Genetic Basis of Behavior in Temnothorax Ants

Dissertation zur Erlangung des Grades
Doktor der Naturwissenschaften

Am Fachbereich Biologie der
Johannes Gutenberg Universität Mainz

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geb. August 17, 1987
Tyler, Texas, United States of America

Mainz, 2018

Dekan:

1. Berichterstatterin:

2. Berichterstatterin:

Tag der mündlichen Prüfung: November 27, 2018

“It is not the strongest of a species that survives, nor the most intelligent, but rather the one most adaptable to change.”

-Unknown

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Summary

Parasitism is an extremely prolific and successful mode of life, as measured by both the number of parasitic species and their evolutionary origins. The transition to parasitism is a drastic shift in lifestyle associated with major changes in gene structure, expression, and function, resulting in alterations of the selective regime. In parasites, genes involved in circumventing host defenses are more likely to be under selection, while genes involved in evading or blunting parasite attacks are more likely to be under selection in host species. Thus, after the establishment of antagonistic relationships, parasite and host become entangled in an *evolutionary arms race*, co-evolving with one-another through reciprocal adaptations. Antagonistic interactions between parasite and host are among the strongest and most persistent forces in evolution.

Here, I elucidate the genetic underpinnings of behaviors associated with slavemaking in social parasites, and their counterpart behaviors within non-parasitic hosts. I focus on *Temnothorax* ants; a genus comprised of socially-parasitic slavemaking species that exploit the workforce of closely-related, non-parasitic host species. Social parasites, including slavemaking ants, exploit the social behavior of their hosts. Slavemakers steal heterospecific host brood during raids and incorporate it into their own colonies. While socially-parasitic *Temnothorax* species all employ raiding strategies to infiltrate host colonies and abduct brood, the precise mechanisms utilized to effectively carry out these raids differs between species. However, despite radically different lifestyles, slavemaker and host also share a number of outwardly like behaviors; raising the question as to whether or not shared molecular mechanisms underlie these behaviors.

1] Building upon previous, purely behavioral, studies - which established that raiding strategies between *Temnothorax* slavemakers diverge while host defense portfolios shift similarly under parasite pressure - the **First Chapter** of this thesis investigates and confirms this observation at the molecular level. Utilizing transcriptomic and gene expression methodology on a dataset of three socially parasitic and three host *Temnothorax* species, we found that slavemaking species exhibit a wider variety of genes with species-specific patterns of expression within their raiding phenotypes, whereas expression similarity is commonly found during the non-raiding phenotype. Conversely, host species response to slavemaker aggression is indicated by strong changes in the expression of a relatively few number of genes. The expression of individual genes such as *Acyl-CoA-Delta(11) desaturase* and

Trypsin-7 is strongly associated with the raiding phenotype of all three slavemaking species, suggesting at least some measure of molecular similarity between the raiding behaviors of *Temnothorax* slavemakers. However, taken as a whole, lineage-specific evolutionary patterns dominate the molecular patterns associated with behavior in both slavemakers and hosts.

2] **Chapter Two** of this thesis complements Chapter One, also exploring - though using different methodology – the importance of specific genes in the ongoing evolution of parasites and hosts in *Temnothorax*. To further investigate the genetic basis of slavemaker-host co-evolution, we analyzed the selective forces associated with the slavemaker and host lifestyles in *Temnothorax*. Utilizing orthologous clusters from the same three slavemaker and three host species as in Chapter One, we identified genes with strong selective signatures, with these genes being examined further for functional enrichment and involvement in metabolic pathways. The first step, a phylogenetic analysis based on over 5000 orthologous sequences, revealed that two slavemakers were sister species, as well as two host species. Furthermore, identified were 309 genes with signatures of positive selection on branches leading to slavemakers and 161 on branches leading to hosts. Genes possessing signatures of positive selection varied widely in their functions, ranging from involvement in cuticular hydrocarbon synthesis to circadian clock functionality. However, between species we identified little overlap in genes with signatures of positive selection; with unique genes, pathways, and functions under positive selection in each species. Thus, as in Chapter One, the results presented here suggest that - even among closely-related species - the path to social parasitism may not be fixed.

3] Where we identified the genes involved in the seasonal raiding behavior of *Temnothorax* slavemakers in Chapter One, and followed this up by investigating those genes exhibiting signs of selection in Chapter Two, neither of these approaches elucidates the precise and present role of specific genes involved in *Temnothorax* behavior. Complex behaviors, like that of *T. pilagens'* raiding phenotype, are the amalgamation of simpler behaviors controlled by single or smaller numbers of genes. While these genes may retain aspects of their ancestral functions, their involvement in taxa-specific networks may result in novel functions. The **Third Chapter** of this thesis details our investigation into the specific role of *Trypsin Inhibitor* within the raiding behavior of *T. pilagens*. We found that while the knockdown of *Trypsin Inhibitor* – accomplished through the oral application of dsiRNA – affected neither the onset of raiding nor behavior during scouting, raiders that did manage to

discover and enter the host nest often remained inside, with no attempt to steal brood or forcefully expel hosts. Consequently – and not unexpectedly – these raiders had far lower raiding success, as measured by the number of brood items successfully stolen. This marks the first evidence linking a single gene – *Trypsin Inhibitor* – to raiding success in ants, and - as raiding is not an ancestral function for trypsin-type genes and their inhibitors - strongly suggests a re-purposing of *Trypsin Inhibitor* in *Temnothorax*.

4] The **Fourth and final Chapter** of this thesis investigates the degree of molecular similarity underlying slavemaker raiding-party and host tandem-running behavior. These behaviors are utilized in numerous contexts: from foraging and nest relocation in host *Temnothorax* species to locating hosts and establishing raiding parties in slavemakers. Here, we elucidate the transcriptomic basis of scouting, tandem-leading, and tandem-following behavior across the slavemaker *T. americanus* and its preferred host *T. longispinosus*. Analysis of gene expression data from brains revealed that only a small number of unique differentially-expressed genes are responsible for scouting and tandem-running behaviors. Comparison of orthologous genes between *T. americanus* and *T. longispinosus* suggests that tandem-running is characterized by species-specific patterns of gene usage. However, within both species, tandem-leaders showed gene expression patterns median to those of scouts and tandem-followers. This is not unexpected, as leaders can be recruited from either of the other two behavioral states. Most importantly, a number of differentially-expressed behavioral genes were found, with functions relating to learning and memory formation in other social and non-social insect species; including glutamate and tyramine receptor genes. Learning and memory genes were specifically up-regulated within scouts and tandem-followers, which use spatial learning extensively to navigate novel environments. As such, results of our analyses suggest that tandem-running in *Temnothorax* ants involves learning of novel information by following individuals and possibly even teaching by leading individuals.

To conclude, this thesis demonstrates that molecular evolution of lifestyle-specific behaviors is lineage-specific within the *Temnothorax* genus. Slavemakers specifically display a variety of mechanisms during raiding, and this is reflected on the molecular level, with very few genes sharing expression patterns before and during raiding behavior and no homologous genes sharing signatures of positive selection between slavemaking species. As such, further functional analyses into genes responsible for raiding behavior in *Temnothorax* will be likely require species-specific approaches, although the gene *Trypsin Inhibitor* does appear to have a strong impact on the raiding behavior and effectiveness of *T. pilagens*.

GENERAL INTRODUCTION

Parasitism, Social Parasitism, and Slavery

Austin Alleman

Parasite and Host

With almost half of all extant animal species on earth being classified as parasitic in some capacity, parasitism is one of the most successful forms of life (Price 1977, Price 1980, Poulin and Morand 2000). The parasite lifestyle is present in all taxa and all levels of biological organization, from micro-parasites such as viruses and selfish genetic elements to macro-parasites including worms, arthropods, and vertebrates. Among the larger macro-parasites, strategies for host exploitation vary dramatically, including the acquisition of resources directly from the host to the less direct brood- and social parasites (Davies *et al*.1989, D'Ettorre and Heinze 2001, Kilner and Langmore 2011, Schmid-Hempel 2011).

Antagonistic parasite-host interactions are associated with a parasite-induced loss in host fitness, the strength of which vary wildly depending on numerous factors including local environmental conditions and the age of the host-parasite relationship (Toft and Karter 1990, Ewald 1995). Ideally for the parasite, strategies hinge upon the highest acquisition of resources without killing the host or inducing a violent host response (Brown 1987); though such dynamics are often highly complex, with the impact of parasites often extending well beyond the individual affected (Beros *et al*. 2015). As such, there is constant interplay between parasite-induced fitness loss in the host and the success of parasite transmission.

Parasite-host relationships are universally characterized by mutual adaptation between parasite and host, resulting in many theoretical co-evolutionary dynamics, trajectories, and outcomes (Dawkins and Krebs 1979). Indeed, the Red Queen theory of co-evolution – where both parasite and host (or predator and prey, etc.) must continually evolve simply to stay competitive – is a common evolutionary outcome of parasite-host interactions (Van Valen 1973). Evolutionary dynamics often result in parasite and host becoming entangled in an evolutionary arms race, co-evolving with one-another through reciprocal adaptations. Alternatively, parasite pressure can drive host diversity through frequency-dependent selection (Livey 2001). Such relationships can persist indefinitely, or resolve entirely if hosts develop effective defenses against their parasites or if the parasite eradicates the host population (Thompson 2005, Nuismer and Thompson 2006, Soler 2014). However, recent evidence suggests that co-evolution might result in drastically different outcomes between otherwise similar parasite-host systems; as is the case in avian brood parasites where host species develop unique resistance or coping mechanisms to the same parasite species (Soler 2014).

Social Parasitism

One particularly intriguing form of parasitism is the exploitation of the social behavior of a host species by another social species (Buschinger 1993). In some cases, these social parasites can manipulate and exploit entire host societies (Hölldobler and Wilson 1990). While social parasitism is most commonly understood by the layman in reference to avian brood parasites such as cuckoos and cowbirds - which avoid the cost of parental care by exploiting the brood behavior of other bird species (Davies 2000; Kilner and Langmore 2011) - social parasitism is also highly prevalent among social insects. Here, it is commonly defined as the co-existence of two species within the same nest, of which one species is parasitically dependent upon the other (Hölldobler and Wilson 1990). Within ants alone there are over 230 described socially-parasitic species, primarily within Myrmicinae and Formicinae (Bolton 2003, Buschinger 2009); with evidence for co-evolutionary arms races between many of these species and their hosts (Soler and Moller 1990, Davies 2000, Foitzik *et al*. 2003, Brandt *et al*. 2005, Bauer *et al*. 2009, Foitzik *et al*. 2009, Pennings *et al*. 2011, Jongepier *et al*. 2014, Cini *et al*. 2015, Jongepier *et al*. 2015). Most socially-parasitic social insects evolved from closely related, non-parasitic species, with the non-parasitic species often serving as primary host for parasites; in keeping with Emery's rule (Emery 1909, Savolainen and Vepsäläinen 2003). Indeed, it is this close relatedness between social parasite and host that allows for the subversion of nestmate recognition and communication mechanisms (D'Ettorre and Heinze 2001).

Slavery

Dulosis - more commonly termed slavery - is an often-permanent form of social parasitism specific to ants (Hölldobler and Wilson 1990, D'Ettorre and Heinze 2001), where workers of one species specialize in the abduction and incorporation of a different species into mixed-species colonies, with the "enslaved" individuals carrying out normal worker tasks for the comparatively inactive slavemakers. The workers of many slavemaking species have even lost all ability to care for themselves, to the extent that these individuals would starve without the presence of their slaves. Consequently, slavemaking is often obligatory (Wheeler 1910). This novel lifestyle has fascinated researchers for generations (Huber 1810): Charles Darwin even made note of slavemaking ants in the Origin of Species (Darwin 1859). Primarily arising within the tribes Formicoxenini (Myrmicine) and Formicini (Formicine), social

slavery has been described in approximately fifty ant species (D'Ettorre and Heinze 2001). Mounting evidence suggests that the slavemaking lifestyle is highly variable and taxa-specific, with at multiple unique origins within ants (Beibl *et al*. 2005, Feldmeyer *et al*. 2018).

The slavemaking lifestyle itself differs markedly from other forms of social parasitism, and is especially divergent from more typical lifestyles found within ants. Strategies for acquiring slaves vary, and are often species specific. Generally, however, the process begins with a large, mated slavemaker queen founding her own nest, and subsequently producing workers which then raid nearby host colonies. Within some genera (*Temnothorax*, *Harpagoxenus*) the queen will instead take over an existing host colony; killing or driving out all host queens and leaving only developing brood within the nest (Herbers and Foitzik 2002, Pamminger *et al*. 2012). In such cases, the developing brood will become the first generation of slaves to the slavemaker queen. However, as the host queen is no longer inside the nest to replenish the slave workforce, additional slaves must be acquired from outside the nest. The slavemaker queen produces slavemaker workers, who do not perform typical nest maintenance and nestmate care tasks but instead specialize in raiding nearby host colonies. Slavemaker workers perform raids during which they abduct host brood (and sometimes even adult workers) in order to bolster the slave workforce of the slavemaker colony. These captured slaves perform normal nest tasks, generally oblivious that their efforts are benefiting the slavemakers. However, in some cases, enslaved individuals detect that the colony in which they live is not their own, and rebel against their slavemakers by killing slavemaker brood (Achenbach and Foitzik 2009, Achenbach *et al*. 2010, Metzler *et al*. 2016).

Temnothorax

This thesis focuses exclusively on six ant species within the genus *Temnothorax*, which belong to the North American Formicoxenine clade. Three of these species – *Temnothorax americanus*, *T. duloticus*, and *T. pilagens* – are obligate slavemakers, with the non-slavemaking species *T. longispinosus*, *T. curvispinosus*, and *T. ambiguus*, respectively, serving as primary hosts to the slavemakers (Wesson 1939). Within these six *Temnothorax* species alone, the slavemaking lifestyle has arisen twice independently (Feldmeyer *et al*. 2017). Curiously, while the strict form of Emery's rule is not well supported in *Temnothorax* in general, it does hold for certain species including the slavemakers examined here (Prebus 2017). However, there is room for interpretation of Emery's rule in this case, specifically with

regard as to what constitutes “closely-related”, as Emery does not elaborate on how phylogenetically similar two species must be in order to be considered closely-related (Emery 1909). Indeed, two species do not necessarily need to be sister-species to be closely-related enough for Emery's Rule to still hold true. As such, applying Emery's Rule to slavemakers and hosts in *Temnothorax* becomes more academic than practical, and care should be taken when drawing conclusions in light of Emery's rule in this context. Of course, not all interactions among *Temnothorax* are antagonistic, as recent evidence, for example, points towards resource partitioning among some non-slavemaking species (Prather *et al*. 2018), suggesting cooperative benefits of reducing inter-specific competition.

