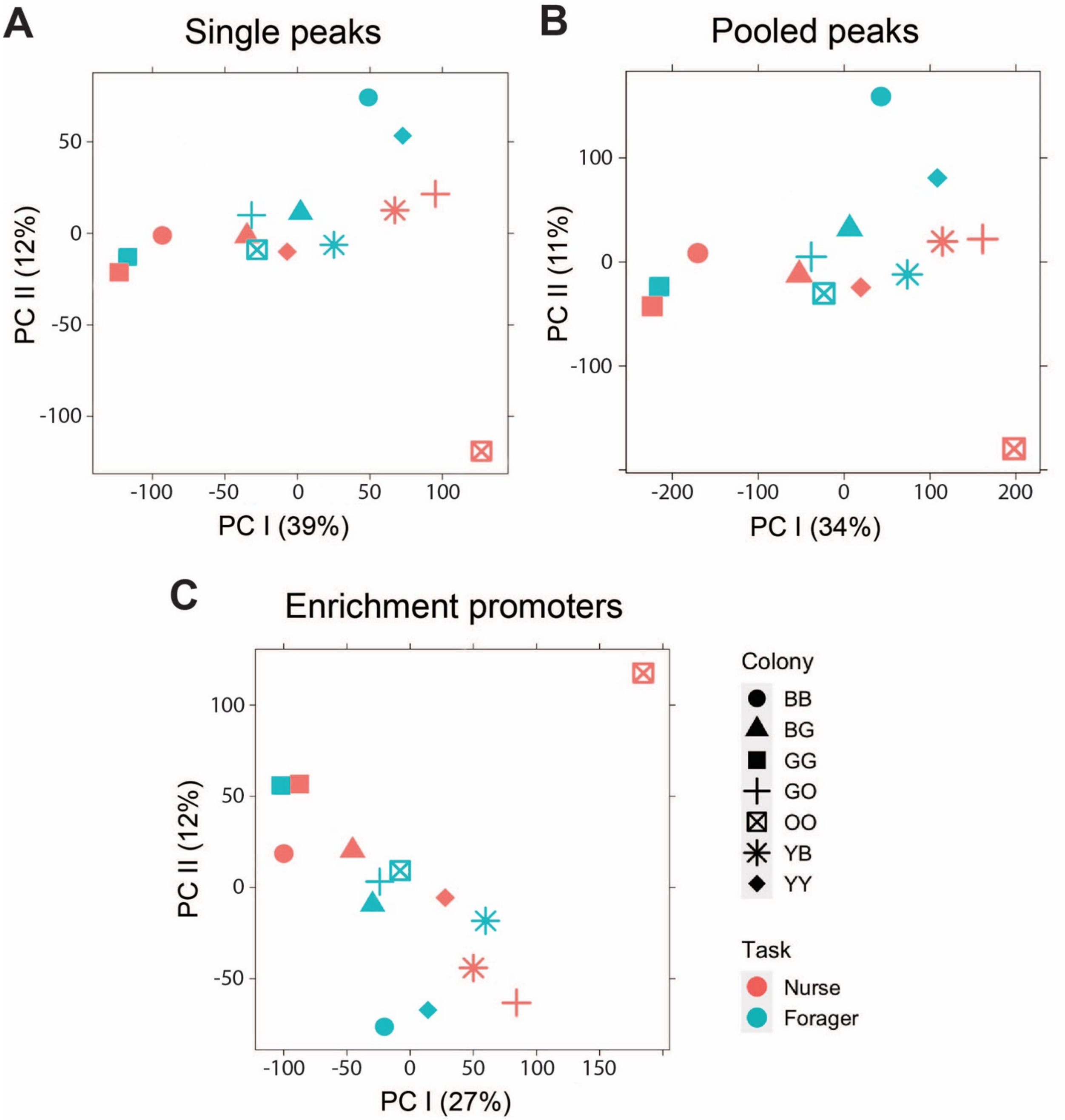


Figure 4. Principal component analysis (PCA) of H3K27ac mark between nurses and foragers. Data was generated based on (**A**) calling peaks for each individual sample, (**B**) pooled calling peaks of all the samples, and (**C**) enrichment promoters regions produced from the gff file.