Division of labor in these slavemakers differs substantially from that of host colonies. Within the non-slavemaking *Temnothorax* species, older and more expendable individuals leave the relative safety of the nest to forage, while younger individuals take over brood care and nest maintenance tasks (Kohlmeier *et al*. 2017, Kohlmeier *et al*. 2018). However, in slavemaking species, infertile workers leave the nest during the summer to search for and subsequently raid nearby host colonies, while fertile workers remain inside the host nest and produce male offspring (Franks and Scovell 1983, Foitzik and Herbers 2001, Blatrix and Herbers 2004, Pohl *et al*. 2011). Host species, on the other hand, respond to parasite intrusion by employing more conventional nest-defense behavior, though the exact strategy employed by a host does vary by population in response to parasite pressure (Foitzik *et al*. 2001, Foitzik *et al*. 2009, Jongepier *et al*. 2014, Kleeberg *et al*. 2015).

Scouting and Raiding in Temnothorax

Despite drastically different lifestyles *Temnothorax* slavemakers and hosts do share some functional homologous behaviors, the most interesting of which is tandem-running. Externally, slavemaker raiding parties resemble the tandem-running behavior employed by non-parasitic *Temnothorax* species, where recruited (i.e. naive) individuals learn the location of a resource by following a leading individual whom has prior information. In such instances, leading individuals travel through a start-stop motion, which allows following individuals to keep track of leaders through direct antennation of the leader's gaster. However, this is where similarities between slavemaking and non-slavemaking raiding/tandem-running behavior ends. *Temnothorax* slavemakers utilize mixed-species raiding parties, often comprised of both slavemakers and slaves. Further separating raiding parties from tandem-

running, slavemaker raiding parties seek out only host colonies and brood for the purpose of enslaving hosts and expanding the enslaved workforce, and are never utilized for food acquisition or nest relocation as in non-slavemaking species.

Broadly, slavemaker raiding and non-slavemaker tandem-running behaviors are characterized by three distinct behavioral components: a scouting component, a leading component, and a following component (Figure 1, Möglich, *et al*. 1974). Scouts perform the highly risky task of searching for and reporting back the location of resources of interest such as food or nesting sites. Like in other social Hymenopterans, scouts are often the oldest and thus most expendable individuals of the colony (Seeley 1983, Dreller 1998), which face many dangers when exploring novel environments. Additionally, prior experience is invaluable for exploration (Seeley 1983, Dreller, 1998). Scouts that do locate a resource of interest then return to their home nest to recruit additional workers to the resource, effectively becoming leaders of the ensuing tandem-runs. Tandem-run leaders physically guide one to a handful of nestmates – followers – to the new resource (Wilson 1959). While leading individuals tend to have first been scouts, those followers that reached the resource of interest via tandem-run can return to the home nest and become leaders by recruiting additional nestmates. Indeed, there is some evidence from *T. albipennis* that the follower orients in a way that best facilitates the learning of the path taken to the destination, and afterwards is then able to effectively guide new nestmates (Franks and Richardson 2006, Franklin and Franks 2012).

Slavemaking species within *Temnothorax* employ a fascinating series of raiding behaviors (Box 1) in order to obtain the brood (and sometimes even adults) of nearby non-slavemaking *Temnothorax* colonies, thereby strengthening their own workforce without incurring the costs associated with raising their own brood. Among the different slavemaking *Temnothorax* species, however, the specific mechanisms employed while raiding a host colony differ: *T. americanus* raiders for example rely upon chemical similarity, mimicking the cuticular hydrocarbon profile of their host population in order to avoid host detection and thus bypass host defensive responses (Brandt *et al*. 2005). *T. americanus* may also release a secretion from the Dufours' gland that elicits confusion within the host colony (Brandt *et al*. 2006); and while it rarely stings to death host defenders, it will use its powerful mandibles in self-defense (Alloway 1979). In stark contrast, however, *T. duloticus* and *T. pilagens* utilize their sting much more often during raiding, often decimating host colonies (Alloway 1979,

Raiding in Temnothorax Box 1

The raiding phenotype is highly complex in *Temnothorax* slavemakers, encompassing countless strategies and behaviors (Figure I.1, Kleeberg *et al*. 2016). While each slavemaking species utilizes a number of species-specific mechanisms when raiding host colonies, *Temnothorax* slavemakers adhere to broadly similar raiding patterns comprised of distinct phases. Thus, the raiding phenotype of *Temnothorax* slavemakers may be generally characterized; though there remains a large degree of variability to slavemaker raids, even within the same species.

Scouting (Figure I.1, Frame 2): Non-reproductive slavemakers raiders must first locate a suitable host nest containing offspring in their pre-pupae and pupal stages. This scouting phase is comprised of a number of host-seeking and path-finding elements, and is characterized by elevated activity levels in scouting individuals. Scouting individuals are not always successful in locating a host nest, and the time required for each scout to locate a host nest varies wildly.

Recruitment of Nestmates (Figure I.1, Frame 3): Once a host nest is located, slavemakers return to their own colony in order to recruit nestmates into a raiding party. The focal scout utilizes antennation and chemical cues in order to stimulate nestmates into action. While it is perhaps expected that scout slavemakers recruit other slavemakers from their colony to form a raiding party, slaves are also recruited for raiding parties. Time taken to recruit nestmates after the scout returns to its home colony varies from minutes to hours.

Deployment of Raiding Party (Figure I.1, Frame 4): Recruited individuals form a raiding party, headed by the original scout which found the host nest. The original scout leads the group of raiders back to the host colony, with the party staying together through repeated physical contact between members where following individuals antennate the gaster of the leading individual. Raiding parties are not always successful, with trailing members often becoming separated and/or lost. Reformation of the party while *en route* does occur, though when a party becomes broken up, a new party is often formed back at the slavemaker nest. It is not uncommon for only one slavemaker individual to make it to the target host nest, under which circumstances the raider might even choose to go forward with the raid.

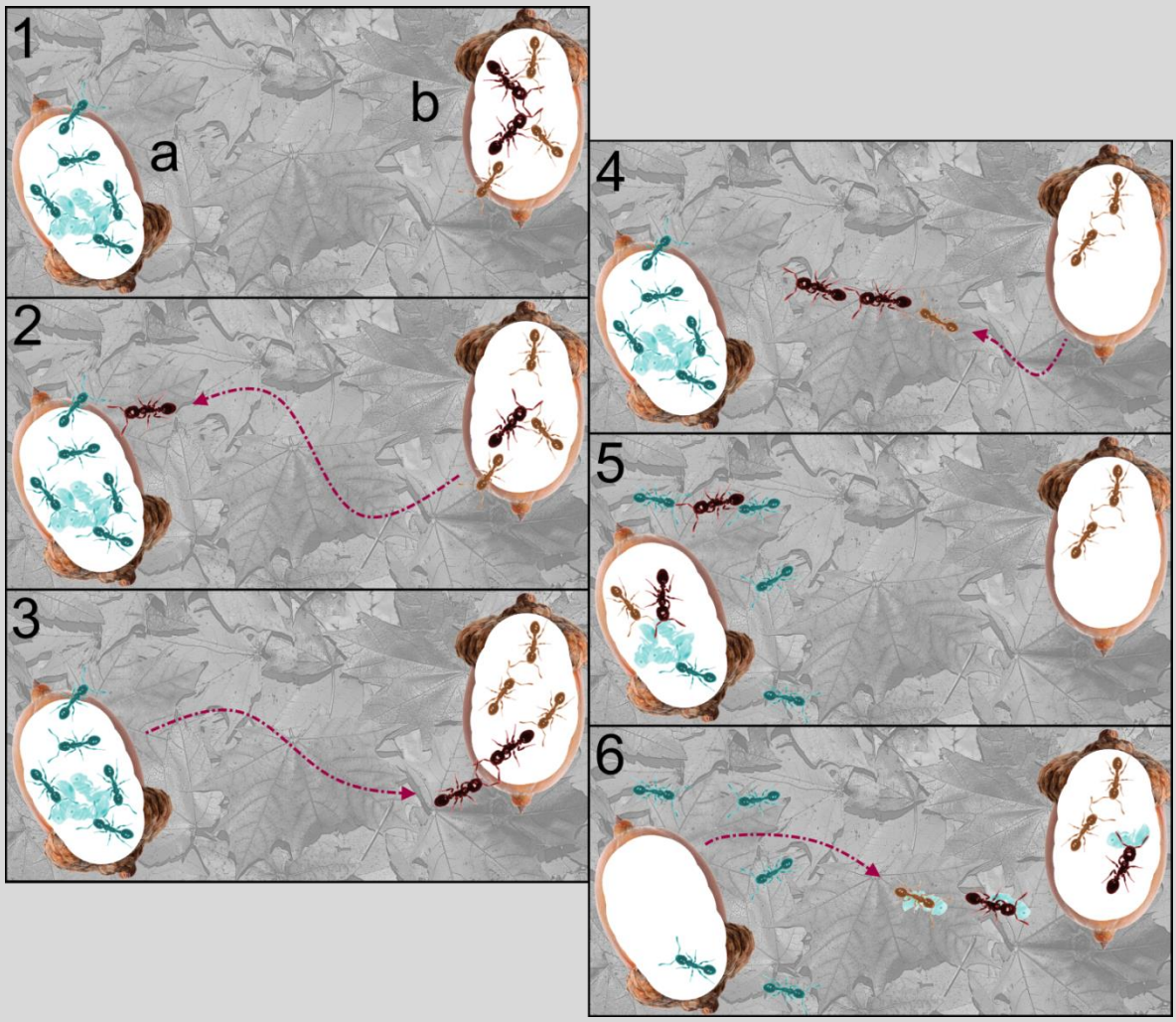

Figure I.1: Stages of a typical raid carried out by *Temnothorax* slavemakers. **Frame 1**: host colony (a) with host individuals (light blue ants) and host brood (light blue pupae), and slavemaker colony (b) with slavemaker workers (red ants) and enslaved host workers (orange ants). **Frame 2**: Scouting by slavemaker workers in order to locate a suitable host colony for raiding. **Frame 3**: Recruitment of nestmates into a raiding party. **Frame 4**: Raiding party, where recruited nestmates follow the original slavemaker scout to a previously-located host colony. **Frame 5**: Execution of raid by slavemakers and slaves against the host colony. **Frame 6**: Transport of brood items by slavemakers and slaves back to the nest.

Execution of Raid (Figure I.1, Frame 5): Raiders that do manage to find the host nest then attempt to avoid or overcome host defenses and steal host brood. Strategies for circumventing nest defenses vary by species, with some species avoiding detection or identification and others killing or driving out host defenders and queen(s). As with the other phases comprising the raiding process in *Temnothorax*, raids are not always successful, and show wide variability in duration and outcome. Utilizing sheer numbers, host defenders can

overcome and immobilize invading slavemakers and slaves. However, once a slavemaker raiding party has located its target host colony, repeated raiding attempts are made until successful.

Transport of Host Brood (Figure I.1, Frame 6): Slavemakers and slaves carry host brood back to the slavemaker colony. Occasionally transportation of host brood occurs while the unsuspecting hosts are still in their nest, though more often this phase takes place after host defenders have been killed or driven from their nest. Time taken to transport host brood varies greatly depending on the number of raiders, number of brood items, distance to slavemaker colony, etc.

Seifert *et al*. 2014, Kleeberg and Foitzik 2015). Curiously, host deaths during raiding result primarily not from host encounters with slavemakers, but from fights between host and slaves of the raiding party (Foitzik *et al*. 2001). Comparative field manipulation studies have shed light onto the differing affects that these slavemakers have upon their hosts, with Hare and Alloway (2001) showing that host populations suffered more from raids by *T. duloticus* than those performed by the phylogenetically older parasite *T. americanus* (Beibl *et al*. 2005); though a later manipulation study revealed that the impact of *T. americanus* upon its host can be quite severe and in fact varies between host populations (Foitzik *et al*. 2009). However, despite differing strategies, the main objectives of raids of all three slavemaking species remain the same: the abduction of live host pupae, which may be incorporated into the slavemaker colony and thus bolstering the workforce.

Much is presently known about the lifestyle, behaviors, and strategies of *Temnothorax* slavemakers. However, much less is known about the molecular mechanisms and patterns associated with this lifestyle. Are there patterns of gene usage which characterize antagonistic behavior within *Temnothorax*? Which genes primarily drive this antagonistic relationship between parasite and host? And do some genes play a larger functional role in slavemaing behavior than others?

Molecular Basis for Behavior

One of the fundamental objectives of evolutionary biology is to elucidate the genetic and molecular mechanisms underlying the origin and diversity of complex traits. It is generally accepted that the evolution of novel complex traits is precluded by gene duplication and sub-functionalization, as well as co-optation of pre-existing networks – rather than the structural alteration of associated genes (Shubin *et al*. 2009). Indeed, the results of numerous studies certainly seem to point towards a "genetic toolkit" of common gene sets repurposed in like-behaviors (e.g. Toth and Robinson 2007; Schrader *et al*. 2015, Rittschoff and Robinson 2016, Qiu *et al*. 2017). While explorations into the development of behavioral phenotypes is far less common, elucidation of the development of morphological characteristics does lend credibility to the hypothesis that the neo-functionalization of existing genes into novel pathways results in the diversification of complex phenotypes (Erclik *et al*. 2008, Kozmik *et al*. 2008, Johanson *et al*. 2007, Emlen *et al*. 2007, Pueyo *et al*. 2008). To complicate matters further, however, recent evidence suggests that behavioral phenotypes are often influenced by epigenetic changes (Ben-Shahar 2017, Kohlmeier *et al*. 2018), adding another layer of complexity onto an already labyrinthine field.