Discussion

The identification and characterization of the genome-wide locations of transcription factors, chromatin-modifying enzymes, and histone modification status is imperative for comprehensive understanding of the transcriptional regulation of task specialization in the DOL in social insects. The application of ChIP-seq analysis has propelled efforts to understand the molecular mechanisms underlying social behaviors, caste determination, and environment adaptation in ants and bees (Glastad et al. 2020; Lowe et al. 2022; Shields et al. 2018; Simola et al. 2013a, 2016; Wojciechowski et al. 2018; Zhang et al. 2023). In this study, we develop a ChIP-seq protocol in order to link the behavioral phenotype to gene expression and regulatory mechanisms in the ant *T. longispinosus*. Our results showed a high variation of peak numbers, low signal, and background noise across samples. Because ChIP-seq analysis is sensitive to the quality of input samples and it is difficult to adjust protocols for the quality of each sample, samples with insufficient quality had to be automatically rejected. Several artifacts could arise during the steps of experimentation that need to be considered for future generation of high-quality genome-wide data, including antibodies quality, sonification, controls, library complexity, and experimental replication.

Antibody quality

ChIP-seq crucially depends on the antibody used for the immunoprecipitation which contributes to the quality of the data generated. A sensitive and specific antibody is necessary for ChIP-seq because it allows the detection of enrichment peaks without substantial background noise, which makes it easier to detect binding events (Park, 2009). The clonality, or heterogeneity, of the antibody should be considered in the selection of an antibody. Monoclonal antibodies recognize a single epitope on an antigen, which may be beneficial for diminishing background noise in ChIP studies. However, the use of monoclonal antibodies may result in a lower signal if the epitope is masked by surrounding chromatin components or if the protein is part of a larger protein complex (Kidder et al., 2011). Although epitope recognition may be problematic for any antibody in ChIP studies regardless of its clonality, polyclonal antibodies offer the flexibility of the recognition of multiple epitopes, which may boost the signal in cases in which epitopes are masked by surrounding material (Kidder et al., 2011). Therefore, there is a higher probability that a polyclonal antibody will work in ChIP when compared to a monoclonal antibody. In our protocol, we used the rabbit polyclonal H3K27ac antibody (ab4729, abcam) previously tested in some species of insects such as *Drosophila*, *Heliconius erato* butterfly, and *C. floridanus* ant (Glastad et al. 2020; Lewis et al. 2016; Simola et al. 2016; Tie et al. 2009). Because there is no definitive rule for choosing the appropriate clonality of an antibody for ChIP studies, we recommended to test several antibodies. This will provide greater confidence that the peaks identified are true positive results. A simple way to screen multiple antibodies is to compare their immunoprecipitation efficiency on fixed versus unfixed samples by dot blot or western blot (Egelhofer et al., 2010).

The abundance of the protein or histone modification to be investigated and the quality of the antibody should be considered when determining the number of cells to begin ChIP-seq analysis. As the signal-to-noise ratio is directly correlated with the cell number, the use of more cells tends to produce a higher signal-to-noise ratio (Kidder et al., 2011). Therefore, it is important to empirically determine the minimum number of cells that can be used. ChIP-seq experiments typically require 1×10^6 to 1×10^7 cells, which results in 10–100 ng of ChIP DNA. The former (1×10^6 cells) is usually sufficient for the analysis of abundant proteins such as RNA polymerase II and localized histone modifications such as trimethylation of histone H3 at Lys4 (H3K4me3), whereas the latter (1×10^7 cells) may be required for the analysis of less-abundant proteins or diffuse histone modifications (Kidder et al. 2011). However, the precise amount of ChIP DNA and the number of cells needed depend on the abundance of the chromatin-associated protein targets or histone modifications (Park, 2009). The brain of the ants could have a range from 5×10^4 to 2×10^5 cells (Godfrey et al., 2021). Here, we pooled seven ant brains belong to the same task and colony to obtain samples with approximately 35×10^4 to 1.4×10^6 cells. The samples were then split into two parts for RNA and Chip-seq analyzes, resulting in an unequal decrease in the number of cells per sample. This difference between samples could explain the high variation found in the number of peaks. Several alternative protocols have been designed that require fewer cells (1×10^4 to 1×10^5) for the profiling of genome-wide distributions of histone modifications, although these methods have not been demonstrated yet to work well for transcription factors (Adli et al., 2010). The advantage of this approach is the ability to use 1–10% as many as cells as are used in conventional ChIP-seq protocols, which may be beneficial for the study of low input samples. Finally, determining the optimal antibody concentration can significantly improve the signal to background ratio. The amount of antibody required per ChIP typically ranges from 2–10 μg of antibody for every 25 μg of chromatin, while in our experiment the concentration used was 4 μg. Although more antibody does not always equal stronger signal, we suggested that to get the best ChIP signal the amount of antibody be titrated.

Sonification

Sonication of the chromatin is a key step in the ChIP protocol as it renders the chromatin soluble. The extent to which one can fine-map the location of a specific protein in the genome depends on the extent of DNA fragmentation. Although chromatin fragments of different sizes may work well for ChIP-PCR assays, the optimal size range of chromatin for ChIP-seq analysis should be between 200 to 600 bp (Park, 2009). In our protocol, DNA fragmentation conditions were optimized and performed with Covaris E220, which only allowed to processed one sample at a time. This resulted in different fragment sizes per sample (range from 400 to 700 bp). To obtain reproducible results, we suggest try to keeping all the parameters constant by processing all samples with a multi-sample sonicator.

Control experiment

The experimental steps in ChIP involve several potential sources of artefacts. For example, in terms of chromatin fragmentation, open chromatin regions are easier to shear than are closed chromatin regions and thus the former may be associated with higher background signal (Teytelman et al., 2009). An important part of ChIP-seq experimental design is determining which controls to use. Therefore, a peak in the ChIP–seq profile should be compared with the same region in a matched control sample to determine its significance. There are three commonly used types of control sample: Input DNA (a portion of the DNA sample removed prior to immunoprecipitation (IP)); mock IP DNA (DNA obtained from IP without antibodies); and DNA from nonspecific IP (IP performed using an antibody, such as immunoglobulin G (IgG), against a protein that is not known to be involved in DNA binding or chromatin modification) (Park, 2009). Although we have used Input DNA as a control in this study, other controls can be used. The predominant Input DNA control corrects for uneven sonication, but not for nonspecific interactions of the IP antibody. The other type of control, mock IP DNA, corrects for both of the issues, but is not widely used because it is considered susceptible to technical noise. In complex samples, the abundance of spurious sites is substantial using Input control, whereas using mock IP control results in more accurate and comparable binding sites across samples (Xu et al., 2021). Although both nonspecific IgG antibodies and Input chromatin have been used as controls, IgG may be less desirable in certain circumstances because IgG antibodies usually immunoprecipitated much less DNA than specific antibodies, and thus limited genomic regions from the control may be overamplified during the library construction step. In this case, the resulting reads will not cover the genome as sufficiently as a background model would for peak identification. Therefore, Input control serves as a better control for bias in chromatin fragmentation and variations in sequencing efficiency, providing greater and more evenly distributed coverage of the genome (Kidder et al., 2011). These three types of control samples test different types of artefacts and there is no consensus on which is most appropriate. As more ChIP-seq experiments with appropriate controls are performed, the deviation in coverage is going to become an increasingly powerful way to identify distinct chromatin states.

Library complexity

ChIP-seq generates sequences from regions specifically, or indirectly, bound to the antibody target (the signal) as well as from background binding of genomic DNA and regions non-specifically bound to the antibody (the noise). Consequently, ChIP-seq libraries need to be sufficiently complex, consisting of billions of unique molecules with distinct 3’ and 5’ ends (Guide, 2007). Library complexity is defined as the fraction of DNA fragments that are nonredundant. With increased depth of sequencing of a library, a point is eventually reached where the complexity will be exhausted and the same PCR-amplified DNA fragments will be sequenced repeatedly. Low library complexity can occur when very low amounts of DNA are isolated during the IP or due to problems with library construction (Landt et al., 2012). One proxy for library complexity is to look at the sequence duplication levels on the FastQC report. An elevated number of duplicates obtained indicates that too little DNA was isolated by immunoprecipitation or that there were problems with library construction. We found 56% (range from 27 to 80%) and 30% (range from 15 to 61%) of duplication reads for IP and Input samples in the FasQC report, respectively. The high duplication reads for the very little starting material samples extracted from the pooled brains (seven ants) may have affected the complexity of the libraries. We suggest an increase in the number of ant brains to obtain higher quality samples in future ChIp-seq experiments.