As such, complex behaviors - such as those employed during raids within *Temnothorax* slavemakers – offer a model for elucidating molecular patterns associated with behavior as well as the opportunity to determine the functional impact of individual genes (Thompson 2005). As they are controlled largely through variable gene expression, complex behavioral phenotypes may in general be shaped by a range of internal and external factors. Indeed, previous studies have shown that the modification of molecular pathways of simple behaviors can result in the evolution of highly complex behavioral phenotypes, such as social behavior in insects (Toth and Robinson 2007) and the subsequent exploitation of that sociality (e.g. Aumer *et al*. 2018). However, many described patterns of gene expression influencing behavior are taxa-specific (Cresco *et al*. 2004, Colosimo *et al*. 2005), and single genes can have a strong impact on otherwise complex behaviors. For example, the gene *foraging* alone is responsible for multiple behavioral phenotypes in *Drosophila melanogaster* (Osborne *et al*. 1997), and is also involved in age-related task switching in honey bees (Ben-Shahar *et al*. 2002). Within *Temnothorax* even, the *Vitellogenin-like A* gene strongly affects nestmate response to brood stimulus (Kohlmeier *et al*. 2017). In many cases, however, specificity of gene usage reduces the applicability of findings to large-scale conclusions and necessitates further exploration of novel behavioral phenotypes; as is likely the case for

Temnothorax, which is distantly related at best to most model organisms.

Bioinformatics, Transcriptomics, and RNAi

The exploration of gene expression patterns associated with complex behavior requires the use of some advanced exploratory techniques (Ekblom and Galindo 2011, Nederbragt 2012). Transcriptomic analyses involves next-generation sequencing of the RNA content of cells, tissues, and individuals. Cheaper than DNA-centric genomic analyses, these RNA-Seq approaches allow for the quantification of gene activity at a single point in time. This measurement of gene expression allows for further analyses utilizing bioinformatic/computational tools (Singhal 2012). However, an RNA-Seq approach does have its limitations, including transcriptomic artifacts (such as multiple splice variants per gene) and patterns introduced by *de novo* assembly of reads. This is especially prevalent in non-model organisms where no reference genome exists.

In addition to the more basal gene expression analyses, next-generation sequencing and RNA-Seq open the door for additional downstream analyses, such as functional enrichment analysis through assignment of Gene Ontology (GO) terms, Weighted Gene Co-expression Network Analysis (WGCNA), and RNA-mediated gene knockdown (RNAi). RNAi in particular offers the ability to determine the exact function of specific genes within – in this case – the context of slavemaker raiding behavior. The species-specific sequences of a single gene combined with the expression information of that gene – both obtained through previous RNA-Seq analyses - allows for the precise targeting and knockdown of genes showing high levels of activity during a focal behavior.

This Thesis

Parasites and their hosts are ideal biological systems for the investigation of ecological specialization and co-evolution (de Meeûset *et al.*1998). To date, very little work has focused on exploring the molecular basis of antagonistic interactions between slavemakers and hosts in the ant genus *Temnothorax*. As such, I utilized combined molecular and computational techniques in order to elucidate the co-evolutionary dynamics of *Temnothorax* slavemakers *T. americanus*, *T. duloticus*, and *T. pilagens* and their

respective host species *T. longispinosus*, *T. curvispinosus*, and *T. ambiguus*. In **Chapter One** of this thesis, I describe the implementation of an RNA-Seq approach for the assembly of species-specific transcriptomes for each of the six *Temnothorax* species in order to elucidate the gene expression patterns underlying the raiding phenotype (Figure I.1) in three slavemaking *Temnothorax* species (*T. americanus, T. duloticus, T. pilagens*) and the reciprocal defensive behaviors in three host species (*T. ambiguus, T. curvispinosus, T. longispinosus*). These transcriptomes served as the basis for additional computational investigations: gene annotation, gene expression analysis, assignment of GO terms and functional enrichment, and WGCNA. Transcriptomes were produced by extracting RNA from slavemaking species during their respective raiding phenotypes, and then again after the end of the raiding season. For hosts, RNA was extracted during normal, non-antagonistic behavior and later during a direct encounter with a raiding slavemaker. This method of RNA extraction allows for a differentiation of the various aspects of the molecular mosaic underlying the complex slavemaker raiding and host defensive behaviors.

However, this identification of important behavioral genes offers no insight into the present evolutionary trajectory of co-evolution between *Temnothorax* host and slavemaker. As such, **Chapter Two** builds upon Chapter One, utilizing the same six transcriptomes for the identification of homologues between species; a necessary first step for subsequent detection of signatures of selection. Analysis of selection provides key insights into the genes most important to the continuous co-adaptation between parasite and host in *Temnothorax*. Additionally, genes showing strong signatures of positive selection are suspected to also have important functionality within the focal phenotype.

In addition to providing valuable insight into the molecular patterns associated with slavemaker raiding and host defensive behavior, the results of these analyses informed additional functional and behavioral experiments. Indeed, we suspect that those genes showing particularly high expression activity during the raiding phenotype (as determined in Chapter One) play an important role within that context. While predictions about the function of those genes may be made based upon the functional annotations from other species, more precise functional classification may only be carried out through context-specific experimentation. **Chapter Three** further explores gene function within the context of slavemaker raiding behavior. We discuss the RNAi-mediated knockdown of the *T. pilagens* version of *Trypsin Inhibitor* during raiding behavior, and the impact that this knockdown has upon the raiding phenotype - and ultimately - raiding effectiveness of *T. pilagens*. The way in

which raiding success is affected allows for the elucidation of the function of *Trypsin Inhibitor* within the context of slavemaker raiding behavior.

Finally, in **Chapter Four** we take a closer look at the molecular similarity between the externally-similar recruitment behaviors of *Temnothorax* hosts and slavemakers: tandem-running and raiding, respectively. Here, we performed tandem-running/raiding behavioral experiments with *T. americanus* and its primary host *T. longispinosus*, followed by RNA-Seq analyses upon brain tissue only in order to isolate genes with significant up-regulation during scouting, leading, or following behaviors. Given the shared evolutionary origin of *T. americanus* and *T. longispinosus*, as well as the external similarity of these behaviors, we did initially expect that many genes share expression patterns between these two species. In addition to this, as tandem-running behavior represents a form of information exchange, teaching, and learning, we also expected that genes found in other social organisms to be involved in these processes to be similarly differentially-expressed in *Temnothorax* as well.

Chapter 1

Comparative Analyses of Co-Evolving Host-Parasite Associations Reveal Unique Gene Expression Patterns Underlying Slavemaker Raiding and Host Defensive Phenotypes

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Based on
Scientific Reports **8**:1951 (2018)

Abstract

The transition to parasitism is a drastic shift in lifestyle, involving rapid changes in gene structure, function, and expression. After the establishment of antagonistic relationships, parasites and hosts co-evolve through reciprocal adaptations, often resulting in evolutionary arms-races. Repeated evolution of social parasitism and slavery among *Temnothorax* ants allows us to examine those gene expression patterns that characterize slavemaker raiding and reciprocal host defensive phenotypes. Previous behavioral studies have established that raiding strategies between *Temnothorax* slavemakers diverge, while host defense portfolios shift similarly under parasite pressure. We are the first to confirm this at the molecular level, revealing that slavemaking species exhibit a wider variety of genes with species-specific patterns of expression within their raiding phenotypes, whereas expression similarity is commonly found during the non-raiding phenotype. Host species response to slavemaker aggression, however, is indicated by strong changes in the expression of a relatively few number genes. Additionally, the expression of individual genes such as *Acyl-CoA-Delta(11) desaturase* and *Trypsin-7* is strongly associated with the raiding phenotype of all three slavemaking species. Here, we provide novel insight into the gene expression patterns associated with raiding and nest defense behavior in *Temnothorax* ants, suggesting lineage-specific evolutionary patterns among both slavemakers and hosts.

Keywords: evolutionary arms-race, gene expression, transcriptomics, social parasites, *Temnothorax*

Background

Understanding the processes that shape the evolutionary trajectories of organisms is a long-standing goal of the biological sciences. Parallel and convergent evolutionary patterns are of particular interest, raising questions as to the predictability and repeatability of evolution. Understanding the molecular mechanisms leading to the repeated evolution of similar phenotypes allows for the elucidation of factors that shape biological diversity. Phenotypic convergence can arise through many molecular mechanisms, where similarities can occur at a number of different hierarchical levels (nucleotide, gene, pathway, etc., Rosenblum *et al*. 2014). As genetic constraints can strongly influence the probability of convergent or parallel evolution, the occurrence of similar phenotypes is more likely within closely-related lineages containing similar genetic and molecular repertoires. Co-evolution among parasites and their hosts offers a unique and ideal system in which to investigate how convergent and parallel evolution affect ecological diversification (de Meeûs *et al*. 1998). Relatedness between parasite and host can vary widely between taxa. While some parasites are only distantly related to their hosts, such as viruses and their human hosts, many parasites – such as avian brood parasite – share close phylogenetic ties (Davies and Brooke 1989). There are few systems where closely-related parasites and hosts occur as frequently as in social insects. Characterized by variable phenotypes and complex social systems, insect societies are particularly susceptible to exploitation by closely-related taxa (Buschinger 1993, Kurze *et al*. 2015). Often occurring as a mechanism of circumventing the cost of parental care, social parasitism is widespread within bees (Alford 1975, Kupper and Schwammberger 1995, Cameron *et al*. 2007, Hines and Cameron 2010, Tierney *et al*. 2008, Smith *et al*. 2013, Gibbs *et al*. 2012) and ants (Seifert 2007, Buschinger 2009) and not uncommon within wasps (Choudhary *et al*. 1994, Cervo 2006, Carpenter and Perera 2006).

Within the ant genus *Temnothorax,* social parasitism has evolved multiple times independently (Beibl *et al*. 2005, Feldmeyer *et al*. 2017, Prebus 2007). As such, many characteristics ideal for investigating the molecular basis of phenotypic traits associated with social parasitism may be found within this genus. 1] *Social parasites are often closely-related to their hosts, and as such share genetic ancestry*; and *Temnothorax* is no exception. One small North American clade is comprised of three slavemakers (*Temnothorax americanus*, *T. pilagens*, and *T. duloticus*) and their three closely related host species (*T. ambiguus*, *T. longispinosus*, and *T. curvispinosus*). The obligate slavemaking species of this taxon, *T. americanus* (genus name recently changed from *Protomognathus* (Ward *et al*. 2015)), *T.*

duloticus, and *T. pilagens,* all display active raiding and slavemaking behavior (Wesson 1939, Alloway 1979, Kleeberg and Foitzik 2016).

2] *Socially-parasitic species have lost many traits essential for a free-living lifestyle*. Slavemakers reside within their own mixed-species nests, but carry out destructive *'slave raids*' against nearby host colonies in order to steal brood and thus strengthen their own workforce (Buschinger 1993). From these stolen host larvae and pupae, a new generation of slaves will develop, which will subsequently carry out all routine worker tasks – such as brood-care and foraging - in the slavemaker nest. In stark contrast, slavemaker workers have almost completely lost the ability to work, and instead are highly specialized for raiding (Hölldobler and Wilson 1990). Slavemaker workers and queens alike have developed mechanisms to subvert, disrupt, or otherwise bypass ordinary host recognition and communication systems (D'Ettorre and Heinze 2001, Brandt *et al*. 2005, Achenbach *et al*. 2010, Kleeberg *et al*. 2017). In addition to slavemaker-specific morphological characteristics such as powerful mandibles (Wesson 1939), potent stingers (Alloway 1979, Kleeberg *et al*. 2016), and enlarged petioles (Hölldobler and Wilson 1990), slavemakers often employ the Dufour's gland and other glandular secretions to manipulate hosts (Brandt *et al*. 2006, Jongepier and Foitzik 2015), mimic host profiles (Guillem *et al*. 2014), or obtain recognition cues directly from hosts in order to camouflage themselves (Johnson *et al*. 2001).

Indeed, this rapid diversification of species-specific mechanisms and strategies within social parasites is a direct indication of 3] *increased phenotypic diversity, a result of loosened phenotypic constraints associated with the transition away from a free-living lifestyle* (Cini *et al*. 2015). The most phylogenetically distant *Temnothorax* slavemaker, *T. americanus* – which split from its non-parasitic ancestors between 22 and 12 million years ago (Prebus 2007) – is also the most behaviorally and morphologically distinct (Figure 1.1). This social parasite is able to exploit all three host species within this clade, *T. longispinosus*, *T. ambiguus*, and *T. curvispinosus;* though it clearly prefers *T. longispinosus* (Brandt and Foitzik 2004). *T. americanus* utilizes glandular secretions to manipulate host defenders (Alloway 1979, Brandt *et al*. 2006, Jongepier and Foitzik 2015), whereas raids conducted by *T. duloticus*, primarily against *T. curvispinosus*, tend to be more destructive since *T. duloticus* workers are much more prone to stinging host defenders to death (Alloway 1979, Hare and Alloway 2001, Johnson and Herbers 2006, Johnson 2008). *T. pilagens* preferentially raids *T. ambiguus* colonies, though will opportunistically target *T. longispinosus* as well (Seifert *et al*. 2014). Unique among *Temnothorax* slavemakers, raids by *T. pilagens* often remain peaceful,

as hosts appear to be unable to recognize invading *T. pilagens* raiders (Kleeberg and Foitzik 2016). In such cases, slavemakers are even able to lead adult host workers into slavery. In those raids where it is recognized as an enemy, *T. pilagens* responds violently to host attacks, stinging defending hosts and imparting high mortality upon the host colony (Kleeberg and Foitzik 2016).