Experimental replication

ChIP replicate experiments are recommended to account for variation between samples and to verify the fidelity of experimental steps to ensure reproducibility of the data.

Biological replicates are essential to understand variation and for differential binding analysis. It is suggested that a sufficient sequencing depth of 20 million reads is a minimum for typical transcription factors (TFs) in humans (Landt et al., 2012), 60 million reads may be required for broad histone markers (Chen et al. 2012), and 40–50 million reads as a practical minimum for most broad histone markers (Jung et al., 2014). In our study we used ~74 million reads per sample. More replicas are generally preferable to greater depth. However, because our samples showed a low concentration of immunoprecipitation, it could be suggested sequence a high-quality sample at a lower depth than a low-quality sample at a higher depth. Assuming such samples are deeply sequenced, two concordant replicate experiments are usually sufficient, as a third replicate appears to add little value (Rozowsky et al., 2009).

Conclusion

Understanding the role of histone modifications in social insects is a growing area of research and offers insights into the remarkable adaptability and resilience of these complex societies. ChIP-seq is a powerful technology with which to evaluate protein-DNA interactions on a global scale. The findings obtained by ChIP-seq have improved the knowledge of the epigenetic landscapes that regulate chromatin structure and transcriptional profiles. Because data sets generated by ChIP-seq studies are valuable resources for the community, it would be beneficial if data sets generated from different laboratories could be compared directly. For this purpose, experimental conditions must be optimized and standardized. Experimental challenges for the future include the careful validation of antibodies, the development of methods for working with a small number of cells and single-cell-level characterization. As ChIP-seq protocols are further improved and data-analysis platforms become more manageable for experimental biologists, the application of this technology will help comprehend the epigenetic mechanisms that allow social insects to rapidly respond to changing environmental conditions and maintain the efficiency of colonies to survive.

General Discussion

Marcel A. Caminer

Summary of findings

In this work we aim to gain deeper insights into the molecular regulation of the non-reproductive division of labor (DOL) in the acorn ant *Temnothorax longispinosus* by analyzing not only changes in gene expression but also their epigenetic programming. **Chapter 1** provided novel insights into the hypothesis that social insect workers perform different tasks because they differ in their ability to detect task-related stimuli. We reported that nearly half of all odorant receptor (OR) genes were differentially expressed between nurses and foragers. Workers specializing in brood care overexpressed OR genes from the 9-exon subfamily, which is predicted to underlie chemical communication between nestmates, while foragers overexpressed a more diverse set of OR genes. We proposed that DOL models would benefit from considering odor sensitivity as a potential upstream sensory filter that may affect task specialization. We also found differentially expressed genes (DEGs) associated with biogenic amines, insulin/insulin-like signaling (IIS), vitellogenin (Vg) juvenile hormone (JH), and target of rapamycin (TOR) pathways in the brain and antennae between both behavioral phenotypes, suggesting that these modulators and hormones may be involved in task-associated behavioral changes and regulation of the sensory filter. Among the DEGs, *Vg-like-A* was overexpressed in nurse brains compared to foragers. Our results, together with previous studies (Kohlmeier et al. 2018), suggest that *Vg-like-A* might be altering the expression of ORs in the antenna. In **Chapter 2** we established downregulation of *Vg-like-A* by injecting dsRNA directly into the gaster of the ants. Our results showed weak behavioral changes over 10 days, but no evidence of significant changes in transcriptional activity of the antennae or brain of treated brood care workers. These weak and pronounced changes in behavior probably could have occurred because the injection decreased the motivation of all workers to perform brood care, including those in the control treatment. In **Chapter 3** we developed a ChIP-Seq protocol to study histone acetylation and, although our analyses were ultimately inconclusive, we discussed important experimental design issues and proposed a number of adaptations to improve this technique for future studies in ants. These first steps to a histone acetylation analysis in *Temnothorax* will become useful as recent studies have added to the growing body of experimental evidence that histone acetylation is involved in the regulation of behavior and reproduction in ants (Choppin et al. 2021; Kohlmeier et al. 2023; Libbrecht et al. 2020; Simola et al. 2013a, 2016). It is even conceivable that this epigenetic regulatory mechanism is responsible for the regulation of odorant receptors in the antennae, which likely acts as a sensory filter for the DOL (Caminer et al., 2023).

Molecular basis of the division of labor in insect societies

In solitary and subsocial insects, females exhibit reproductive and non-reproductive stages that are strongly linked to physiology (Page et al. 2009). During the evolution of eusociality and the associated DOL, the genetic and hormonal networks associated with reproduction in subsocial ancestors were decoupled from the physiological networks and instead were designed to additionally regulate behavior (Amdam et al. 2004, 2006; Kapheim & Johnson 2017; Weitekamp et al. 2017). The ancestral non-reproductive stage in eusocial insects is mainly represented by foraging and is linked to endocrine signaling, low nutritional stores, and decreased fertility (Ament et al. 2008; Morton et al. 2006), whereas fertility stage was evolutionarily predisposed to regulate the behavior of sterile workers that remain within the nest (Blanchard et al., 2000; Porter & Jorgensen, 1981). This resulted in a colony-wide selection in which the young, fertile workers and with the highest lipid content took over brood care, while workers with shortest residual life span, lowest lipid reserves, and lowest reproductive potential were recruited for tasks outside the nest such as foraging. Our study indicates that understanding the differences in the molecular mechanisms between nurses and foragers can provide important and novel insights into how these behavioral phenotypes could have evolved.

Social insects display molecular caste differentiation due to various environmental cues and genetic factors. This differentiation occurs primarily through changes in gene expression influenced by diet, hormones, and social interactions during development. JH is one of the most important hormones for the regulation of behavioral plasticity in social insects (Bloch et al., 2009; Korb, 2015). Recently, JH has been shown to mediate long-lasting genomic changes or signal transduction cascades related to the central nervous system (Wheeler et al., 2015) or peripheral nervous system (Wang et al. 2012), which could lead to behavioral changes. Low JH titers and high expression levels of Vg genes activate brood care behavior (Amdam et al. 2003; Kohlmeier et al. 2018), whereas elevated JH titers and Vg downregulation shifts behavioral states of workers to foraging (Korb, 2015; Marco Antonio et al., 2008). Our results reveal differential expression of genes in the brain and antennae associated with the JH pathway (**Chapter 1**), such as *venom carboxylesterase-6*, gene that has also been found expressed in the brain of *Camponotus floridanus* nurses (Das & de Bekker, 2022). The protein encoded by *venom carboxylesterase-6* is a JH esterase enzyme (JHE) that degrades and regulates JH titers and caste-associated behaviors in ants (Kamita et al., 2003; LeBoeuf et al., 2018). We also found differential expression of takeout genes that have been confirmed to be involved in behavioral changes (Guo et al. 2011). Takeout genes are part of the hemolymph juvenile hormone binding protein (JHBP) superfamily and were found to be specifically expressed in antennae and labella, suggesting functions in chemical perception (Fujikawa et al. 2006; Guo et al. 2011). Among the overexpressed genes found in foragers were *Insulin-like growth factor I* (IGF1; sometimes referred to as Ilp1, see Chandra et al. 2018) and *allatostatin A-like*. IGF1 is expressed in the neural and peripheral nervous systems, can increase with JH, and is positively correlated in harvester ants and honeybees (Gospocic et al., 2017; Nilsen et al., 2011; Yan et al., 2022), while allatostatins are essential for inhibiting JH biosynthesis (Altaratz et al., 1991; Stay & Tobe, 2007). The relationships between diet, Vg, JH, and behavior have been linked to IIS, which involves insulin-like peptides (ILPs) (Corona et al., 2007), and the overlapping TOR (a nutrient-sensitive kinase) pathway (Patel et al., 2007).