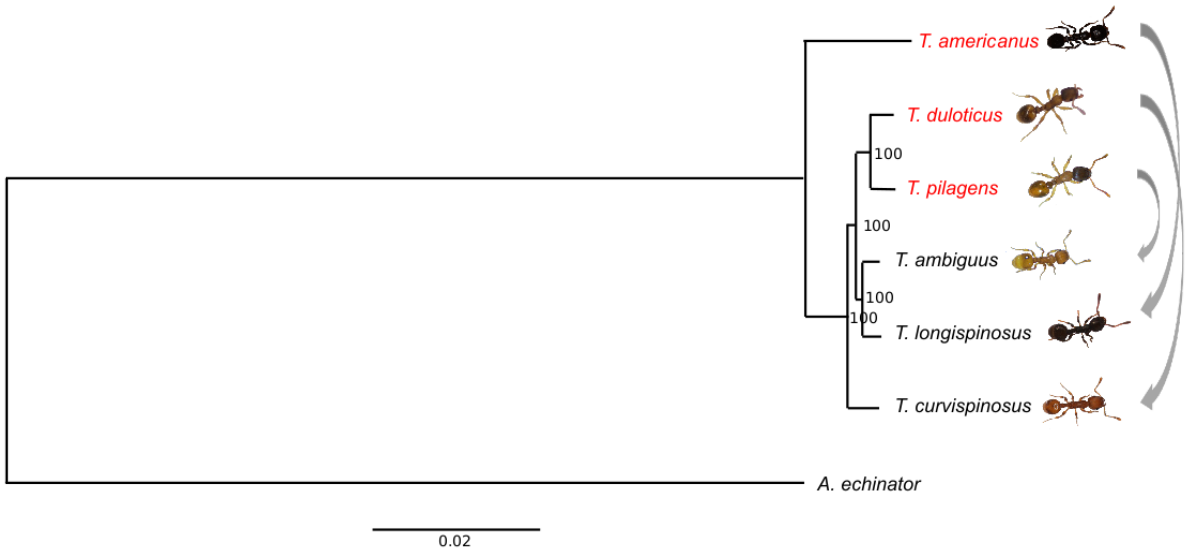

Figure 1.1: Maximum Likelihood phylogenetic relationship between the six *Temnothorax* species herein examined, with *Acromyrmex echinatior* as outgroup (used with permission: Feldmeyer *et al*. 2017). Produced using RaxML based on 5199 orthologous gene clusters (ML boostrap values given at each node). Slavemaker *T. americanus* preferentially parasitizes *T. longispinosus*, *T. duloticus* preferentially parasitizes *T. curvispinosus*, and *T. pilagens* preferentially parasitizes *T. ambiguus*.

Presented with ever-evolving mechanisms of subversion and aggression by slavemaking species, hosts respond through defensive mechanisms that minimize the loss of individuals to raiding or aggression (Dawkins and Krebs 1979, Jongepier *et al*. 2014, Kleeberg *et al*. 2015), highlighting that 4] *parasite and host are engaged in an evolutionary arms race*. During slave raids, host colonies not only lose their brood, but workers and queens often die during defense of the colony. Accordingly, these raids can exert high fitness costs upon host colonies (Hare and Alloway 2001, Foitzik and Herbers 2001, Foitzik *et al*. 2003, Foitzik *et al*. 2009). This results in continuous adaptation and counter-adaptation on both sides, with parasites having the advantage in some cases (Grasso *et al*. 1992, Foitzik *et al*. 2001), and hosts in others (Foitzik *et al*. 2001, Molina-Morales *et al*. 2014).

Additionally, *Temnothorax* slavemakers and hosts alike display complex behaviors that are easily observed and recorded in a laboratory setting. This facilitates smooth integration of both molecular and phenotypic studies. These above factors, taken together, allow for effective elucidation of the molecular components underlying the behavior and physiology of organisms with shared evolutionary history, as well as the examination of similarities and differences in phenotypes arising within the same environmental context.

The objectives for this project are informed by three primary assumptions about the molecular evolution of social parasitism, first outlined by Cini *et al*. (2015): 1] Firstly, *trait novelty or commonality will be reflected at the molecular level.* Within closely-related species, phenotypic diversification often operates through gene-regulatory shifts, rather than sequence alterations to protein-coding genes (Britten and Davidson 1969, King and Wilson 1975). 2] *Novel molecular processes underlie lineage-specific phenotypes*. While groups of conserved genes associated with convergent social behaviors have been found in a number of eusocial insects (Toth and Robinson 2007, Fischman *et al*. 2011, Woodard *et al*. 2011, Toth *et al*. 2014), more recent work has also revealed that eusocial lineages can harbor novel genes that are associated with eusocial behaviors (Ferreira *et al*. 2013, Simola *et al*. 2013, Feldmeyer *et al*. 2014, Sumner 2014). 3] Lastly, that *conserved regulatory processes underlie the response to a shared environment*. In contrast to raiding behavior, *Temnothorax* slavemakers display much more behavioral similarity when out of raiding season, universally possessing a reduced capacity for normal nest-work and a less active lifestyle. Given this, we might expect that gene regulation in this phenotype may indeed be more similar between species, certainly more so than expression patterns during the raiding phenotype. Here, we utilized an RNA-Seq approach following behavioral experiments in order to identify regulatory patterns involved in slavemaker raiding behavior and host defensive behavior by focusing on the three slavemakers (*T. americanus*, *T. pilagens*, and *T. duloticus*) and their three preferred host species (*T. ambiguus*, *T. longispinosus*, and *T. curvispinosus*). Workers of these species were collected during two different behavioral phenotypes: a raiding and a non-raiding phenotype. For hosts, the raiding phenotype is typified by active nest defense against a slavemaker raid, and the non-raiding phenotype characterized by a normal, non-antagonistic nest-life. For slavemakers, the raiding phenotype is typified by active slave raiding behavior aimed at subverting host defenses and stealing host brood, and the non-raiding phenotype characterized by slavemakers within their own nests outside of raiding season. Workers of host species were collected 1] before any contact with slavemakers, and 2] during active nest

defense from raiding slavemakers; and slavemakers workers collected 1] out of raiding season, and 2] during a slavemaking raid. The use of separate behavioral phenotypes allows for the disentanglement of intra-species (between behavioral phenotypes of a single species) and inter-species (within behavioral phenotypes across species) signatures of differential gene expression. Thus, by comparing three slavemaker and three host species, we were able to elucidate whether or not raiding or defensive strategies evolved along independent, species-specific trajectories, or whether these behaviors evolved in parallel within this genus.

Methods

Sample Collection and Raiding Experiment

Ant colonies were collected in spring 2012 and 2013 at three locales in the Northeastern US (Supplementary Table S9, see source publication) and transported in Ziploc bags within their natural nest sites. Upon arrival to the laboratory at the Johannes Gutenberg University in Mainz, each colony was transferred into its own plaster-floored nesting box, containing a single slide-nest into which the colony relocated. A slide nest is an artificial nesting site comprised of a small Plexiglas cavity sandwiched between two glass microscope slides. Colonies were then kept under a constant 20°C, 14L:10D light cycle and were fed twice weekly with honey and crickets. All colonies used in raiding experiments were transferred to 25°C, 14L:10D light cycle conditions one week prior to the onset of the raiding experiment in order to promote an increase in scouting and raiding activity in slavemakers.

Each slavemaker species was allowed to raid colonies of its preferred host species from the same community. Raids using colonies of *T. americanus* vs *T. longispinosus* from the New York site, and *T. duloticus* vs. *T. curvispinosus* from the Ohio site were conducted in 2012, whereas raids involving *T. pilagens* vs. *T. ambiguus* from Michigan were conducted in 2013. All 36 raids, i.e. 12 per host-slavemaker pair, were performed in the year of collection. On each day of the raiding experiments, five raiding arenas were set up in the laboratory, into which a host and a slavemaker colony were placed. This setup gave slavemakers the opportunity to raid a host nest. If a slavemaker performed no raid on a specific day, both host and slavemaker colonies were placed back in their respective nest boxes overnight, and the experimental procedure repeated again on the following day until a successful raid occurred (for a maximum of 14 days). On the first day of each raiding experiment, two foragers (outside individuals) were removed per host colony as “before raid” individuals for later

transcriptome analyses. Then one host colony and one slavemaker colony (residing in their slide-nest) were transferred to diagonally-opposite corners of a 30 x 40 cm plastic box with plastered floor. The plaster was kept moist throughout the experiment to prevent desiccation. Within this raiding chamber, opposite the slavemaker colony, honey and water was provided. Colonies were observed continuously until the onset of a raid. We waited until slavemaker scouts had returned to their mother nest and recruited additional raiders in order to infiltrate the host nest, and aggressive encounters could be observed between slavemaker and host workers. At this point, two slavemaker and two host workers per colony, directly engaged in aggressive interactions just outside of the artificial nest, were collected. As slavemaker workers show raiding activity - i.e. leave the colony in search for host nests throughout the raiding season from July to September - we decided to wait until mid-October to collect "out of raiding season" slavemakers. Two weeks before sampling in fall, colonies were again moved to 25°C, 14L:10D light cycle conditions, so that environmental conditions were the same as during the raiding experiments in summer. Only infertile slavemakers engage in raiding activity, whereas fertile slavemaker workers remain in the nest during the raiding season (Pohl *et al*. 2011, Blatrix and Herbers 2004), so that we dissected the ovaries of slavemakers to select infertile workers during both sampling points. Laboratory-based raiding experiments were deemed acceptable given the inherent difficulty – due largely to the small size of individuals and low number of workers involved in raiding parties – in facilitating and observing raids within the field. Indeed, even in the field, the foraging and raiding ranges of *Temnothorax* tends to be short (Heinze *et al*. 1996). Additionally, living and laboratory conditions could be standardized for all colonies throughout the experiment.

Unfortunately, the intrinsic nature of our system does not allow for a "clean" experimental set up. Raiding versus non-raiding slavemakers could have been sampled in two different ways: either by comparing younger, fertile stay-at-home slavemakers with older, infertile raiding slavemakers; or by comparing infertile raiders to the same behavioral caste outside of the raiding season. Both approaches introduce method-specific confounding factors: fertility and age using the first approach, and physiological makeup of individuals due to seasonal differences using the latter. The latter method was ultimately chosen, as we expected that seasonal changes would influence gene expression far less strongly than fertility and age - especially when external conditions, such as temperature and humidity, are kept the same for all colonies. As hosts alter their behavior in the long term after a slavemaker encounter, generally in the form of persistent elevated aggression (Kleeberg *et al*. 2015, Pamminger *et al*. 2011), we decided to sample host workers on the day of the raids,

just before slavemaker contact, as well as during raids. All workers collected during this experiment for later transcriptome analysis were transferred directly into 500µl Trizol (Invitrogen) and homogenized before freezing and stored at -80°C. Ants were sampled at different times of day; since it is impossible to plan or instigate a raid, individuals must be collected whenever a raid takes place, independent of time of day. Additionally, daytime variation between individuals should be canceled out by our pooling strategy (see below).

RNA Isolation and Sequencing

As we are less interested in individual differences in gene expression, but in general changes associated with specific behavioral *and* physiological states, we pooled whole bodies of six individuals per replicate (two individuals from three colonies each, Feldmeyer *et al*. 2014). We pooled the same colonies across treatments to keep possible colony-dependent variation constant across treatments. In total, four samples per species per behavioral phenotype were generated, resulting in a total of 48 samples. While pooling does indeed result in a loss of data at the individual level, this elimination of individual variation also strengthens common signals (Feldmeyer *et al*. 2014). For example, here, since time of day (of collection) cannot be controlled for due to the unpredictable nature of raiding, pooling individuals of the same colony mitigates variation due to collection time. Additionally, physiological factors can influence behavior in insects (Blanchard *et al*. 1999, Ben-Shahar *et al*. 2002). As such, focusing our RNA-Seq approach entirely on brains, for example, excludes all physiology-associated molecular signals, which might have important down-stream affects upon behavior.

For RNA isolation, we followed the protocol of the Center for Genomics and Bioinformatics Bloomington (https://dgrc.bio.indiana.edu/include/file/CGB-TR-200610.pdf). In short, 200 µl chloroform was added to each Trizol sample and the mixture shaken vigorously for 15 seconds, and then centrifuged for 15 min at 4°C and 11,000xg. The upper aqueous phase was transferred to a new 1.5 ml RNase-free tube and precipitated with 200 µl of absolute ethanol. The solution was gently pipetted four times and transferred to an RNeasy mini-spin column (Qiagen). Further procedure followed step three onwards of the RNeasy Clean-Up manual (Qiagen). Illumina library preparation with individually marked (MID) libraries was performed through GENterprise Genomics, affiliated with Mainz University (http://www.genterprise.de/), and paired-end sequenced on an Illumina HiSeq 2000. Raw reads were analyzed for quality using FastQC v0.11.2, and Illumina adapters cut from all sequences with Trimmomatic v0.32 (Bolger *et al*. 2014) using the following parameters:

2:30:10:8:TRUE LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:20 HEADCROP:13. Reads may be obtained from NCBI short read archive (Accession Number GSE95604).

De novo transcriptome assembly

After analyzing the quality of our initial data, as obtained from the sequencing facility, we tested a number of *de novo* assembly and analysis approaches to determine which method yielded the best-assembled transcriptomes. Assembly methods examined included standard, short-read assembly approaches using Trinity (Grabherr *et al*. 2011) and CLC Workbench v.7.0.3 (https://www.qiagenbioinformatics.com/), followed by a meta-assembly approach using EvidentialGene (https://sourceforge.net/projects/evidentialgene/) and MIRA (Chevreux 2005). For meta-assembly of the transcriptome, a same-mixed pattern of replicate-matching was utilized (BeforeRaid1-BeforeRaid2, BeforeRaid3-DuringRaid3, DuringRaid1-DuringRaid2, BeforeRaid4-DuringRaid4) for the CLC Workbench phase of the assembly. Using all reads at once decreased assembly quality, and we therefore decided for a step-wise assembly approach. Default parameters were used for these CLC assemblies except for 'bubble size' – auto, and a 'word size' of 35. Final transcriptome assembly was performed with MIRA, using CLC Workbench contigs as input. Trinity and EvidentialGene assemblies were performed using default settings. Transcriptome assembly was followed by the removal of redundant and/or low-confidence contigs from each transcriptome using CD-Hit-Est v.4.6.1 (Li and Godzik 2006).

After the successful assembly of all transcriptomes, we looked at a number of factors to assess assembly quality including 1] total number of contigs, 2] average contig length, 3] percent coverage, and 4] contig BLAST hit rate. Qualimap v.2.1 (García-Alcalde *et al*. 2012) was used to view the .bam output from TopHat v.2.0.13 (Trapnell *et al*. 2009), as well as determine the average number of raw read hits of each base within a contig. Based on the above-mentioned analyses we decided to use the CLC+MIRA meta-assembly for the following analyses. Summary statistics for each assembly can be viewed in Supplementary Table S1.

Differential Gene Expression Analyses

In order to determine which genes were most active during a specific behaviour, we utilized a gene expression approach in which we compared expression levels within a single species, between the two behavioural phenotypes. To accomplish this, we used EdgeR

v3.9.12 (Robinson *et al*. 2009), an add-on package for R v.3 (R Core Team, 2015). TopHat v2.0.13 (Trapnell *et al*. 2009), in conjunction with Bowtie 2 v2.1.0 (http://bowtie-bio.sourceforge.net/) were used to align reads to their corresponding contigs. eXpress v1.5.1 (http://bio.math.berkeley.edu/eXpress/) was used to obtain read count information. After initial expression analyses, PCA and NMDS analyses were performed in R using 'vegan' and 'MASS' libraries to determine whether samples grouped primarily by species or by phenotype (Supplementary Figure S1), and how well samples grouped within a species (Supplementary Figure S2 and S3). While all gene expression analyses are based upon all species-specific contigs, between-species comparisons are based upon lifestyle (slavemaker or host)-specific orthologous clusters. These ortholog sequence clusters were constructed using OrthoMCL 2.0.9 (Li *et al*. 2003) during a sister study (Feldmeyer *et al*. 2017). Thus, to elucidate which differentially-expressed genes (DEGs) were shared between species, we utilized these orthologous clusters, matched with previously-determined DEGs, in combination with Venny v2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/). Next, we elucidated the broad functionality of DEGs through both functional analysis as well as metabolic pathway mapping. Contigs within all assemblies were functionally annotated, using NCBI's BlastX v.2.2.30 against NCBI's November 2014 non-redundant arthropod database. Functional enrichment analyses were performed using the Enrichment Analysis (f-test) functionality of Blast2GO Pro v3.2/3.3 (https://www.blast2go.com/) with default settings. We utilized the KEGG Automatic Annotation Server (KAAS) to assign KEGG Orthology (KO) terms to contigs (Moriya *et al*. 2007). Acquisition of KO terms for contigs was followed by the use of KEGG Mapper (Reconstruct Pathway) to obtain pathways associated with each KO term (http://www.genome.jp/kegg/tool/map_pathway.html).

The number of differently expressed genes and pathways between species were compared using chi-square tests. Differences in log fold change of DEGs were analyzed with a general linear-mixed model (lmer function implemented in the lme4 package, Bates *et al*. 2014. All statistical analyses were conducted in R v. 3.3.1. Finally, weighted gene co-expression network analysis (WGCNA) was performed in R using package WGCNA (Langfelder and Horvath 2008), followed by Kruskal-Wallis tests using package ggpubr in order to determine how the expression of groups of contigs is associated with shifts in phenotype. Initial clustering for the production of dendrograms was carried out using the WGCNA sub-function Hclust with default parameters.

Results

Transcriptome Sequencing and Assembly

In total, we obtained 1.16E+09 raw reads across all six species, with an average of 24-million raw reads per replicate (Supplementary Table S1). Finalized transcriptomes vary from 43,664 contigs (*T. ambiguus*) to 79,227 contigs (*T. curvispinosus*), with N50 values ranging from 2,973 to 3,606 (Supplementary Table S2). The *T. ambiguus* transcriptome is the smallest, whereas the *T. curvispinosus* transcriptome is the largest. Total number of contig BLAST annotations varied between 16,433 and 31,636, with single gene annotations ranging from 10,206 in *T. pilagens* to 18,396 in *T. curvispinosus* (Supplementary Table S1 and S2).

Gene Expression and Weighted Gene Co-Expression Network Analyses

DEGs were determined to be up-regulated in either one of two phenotypes: a *raiding* and a *non-raiding* phenotype. A total of 3,381 genes were found to be differently-expressed between these two phenotypes of the slavemakers (*T. americanus*: 975, *T. duloticus*: 890, and *T. pilagens*: 1616; Figure 1.2) and 697 genes differentially-expressed within the hosts' two phenotypes (*T. longispinosus*: 209, *T. curvispinosus*: 108, and *T. ambiguus*: 380; Figure 1.2; complete lists contained in Supplementary Tables S3 and S4). Examination of each slavemaker-host pair revealed that the ratio of DEGs from all expressed genes was higher in slavemakers than their preferred host species (*T. amer - T. longi:* $\chi^2 = 531.1$; $p < 0.0001$; *T. dul – T. curvi:* $\chi^2 = 1133.6$; $p < 0.0001$; *T. pila – T ambi*: $\chi^2 = 1916.8$; $p < 0.00001$). Slavemaking species also displayed fewer genes up-regulated during raids when compared to the non-raiding phenotype (*T. amer:* $\chi^2 = 20.9$; $p < 0.0001$; *T. dul:* $\chi^2 = 49.2$; $p < 0.00001$; *T. pila.* $\chi^2 = 81.9$; $p < 0.00001$). In contrast, however, we found no difference in the ratio of DEGs between phenotypes within two hosts, and, in the case of *T. longispinosus,* an *increased* number of genes up-regulated during nest defense (*T. longi:* $\chi^2 = 7.4$ $p < 0.01$; *T. ambi.* $\chi^2 = 0.3$; $p = 0.56$; *T. curvi.* $\chi^2 = 0.3$; $p = 0.58$). Additionally, hosts possess a greater proportion of genes up-regulated during their defense when compared to the number of genes up-regulated during the slavemakers raiding phenotype ($\chi^2 = 86.6$, $p < 0.0001$). Lifestyle-specific dendrograms produced during weighted gene co-expression network analysis (WGCNA) (Figure 1.3) also revealed that slavemaker samples clearly cluster first by species and secondarily by raiding phenotype. However, while hosts also cluster primarily by species, secondary clustering by phenotype is far less apparent.

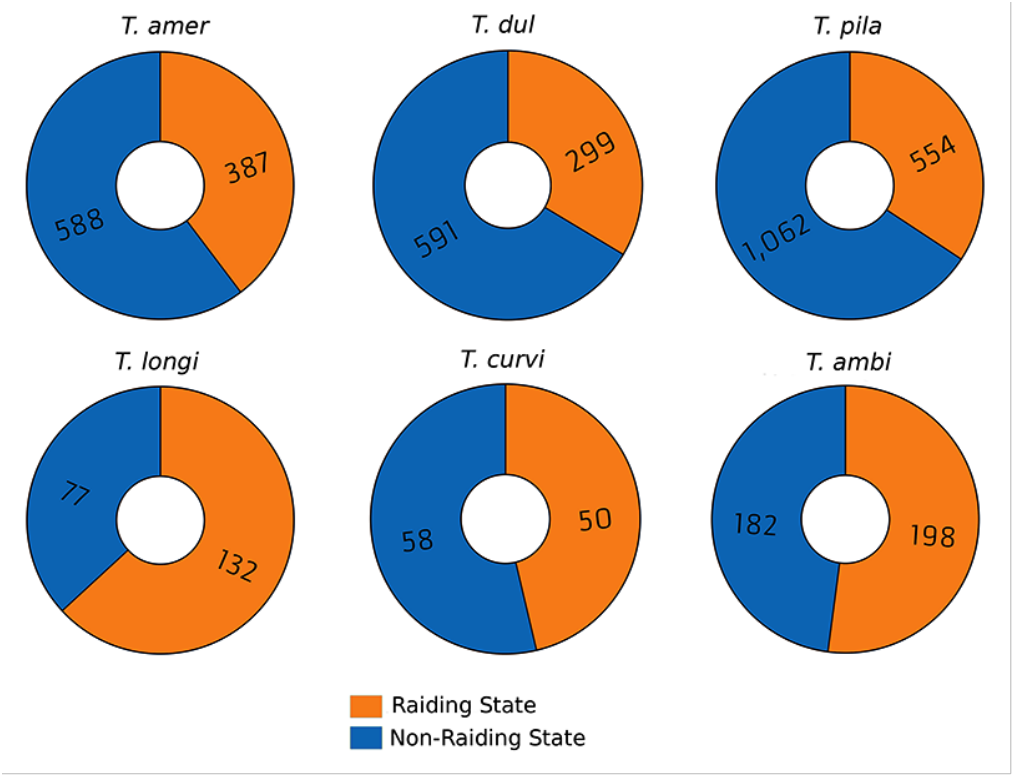

Figure 1.2: Number of genes found to be differentially-expressed within six *Temnothorax* species, up-regulated during either slavemaker or host raiding or non-raiding behavioral states. Upper row: Slavemakers; Bottom row: Hosts. Species abbreviations are as follows: *T. ambi: T. ambiguus, T. curvi: T. curvispinosus, T. longi: T. longispinosus, T. amer: T. americanus, T. dul: T. duloticus, T. pila: T. pilagens.*

Providing additional insight into the differences in expression patterns between slavemaker and host, we also find that while slavemakers had in total more genes differentially expressed, the log fold change of DEGs was *lower* in slavemakers when compared to hosts (lmer: lifestyle: $\chi^2 = 9.14$; $p < 0.0025$; behavior: $\chi^2 = 1.69$; $p = 0.19$; lifestyle *x* behavior, $\chi^2 = 9.44$; $p < 0.0022$; Figure 1.4). Moreover, in slavemakers, the many up-regulated genes during the raiding phenotype shifted their expression *less* than up-regulated genes in the non-raiding phenotype, whereas the opposite holds true for the hosts (Figure 1.4).

In order to determine which genes shared similar expression patterns between species, we utilized gene cluster orthologs produced during a sister study (Feldmeyer *et al.* 2017). The resulting Venn diagrams (Figure 1.5) allowed us to visualize the number of genes possessing species-specific or shared expression patterns between species and phenotype.

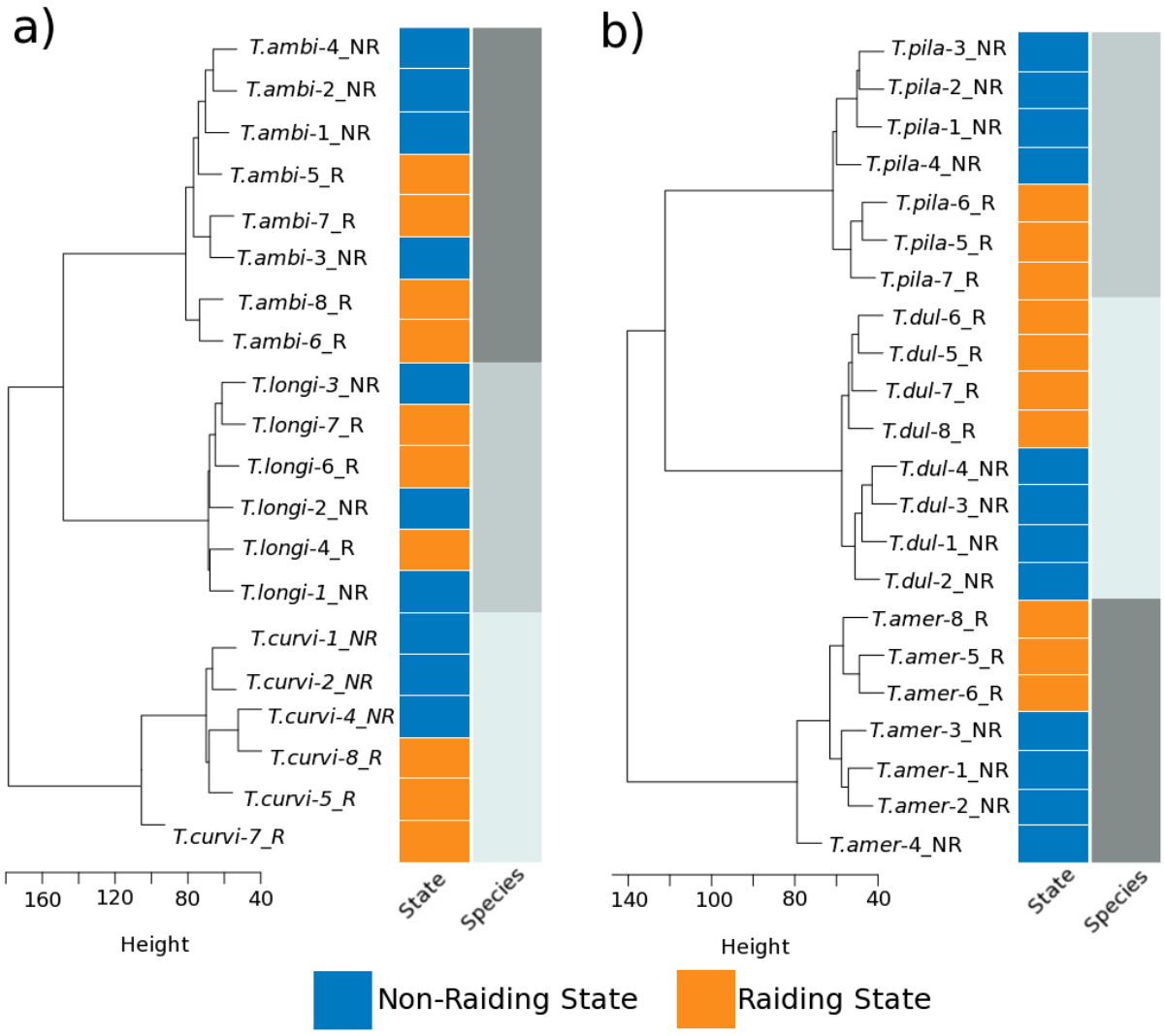

Figure 1.3: Dendrograms resulting from WGCNA of orthologous clusters. (a) WGCNA of host-specific clusters shows that samples group primarily according to species. Phenotype does not appear to have a strong influence on host grouping. (b) WGCNA of slavemaker-specific clusters yields patterns of grouping driven first by species and then secondarily by phenotype. Unlike hosts, phenotype does appear to be associated with more similar gene expression patterns within slavemakers.