Biogenic amines are not only important for learning, memory, and information exchange in insects (Awata et al., 2016; Berry et al., 2012; Blenau et al., 2000; Scheiner et al., 2006; Sitaraman et al., 2008), but also play a role in other behaviors such as foraging (Schulz et al. 2003). Therefore, it is not surprising that we found differential expression of biogenic amines and their receptors in the nervous systems of nurses and foragers. However, we had expected this difference to be more pronounced in the central nervous system, i.e., the brain rather than in the periphery, that is in the antennae, where we found most evidence for it. Yet, biogenic amine activity in the antennae can influence the behavioral plasticity in insects (Guo et al. 2013; Ma et al. 2015). Given the central role of Vg and JH in regulating the DOL in social insects and the variation in biogenic amines detected in the antenna in this study, we hypothesized that these modulators and hormones are involved in the regulation of olfactory pathways in *T. longispinosus*, which is also likely related to the mechanics of the circadian clock.

Sensory filter hypothesis

Animals receive much more sensory information than they could possibly process in their brain. The brain must therefore be selective and filter out certain information that is not so important. The adapted filter hypothesis states that sensory systems develop in such a way that they only perceive the most ecologically relevant stimuli (Barlow 1961; Wehner 1987). Efficient allocation of sensory resources enhances signal detection by maximizing signal-to-noise ratios to facilitate behaviors essential for survival and reproduction (Endler 1993; Lucas et al. 2015). Sensory filters have evolved in response to almost every aspect of animal ecology, from locomotion and navigation to predator avoidance, food acquisition and courtship. They are manifested in all of the senses, in both vertebrates and invertebrates (von der Emde & Warrant, 2016). In small insects, with complex lifestyles but strictly limited energy budgets, sensory filters could be highly beneficial in freeing up energy that could be used for other vital functions. Our results predict that the peripheral nervous system (i.e., antennae) acts as a sensory filter in the ant *T. longispinosus*, allowing nurses and foragers to perceive their environment differently and react to task-specific cues appropriately. This sensory filter remains flexible and can be regulated through changes in physiological conditions such as age, nutrition, and hormones (**Chapter 1**). For example, the sensory filter of nurses may be more fine-tuned to detect brood cues. In the behavioral maturation transition from nursing to foraging, nurses would become less efficient at detecting brood cues via the downregulation of specific OR genes. In contrast, the sensory filter of foragers may also become fine-tuned to detect a more diverse set of task-related odors outside the nest. This sensory filter mechanism would affect the DOL in non-reproductive workers by making them more skilled and efficient at specific tasks, leading to an overall increase in colony productivity.

The sensory filtering function could be modulated by biogenic amine signaling and genes related to physiological condition, fecundity, and lifespan such as IIS, JV, Vg, and TOR pathways. This hypothesis is supported by several studies showing that genes related to these pathways can alter sensitivity to various signals in the insect antennae (Gadenne et al. 2016; Mercer & Menzel 1982; Scheiner et al. 2017) and regulate variation in workers behavior (Ament et al. 2008; Corona et al. 2013; Dolezal et al. 2012; Kamhi & Traniello 2013; Kohlmeier et al. 2019; Libbrecht et al. 2013; Schulz & Robinson 1999; Sullivan et al. 2000) (**Chapter 1**). Based on our and previous experiments of downregulation of *Vg-like-A* in *T. longispinosus* (Kohlmeier et al. 2018), *Vg-like-A* was associated with behavioral changes in responsiveness to chemical cues from larvae and workers, making it a highly candidate behavioral gene in the regulation of the sensory filter (**Chapter 2**). *Vg-like-A* - overexpressed in fat bodies of nurses- is correlated with multiple factors such as age, immunocompetence, physiology and fertility, resulting in behavioral maturation from brood care to foraging (Kohlmeier et al., 2018; Salmela et al., 2016). The behavioral maturation in social insects could be modulated via trophallaxis (Leoncini et al., 2004), a form of food exchange that plays a prominent role as an information channel in various contexts (Farina & Grüter, 2009), and via pheromones produced by larvae that have a similar effect (Le Conte et al., 2001). However, the detailed molecular mechanisms underlying the *Vg-like-A* action in the brain and fat body that are causally linked to the changes in complex olfactory behavior in antennae remains unknown.