Within both slavemaker and host non-raiding phenotypes, the proportion of genes a] sharing expression patterns within at least two species ($\chi^2 = 8.037$; $p = 0.005$), and b] possessing species-specific patterns of expression ($\chi^2 = 13.230$; $p < 0.0001$), was higher in slavemakers. We found no difference in the ratio of commonly to privately expressed genes between slavemakers and hosts during their respective raiding phenotypes ($\chi^2 = 0.662$; $p = 0.416$), though between non-raiding phenotypes, slavemakers show a trend towards a higher proportion of genes up-regulated compared to hosts ($\chi^2 = 3.807$; $p = 0.0511$).

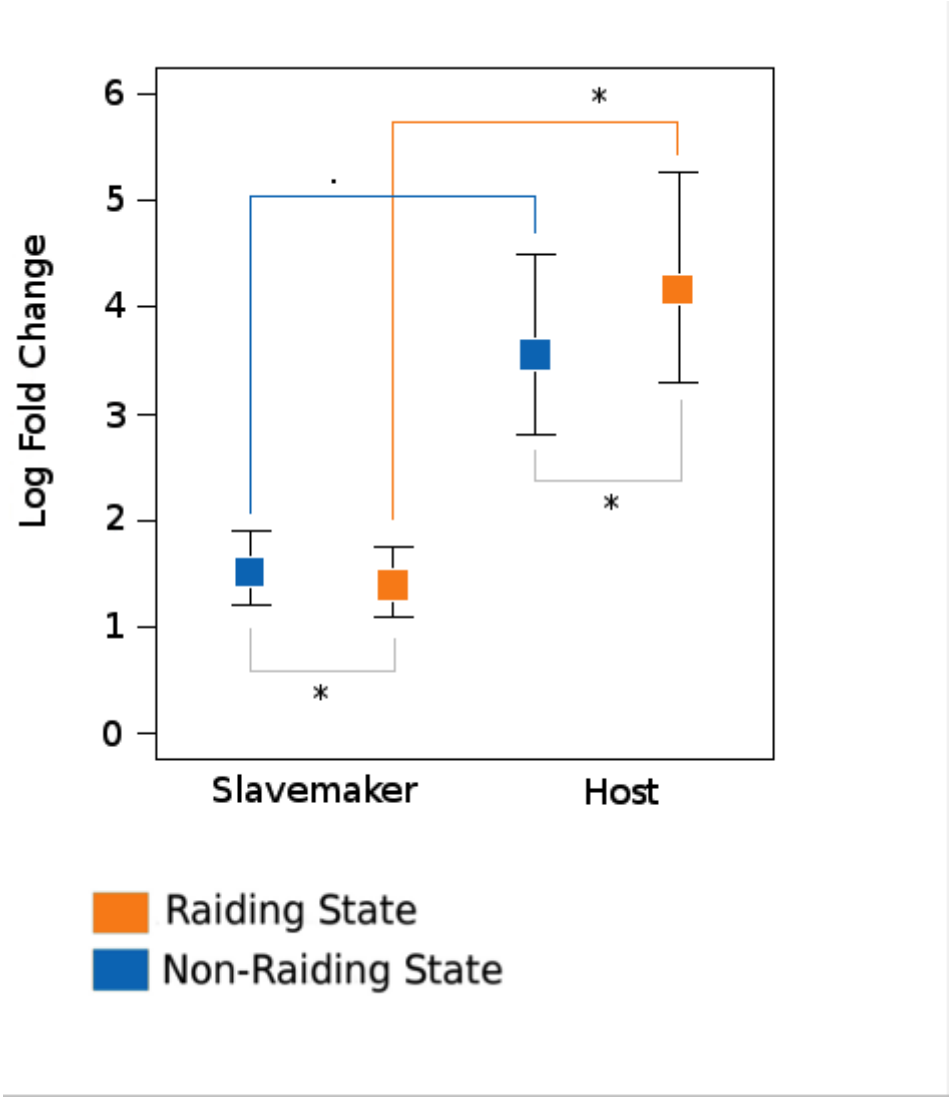

Figure 1.4: Average log fold change of differentially-expressed genes between behavioral phenotypes of hosts and slavemakers herein examined. While non-raiding phenotypes only show a trend towards differentiation between slavemaker and host ($p < 0.059$), the log fold change in expression between slavemaker and host during the raiding/defensive phenotypes ($p < 0.028$) does differ significantly. Log fold change within lifestyle groups between raiding and non-raiding phenotypes differ significantly for both slavemakers ($p < 0.013$), as well as hosts ($p < 0.028$).

Additionally, during the raiding phenotypes of all species examined, we note no significant difference in the ratio of genes commonly up-regulated ($\chi^2 = 3.47$; $p = 0.062$), up-regulated by only two species ($\chi^2 = 0.02$; $p = 0.881$), or privately up-regulated ($\chi^2 = 0.662$; $p = 0.416$), between lifestyles. A list of DEGs and their expression stats may be found in Supplementary Table S4 (see source publication).

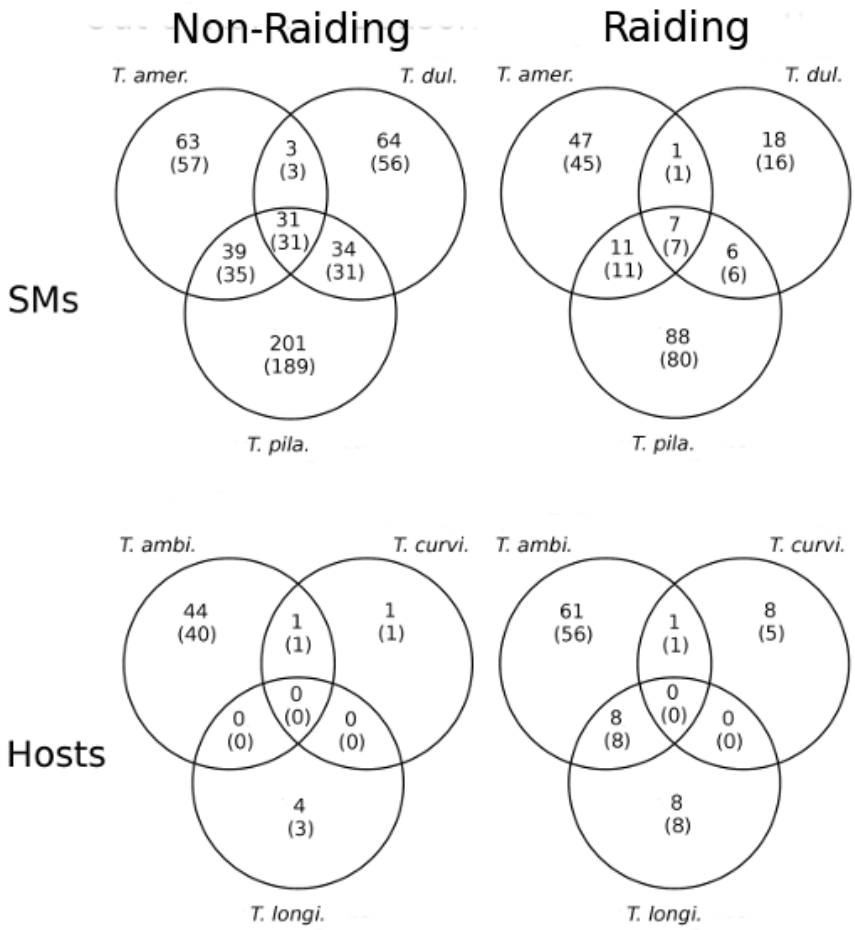

Figure 1.5: Venn diagrams displaying the number of significantly differentially-expressed contigs that are either private to a specific species, or shared by multiple species. Homology determined by cluster analysis. Un-bracketed numbers indicate total number of clusters, bracketed numbers indicate subset of clusters with accompanying functional annotation. For species abbreviations, see Figure 1.1.

WGCNA across all three slavemaking species revealed that one group of contigs with similar expression patterns across behaviors (gene module) is significantly positively associated with the raiding phenotype and one module significantly positively associated with the non-raiding phenotype (Figure 1.6; Supplementary Figure S4). Functional enrichment of contigs within each module reveal that Slavemaker-Module-1 shows a significant bias towards translation and various metabolic functionalities (Supplementary Table S5), while contigs in Slavemaker-Module-7 show a significant functional enrichment of translation, response to oxidative stress, and various additional metabolic functions (Supplementary Table S6). Hosts only show one significantly-enriched module, here positively associated with the raiding phenotype (Figure 1.6b). Functional enrichment of this module (Host-Module-

9)

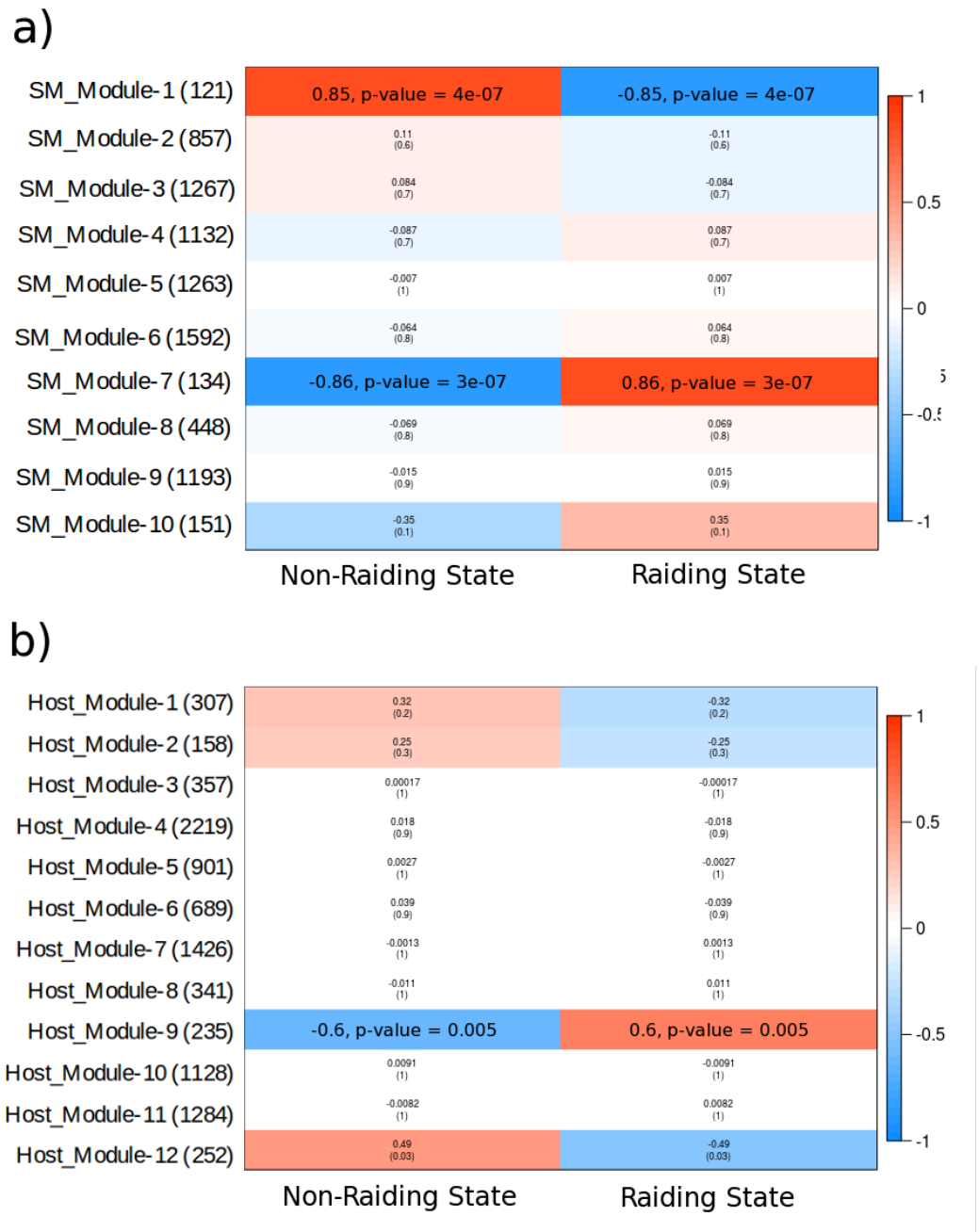

Figure 1.6: Module-Trait relationships within all three slavemaking species (a) and all three host species (b). Contigs within Slavemaker Module 1 are strongly associated with the non-raiding phenotype, where contigs in slavemaker Module 7 are strongly associated with the raiding phenotype. Host contigs within Host Module 9 are positively associated with the defensive phenotype. Numbers to the right of module identifiers indicates the number of contigs within that specific module. Slavemaker Module 10 and Host Module 12 are “leftover” modules, containing random contigs that did not fall into any other module.

reveals that lipid metabolic and isoprenoid biosynthetic processes, among others, are over-represented within these contigs (Supplementary Table S7). Unsurprisingly, given that slavemaker raids and host nest defense show few external behavioral similarities, we find no modules with significant patterns of shared expression when performing WGCNA upon all six *Temnothorax* species herein examined (Supplementary Figure S5).

Additionally, comparative pathway analysis reveals that slavemakers display a greater number of commonly over-represented pathways relative to privately over-represented pathways during both raiding ($\chi^2 = 6.5$; $p = 0.011$) and non-raiding phenotypes ($\chi^2 = 81.8$; $p < 0.0001$) compared to hosts (Figure 1.7).

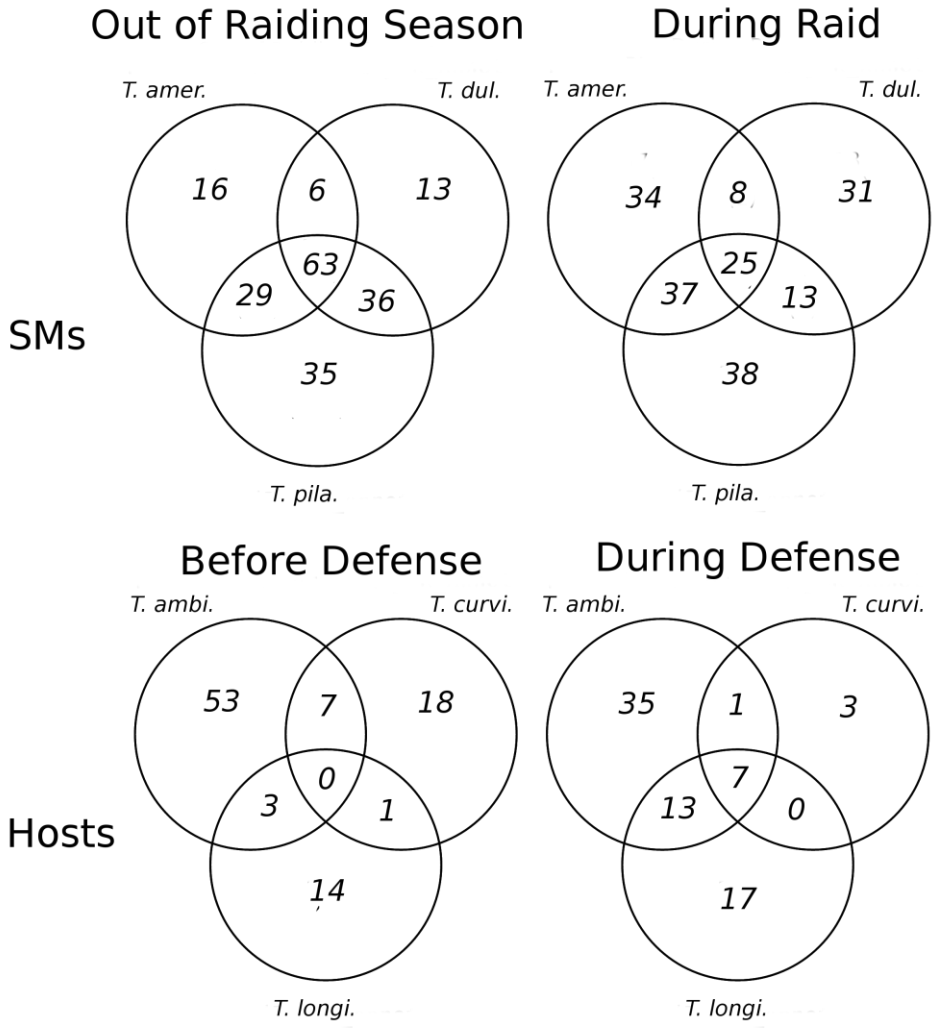

Figure 1.7: Venn diagrams displaying the number of KEGG pathways that are either private to a specific species, or shared by multiple species. Pathways generated by obtaining KO (KEGG Pathway) terms for phenotype-specific, significantly-

differentially-expressed genes. For species abbreviations, see Figure 1.1.

Within slavemakers, fewer pathways shared over-representation across species in the non-raiding phenotype than the raiding phenotype ($\chi^2 = 20.8$; $p < 0.0001$); whereas the reverse is true for the hosts, in which more pathways were commonly up-regulated during the raiding than the non-raiding phenotype ($\chi^2 = 7.3$; $p < 0.007$).

Functional Enrichment

Examination of functional enrichment results, performed on 1] groups of genes with species-specific expression patterns within a single phenotype, 2] groups of genes with expression patterns shared between two or more species within a single phenotype, and 3] all genes differentially expressed by a single species in a specific phenotype, revealed a number of functions over-represented both within and between species (Supplementary Figure S6 – S21). While host species did not possess enough enriched functions for additional comparison, we were able to further analyze those functions found to be enriched within slavemaking species both in their non-raiding (Supplementary Figure S22) and raiding (Supplementary Figure S23) phenotypes. Far more functions appear within all examined slavemaking species in their non-raiding phenotype. Indeed, the entire enriched functional repertoire of *T. duloticus* in its non-raiding phenotype is shared by *T. americanus* and *T. pilagens* (Supplementary Figure S22). Contrasting this, the raiding phenotype is characterized largely by the enrichment of species-specific functions and processes, with no enriched functions being shared between all three slavemaking species (Supplementary Figure S23). GO terms of enriched functions within Supplementary Figure S22 and S23 may be found in Supplementary Table S8.

Discussion

Guided by three primary assumptions about the molecular evolution of social parasitism: 1] *trait novelty or commonality will be reflected at the molecular level;* 2] *Novel molecular processes underlie lineage-specific phenotypes;* and 3] *that conserved regulatory processes underlie the response to a shared environment* (Cini *et al*. 2015) this study utilized an RNA-Seq approach following behavioral experiments in order to identify regulatory patterns involved in slavemaker raiding behavior and host defensive behavior by focusing on the three *Temnothorax* slavemakers and their three primary host species (Figure 1.1).

Indeed, by comparing multiple slavemaker and host species, we can determine whether or not raiding and defensive phenotypes evolved along independent, species-specific trajectories, or if these phenotypes arose in parallel within *Temnothorax*.

Expression Analysis

In keeping with our first prediction - that trait novelty or commonality will be reflected at the molecular level – we were able to detect a number of regulatory differences between species and lifestyles within this transcriptome study. As expected, we find more genes differentially expressed between raiding and non-raiding phenotypes in slavemakers than within hosts. Moreover, slavemakers seem to down-regulate a relatively large number of genes during raiding, suggesting that slavemakers focus their gene expression for the singular and crucial task of raiding. Together, these patterns are likely driven by the highly dissimilar physiological and behavioral phenotypes of slavemakers out of raiding season and hosts before nest defense. Slavemakers switch from their inactive state in spring to a highly active state for a few weeks in summer. Hosts, however, become active after winter and carry out their normal daily chores until they are attacked by slavemakers, which then necessitates a rapid, though short-term, response. Thus, with regard to the disparity in raw number of DEGs between lifestyles, it is likely that we are observing an equipoised gene expression pattern - in conjunction with some short-term changes - within slavemaking species during raiding. This is in direct contrast to host species, where we observe a radical short-term shift in expression of a few genes as host defenders have only minutes or seconds to respond to a slavemaker attack. However, due to differences in physiology between raiding and stay-at-home slavemakers, it must be noted here that the time of sampling for the non-raiding phenotype between host and slavemaker was about two months apart (see Material and Methods for further explanation). Still, taken together, these patterns suggest that gene regulation is fundamentally different between slavemaking and host species within these phenotypes; a finding that is corroborated by an accompanying study investigating genes under selection within these same six species (Feldmeyer *et al*. 2017).

Additionally, we find largely species-specific patterns of expression for most DEGs (Figure 1.4), suggesting that, while all three slavemakers conduct raids, and all host species defend their colonies against such raiding attempts, these responses are largely controlled via unique, species-specific mechanisms. Within slavemakers, this pattern is in keeping with our second prediction that novel molecular processes underlie lineage-specific phenotypes, where the more varied slavemaker raiding phenotypes (Wesson 1939, Alloway 1979, Foitzik *et al*. 2001, Alloway 1990) are reflected in a greater number of genes under differential

regulation between species. By comparison, the relatively low number of DEGs within host species is striking though not unexpected, as this finding is in keeping with our final prediction - that conserved regulatory processes underlie the response to a shared environment and similar evolutionary pressures - and is likely the result of overall mechanistic similarity, and the short transition time, between these two phenotypes in host species. Additionally, hosts share no overlap in those genes that were determined to be differentially expressed between raiding and non-raiding phenotypes, indicating that hosts utilize comparatively minor, species-specific regulatory shifts that correspond with ecological pressure exerted by local social parasites (Jongepier *et al*. 2014, Kleeberg *et al*. 2015, Jongepier *et al*. 2015, Jongepier and Foitzik 2016).

While host species did not possess enough DEGs for effective functional enrichment, functional enrichment analysis of slavemaking species did yield additional insight into the similarity, or lack thereof, of biological processes underlying slavemaker behavior and physiology during raids as well as out of raiding season. When out of raiding season, slavemakers share a large proportion of enriched functions, with the entire functional repertoire of *T. duloticus* mirrored in both *T. americanus* and *T. pilagens* (Supplementary Figure S22). Contrast these findings with the shared proportion of enriched functions between slavemaking species during raiding (Supplementary Figure S23), where we find much less similarity between species. Taken together, these findings strengthen our initial assertion that, while the non-raiding phenotype of slavemakers is characterized by a degree of molecular - and subsequently, functional - commonality, raiding phenotypes are regulated in a species-specific manner.

Genes of Interest

One frequently recurring gene, found up-regulated during raiding within all three slavemaker species is *Acyl-CoA-Delta (11) desaturase* (Table 1.1)*;* a gene found previously to be involved in pheromone biosynthesis (Moto *et al*. 2004). Given that *Temnothorax* slavemakers employ a number of subversive chemical weapons – from Dufour's gland secretions that elicit fighting among host defenders (Jongepier *et al*. 2015), to CHC profile modifications during raiding (Brandt *et al*. 2005, Kleeberg *et al*. 2017) – we speculate that regulatory shifts of esterase and desaturase genes (Moto *et al*. 2004) involved in these mechanisms could impart a number of benefits to raiders: from desiccation resistance during raiding activity out of their own nest to CHC-masking, making chemical detection of

Table 1.1: Expression statistics for genes of interest. Positive log fold change indicates gene up-regulation during the raiding phenotype, negative log fold change indicates up-regulation during non-raiding phenotype. Species abbreviations are as follows: *T. ambi: T. ambiguus, T. curvi: T. curvispinosus, T. longi: T. longispinosus, T. amer: T. americanus, T. dul: T. duloticus, T. pila: T. pilagens.* Note: a single gene can occur multiple times for a single species due to low coverage of certain regions, or due to splice variants.

Gene	*Species*	*Up-Regulated*	*LogFC*	*FDR-Value*
Acyl-CoA-Delta (11) desaturase	*T. amer.*	Raiding State	0.9	<0.01
		Raiding State	2.03	<0.01
	T. dul.	Raiding State	6.2	<0.01
		Raiding State	0.65	0.01
	T. pila.	Non-Raiding State	-1.57	<0.01
		Non-Raiding State	-1.38	<0.01
		Raiding State	0.49	0.04
		Raiding State	0.78	<0.01
	T. ambi.	Raiding State	3.14	<0.01
Vitellogenin-6	*T. dul.*	Raiding State	0.93	0.02
	T. pila.	Raiding State	2.06	0.01
	T. longi.	Raiding State	2.1	<0.01
		Raiding State	2.12	<0.01
Vitellogenin-3	*T. dul.*	Non-Raiding State	-2.4	0.01
	T. ambi.	Raiding State	3.47	0.01
Vitellogenin Receptor	*T. dul.*	Non-Raiding State	-1.94	<0.01
		Non-Raiding State	-2.34	<0.01
		Non-Raiding State	-2.2	<0.01
	T. pila.	Non-Raiding State	-1.06	0.05
	T. ambi.	Raiding State	2.48	<0.01
Trypsin-7	*T. amer.*	Non-Raiding State	-11.07	<0.01
		Non-Raiding State	-9.82	0.02
	T. dul.	Non-Raiding State	-6.71	<0.01
		Non-Raiding State	-7.6	<0.01
	T. pila.	Non-Raiding State	-6.85	<0.01
		Non-Raiding State	-6.39	0.04
	T. ambi.	Raiding State	5.94	0.04
		Raiding State	8.13	<0.01
		Raiding State	8.26	<0.01
	T. curvi.	Raiding State	9.18	0.01
	T. longi.	Raiding State	5.69	<0.01
		Raiding State	8.47	0.03
		Raiding State	8.9	<0.01
		Raiding State	9.3	0.01
		Raiding State	9.54	0.01
		Raiding State	9.92	0.01
Trypsin Inhibitor	*T. pila.*	Raiding State	1.52	<0.01
Kelch	*T. pila.*	Non-Raiding State	-4.4	0.01
	T. longi.	Non-Raiding State	-6.16	0.05

painless	*T. amer.*	Raiding State	1.84	<0.01
	T. curvi.	Raiding State	10.45	0.04
MYG1	*T. amer.*	Non-Raiding State	-1.63	<0.01
	T. curvi.	Non-Raiding State	-8.29	0.04

slavemaker raiders by host defenders less likely (Kleeberg and Foitzik 2016 , Kleeberg *et al*. 2017). However, that *Acyl-CoA-Delta (11) desaturase* was also found to be up-regulated in *T. ambiguus* during its raiding behavioral state is unusual. This pattern of expression across lifestyles does seem to further suggest that *Acyl-CoA-Delta (11) desaturase* is involved in CHC production in *Temnothorax* ants – and not chemical weapons for raiding – as there is no evidence that *T. ambiguus* uses chemical weapons against raiding slavemakers.

Trypsin-7 was also found to possess a particularly interesting pattern of expression: it was universally down-regulated in slavemaking species during raiding while, conversely, was up-regulated universally in hosts during raiding (Table 1.1). A previous study into the function of *Trypsin-7* revealed its role in digestion and, potentially, host-seeking behavior within the malaria mosquito *Anopheles gambiae* (Müller *et al*. 1995). Given the expression data produced here, *Trypsin-7* expression certainly does not appear to be positively correlated with host-seeking as in *A. gambiae.* That *Trypsin-7* expression is strongly negatively correlated with the slavemaker raiding phenotype does seem to suggest that *Trypsin-7* is somehow involved in the control of this behavior. Indeed, even within the context of raiding, *Tyrpsin-7* might retain some of its digestion functionality - as food restriction does trigger an increase in raiding activity in *T. americanus* (Pohl *et al*. 2011); though determining the exact mechanisms involved are beyond the scope of this study. However, given the sampling method of this experiment, it is difficult to disentangle raiding phenotype effects from seasonal or unrelated physiological effects. That an unspecified *Trypsin Inhibitor* was found to be strongly up-regulated during raiding behavior within *T. pilagens* might indirectly shed some light onto the hypothetical role of *Trypsin-7.* Assuming that *Trypsin-7* prevents slavemaker raiding behavior, we postulate that this *Trypsin Inhibitor* is at least involved in the suppression of *Trypsin-7*, in turn facilitating raiding behavior within *T. pilagens*. Again, however, additional gene-specific approaches are required in order to elucidate the importance of *Trypsin Inhibitor* to the raiding phenotype of *T. pilagens*, as well as the precise interplay between *Trypsin Inhibitor* and *Trypsin-7.*

While the exact function of specific genes cannot be determined within the purview of

this study – indeed functional verification of many genes found here could be accomplished through RNA-mediated gene knockdown – the proposed functions of DEGs is nonetheless insightful into the potential processes and mechanisms that define species-specific phenotypes, or are maintained within like phenotypes.