The role of H3K27ac histone acetylation in the regulation of division of labor

Epigenetic plays an important role in social insects where behaviors and traits are not only determined by genetic factors, but are also influenced by environmental cues and social interactions. Understanding epigenetic mechanisms in social insects provides information on behavioral plasticity, adaptation to changing environmental conditions, caste determination, and thus DOL regulation within colonies. The transcriptional coactivator CREB binding protein (CBP) is the main histone acetyltransferase that catalyzes H3K27ac (Jin et al. 2011; Tie et al. 2009). It acts as a coactivator to a large number of transcription factors (TFs) and epigenetically regulates the expression of many genes by acetylating histones (Négre et al., 2011). H3K27ac, TFs binding motifs, and CBP occupancy have been widely used to successfully map enhancers in numerous cell types, tissues, and organisms (Koenecke et al., 2016; Rada-Iglesias et al., 2011; Visel et al., 2009) which may play an important role in circadian rhythm, behavior and worker caste determination in ants and honeybees (Simola et al. 2013a, 2016; Spannhoff et al. 2011; Wojciechowski et al. 2018). In addition, Kohlmeier et al. (2023) found that inhibition of histone acetyltransferases (HATs) in *T. longispinosus* impairs the ability of older workers to switch to brood care, while increasing the ability of young workers to accelerate their behavioral development and switch to foraging. These results suggested that changes in histone acetylation patterns are involved in the regulation of task switching and therefore in behavioral flexibility. According to our sensory filter hypothesis, we suggested that H3K27ac could be regulating the gene networks responsible for the change of odor perception. However, our immunoprecipitation sequencing (ChIP-seq) assay did not allow linking the behavioral phenotype with gene expression and regulatory mechanisms in the ant *T. longispinosus* due to high variation of peak numbers, low signal, and background noise across samples (**Chapter 3**). Different epigenetic systems such as DNA methylation, miRNA and another histone protein posttranslational modifications (hPTMs) could be interacting to affect gene function in caste differentiation; therefore, their possible influence cannot be excluded. Further studies are required to evaluate the presence of direct spatial overlap between different epigenetic marks within genes.

Research perspectives

In our gene expression study, we show that physiological differences between the most distinct behavioral phenotypes of nurses and foragers are less pronounced in the central nervous system than in the peripheral sensory organs. Our results suggest that a sensory filter might play an important role in the regulation of DOL in insect societies in which workers live in a different sensory environment and perform different tasks (**Chapter 1**). Further task reversal experiments with multiple transition points could produce changes in gene and epigenetic activity across multiple time points in nurses, inverted nurses, and stable foragers. These kinds of results could determine how long and which steps a forager needs to change its transcriptional regulation and investigate whether there are traces of an epigenetic memory. On the other hand, few studies have profiled whole-genome histone modification patterns in non-model organisms, leaving the regulatory landscape of histone modifications in the DOL of social insects unclear. These studies have used chromatin profiling techniques such as ChIP-seq to experimentally demonstrate the relevance of epigenetics for caste differentiation in ants and bees (Glastad et al. 2020; Lowe et al. 2022; Shields et al. 2018; Simola et al. 2013a, 2016; Wojciechowski et al. 2018; Zhang et al. 2023). However, Chip-seq may be limited due to the high number of input cells it requires (Kidder et al., 2011) as our results demonstrated (**Chapter 2**). Therefore, newer studies are required that use novel techniques with less input chromatin, shallower sequence depth, reduce the signal-to-noise ratio and can be performed faster, such as cleavage under targets and release using nuclease method (CUT & TAG; Kaya-Okur et al. 2019). Finally, we were able to downregulate *Vg-like-A* gene by establishing a protocol injecting dsRNA directly into the gaster with a low mortality rate, but affecting the ant behavior from the treatment and control (**Chapter 3**). We propose to functionally validate the differentially expressed genes found in our RNA-seq results using a less invasive and continues knockdown method following with behavior assays, which allow the exploration of downstream effects of gene expression that could influence responsiveness and behavior to social cues. Overall, all those new findings will contribute to the understanding of the evolution and molecular regulation of the DOL in social insects and beyond.

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