Conclusions

While it has long been known that slavemakers display lineage-specific chemical and behavioral phenotypes during raids (Wesson 1939, Kleeberg and Foitzik 2016, Foitzik *et al*. 2001, Alloway 1990), here we provide the first evidence for the underlying gene expression patterns governing the raiding phenotype within *Temnothorax* slavemaking ants; as evidenced by the comparatively high number of orthologous genes found to possess species-specific patterns of expression. That this same pattern is observed on the pathway levels as well suggests regulatory and, ultimately, functional divergence of molecular mechanisms underlying the raiding phenotype in *Temnothorax* slavemakers. Not unexpectedly, slavemaking species display a much higher level of regulatory similarity when out of raiding season, where these species are universally inactive and do not engage in normal nest tasks. A similar pattern was also observed in workerless parasite species within the genera *Pogonomyrmex* and *Vollenhovia* (Smith *et al*. 2015). Despite being of different genera, the behavioral differences between these species is not due to sequence change or gene loss, but attributed to differential expression patterns of gene sets. Our results seem to reflect this finding, as behavioral diversification among slavemakers and hosts in *Temnothorax* appear to be typified by species-specific patterns of gene expression; however the extent of gene loss and sequence change, and the precise importance of these mechanisms within the context of *Temnothorax* slavemakers, is beyond the purview of this investigation.

Additionally, we note that hosts possess largely species-specific molecular responses to slavemaker aggression – which are driven by comparatively small shifts in regulatory mechanisms – also suggesting regulatory differences in orthologous genes as well as species-specificity at the pathway level between host species during nest defense.

Functionally, we find a diverse repertoire of DEGs both within slavemakers as well as in hosts. Broad characterization of slavemaker raiding behavior includes the universal up-regulation of *Acyl-CoA Delta (11) desaturase* genes, which are likely involved in the production of olfactory signals in slavemakers or modification of the cuticular hydrocarbon

profile in both slavemakers and hosts. While the raiding strategies of all slavemaking species examined here differ substantially from one another, all rely on an altered chemical secretion to enhance the chance of raiding success (Jongepier and Foitzik 2015). Perhaps most interestingly are the differential expression of *Trypsin-7* and its suspected controller *Trypsin Inhibitor*. Further analysis of genes found here to be significantly differentially-expressed - likely through the use of RNA-mediated gene knock-down (RNAi), followed by extensive behavioral analysis - is necessary in order to more clearly elucidate the exact role of these genes within the context of slavemaker raiding and host nest defense behavior. Taken together, and framed by previous studies (Cini *et al*. 2015, Smith *et al*. 2015), our findings suggest that the evolution of molecular mechanisms underlying slavemaker raiding and host nest defense phenotypes in *Temnothorax* is characterized by high flexibility and lineage-specificity.

Acknowledgements

The authors would like to thank I. Kleeberg and E. Jongepier for their help in ant collection and S. Emmling, M. Kever, and H. Stypa, for their assistance in extracting RNA prior to sequencing. Additional thanks to D. Elsner for contributing to the development of the transcriptome assembly methods and M. Stoldt for performing the WGCNA. This project was funded by a grant to Barbara Feldmeyer and Susanne Foitzik (DFG FE 1333/3-1 and FO 298/17-1) and support from the E. N. Huyck Preserve, Rensselearville NY. Collection permits were obtained at the Parks and Preserves.

Data Accessibility

Raw reads deposited into NCBI's GEO database under Accession Number GSE95604

Chapter 2

Species-specific genes under selection characterize the co-evolution of slavemaker and host lifestyles

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Based on

BMC Evolutionary Biology **17**:237 (2017)

Abstract

The transition to a parasitic lifestyle entails comprehensive changes to the selective regime. In parasites, genes encoding for traits that facilitate host detection, exploitation and transmission should be under selection. Slavemaking ants are social parasites that exploit the altruistic behavior of their hosts by stealing heterospecific host brood during raids, which afterwards serve as slaves in slavemaker nests. Here we search for evidence of selection in the transcriptomes of three slavemaker species and three closely related hosts. We expected selection on genes underlying recognition and raiding or defense behavior. Analyses of selective forces in species with a slavemaker or host lifestyle allowed investigation into whether or not repeated instances of slavemaker evolution share the same genetic basis. To investigate the genetic basis of host-slavemaker co-evolution, we created orthologous clusters from transcriptome sequences of six *Temnothorax* ant species - three slavemakers and three hosts - to identify genes with signatures of selection. We further tested for functional enrichment in selected genes from slavemakers and hosts respectively and investigated which pathways the according genes belong to.

Results

Our phylogenetic analysis, based on more than 5000 ortholog sequences, revealed sister species status for two slavemakers as well as two hosts, contradicting a previous phylogeny based on mtDNA. We identified 309 genes with signs of positive selection on branches leading to slavemakers and 161 leading to hosts. Among these were genes potentially involved in cuticular hydrocarbon synthesis, thus species recognition, and circadian clock functionality possibly explaining the different activity patterns of slavemakers and hosts. There was little overlap of genes with signatures of positive selection among species, which are involved in numerous different functions and different pathways.

Conclusions

We identified different genes, functions and pathways under positive selection in each species. These results point to species-specific adaptations rather than convergent trajectories during the evolution of the slavemaker and host lifestyles suggesting that the evolution of parasitism, even in closely related species, may be achieved in diverse ways.

Keywords: positive selection, social parasites, *Temnothorax,* co-evolution

Background

Parasitism is one of the most successful modes of life, as measured by how often it evolved and by how many parasitic species presently exist (Poulin and Morand 2000). Parasites are a taxonomically highly diverse group, and range from intragenomic 'genetic' parasites, through microparasites (viruses, bacteria and protozoa) and macroparasites (worms, arthropods and even vertebrates) (Viney and Cable 2011), to brood and social parasites (Buschinger 1993, Davies *et al*. 1989).

Parasites are ideal biological models for the study of ecological specialization, speciation mechanisms, diversification, and co-adaptation (de Meeûs *et al*. 1998). The relationship of hosts and parasites is one of mutual adaptation with parasites trying to dupe the host, whereas hosts adapt to defend themselves (Dawkins and Krebs, 1979, Foitzik *et al*. 2001, Calcagno *et al*. 2010, Kilner and Langmore 2011, Grasso *et al*. 1992, Jongepier and Foitzik 2015, Ortolani *et al*. 2010). Co-evolutionary dynamics are shaped by numerous factors including life history traits (Barrett *et al*. 2008, Strauss *et al*. 2016), epidemiological characteristics (Tellier and Brown 2007, González-Tortuero *et al*. 2016), population size (Papkou *et al*. 2016, Gandon and Michalakis 2002), fluctuating environmental changes (Wolinska and King 2009), the presence of multiple parasites (reviewed by Bose *et al*. 2016), and social interactions within the host taxon (reviewed by Kurze *et al*. 2016, Joop and Vilcinskas 2016).

Signatures of balancing selection are expected on immunity genes, playing a major role in the co-evolution between micro-parasites and their hosts, whereas genes encoding behavioral or morphological traits, important in social parasites and their hosts, should show signs of positive selection (Croze *et al*. 2016).

Social parasitism is a special form of parasitism, where the social behavior of the host, rather than its physiology, is exploited (Buschinger 1993). Avian brood parasites, such as cuckoos and cowbirds take advantage of the brood care behavior of other bird species, and thus avoid the costs of parental care (Brooke and Davies 1988, Davies 2000). Several avian brood parasites evolved from non-parasitic ancestors, and started out by exploiting the brood care behavior of their conspecifics (Yamauchi 1995). Similarly, ant social parasites can arise via sympatric speciation from their later host (Leppänen *et al*. 2015). Such a transition to a parasitic lifestyle should lead to the selection of traits important for a parasitic mode of

life, such as host recognition, circumventing the host defence system, and transmission. Indeed, avian brood parasites lost their ability to build nests (Hamilton and Orians 1965), a social parasitic wasp needs a specific host species to be successful as parasite (Fanelli *et al*. 2005), and many slavemaking ants are unable to even feed themselves and completely rely on the care of their enslaved host workers (Darwin 1859).

We are just starting to understand the genomic basis of the parasite lifestyle as such (Zhang *et al*. 2016, Skippington *et al*. 2015, Jackson 2015 and the authors therein), and some first patterns of convergence, gene losses and gains are becoming apparent (Jackson 2015 and authors therein, Poulin and Randhawa 2015). First studies on the genetic basis of social parasitism concentrated either on ant inquilines - social parasites that secondarily lost the worker caste and inquiline queens that live within host colonies (Smith *et al*. 2015) or the Cape honeybee (*Apis mellifera capensis*), in which workers invade other colonies and reproduce clonally (Goudie *et al*. 2015). Identified candidate loci for this latter form of social parasitism include genes involved in ecdysteroid signalling and juvenile hormone and dopamine biosynthesis, which may regulate worker ovary activation (Wallberg *et al*. 2016). A study on three workerless inquiline social parasites of *Vollenhovia* ants in comparison to their *Pogonomyrmex* hosts found little evidence for gene loss, damaging mutations, or shifts in selection regimes, suggesting that regulatory changes – rather than sequence differences – play a role in the evolution of these workerless social parasites (Smith *et al*. 2015). However, the genomic basis of the slavemaker lifestyle and its' peculiarities has never been investigated.

Here we explore the evolution of the slavemaker lifestyle in North American *Temnothorax* ants, a taxon in which slavery evolved several times independently (Beibl *et al*. 2005). We specifically focus on three slavemaker species *T. americanus, T. duloticus* and *T. pilagens*, and their three closely related host species, *T. longispinosus, T. curvispinosus* and *T. ambiguus* (Herbers *et al*. 2002, Seifert *et al*. 2014). The dulotic lifestyle of these three slavemakers is characterized by recurrent and destructive slave raids during summer (Herbers *et al.* 2002). During these raids, slavemaker worker raiding parties search for and attack host colonies to steal worker brood. Upon their emergence as adult workers in slavemaker nests, the social behaviors of these enslaved host workers will be exploited by the slavemakers, whose workers have lost the ability to care for themselves (Alloway 1979). While host nests on average contain around 50 workers (Foitzik *et al*. 2009), the number of workers in slavemaker nests is much lower with on average approximately five workers

(Herbers *et al*. 2002, Seifert *et al*. 2014, Johnson and Herbers 2006). Moreover, slavemaker workers are only active during the raiding season and do not take over normal worker chores such as brood care and foraging (Wilson 1975, Stuart and Alloway 1985). Each slavemaker species exhibits distinct morphological characteristics (e.g. size and colour) and raiding behaviors (Alloway 1979, Winson 1975). *T. americanus* - the most derived parasite in the group in terms of morphology and behavior - mainly uses a propaganda pheromone to induce panic among hosts, preventing organized evacuation or nest defence (Alloway 1979, Brandt *et al*. 2006, Jongepier *et al*. 2015). The strategy of *T. pilagens* is quite variable, and may also depend on the aggressiveness of the host colony (Seifert *et al*. 2014, Kleeberg and Foitzik 2016). In some instances host workers are killed by stinging, while in other cases the raid is seemingly peaceful without any casualties, facilitating the incorporation of even adult host workers into the slavemaker colony (Kleeberg and Foitzik 2016). *T. duloticus* is a fierce slavemaker that mostly stings all opponents to death before taking the brood, resulting in the local eradication of host colonies (Alloway 1979, Johnson and Herbers 2006, Wilson 1975, Hare and Alloway 2001). Each of the three slavemakers can exploit several host species, but has a clearly preferred host. The derived *T. americanus* uses all three *Temnothorax* species, but focuses when possible on *T. longispinosus* (Brandt and Foitzik 2004). *T. duloticus* occasionally attacks *T. longispinosus* but prefers *T. curvispinosus* (Johnson and Herbers 2006) and *T. pilagens* prefers *T. ambiguus* over *T. longispinosus* (Seifert *et al*. 2014, Kleeberg and Foitzik 2016).

Co-evolution between the obligate social parasites and their hosts not only leads to adaptations in slavemakers, but also to counter-adaptations in behavioral, chemical and life history traits in host species and populations (Jongepier and Foitzik 2015, Jongepier *et al*. 2015, Pamminger *et al*. 2011, Scharf *et al*. 2011, Kleeberg *et al*. 2014, Kleeberg *et al*. 2015). Host aggression (Kleeberg *et al*. 2015), as well as host defence strategies (Jongepier *et al*. 2014) are linked to geographic variation in parasite pressure. It is known that adaptations to similar ecological conditions may lead to the evolution of similar (convergent) phenotypes in non-related species. The degree to which parallelism extents to the molecular level has recently experienced an upsurge of interest (Conte *et al*. 2012, Martin and Orgogozo 2013, Elmer and Meyer 2011, Stern 2013, Soria-Carrasco *et al*. 2014). Evidence is ambiguous, with some studies pointing to parallelism, and others to species-specific trajectories (Stern 2013, Lobkovsky and Koonin 2012 and authors therein). Moreover, it becomes clear that the level of organisation plays a major role in detecting convergent evolution, as the degree of parallelism is predicted to increase from the nucleotide level to features of whole organisms

(Lobkovsky and Koonin 2012). The North American *Temnothorax* system, with six closely related slavemaker and host species is ideal to study the genetic basis of repeated evolution of phenotypic traits involved in host-parasite co-evolution.

The main objective of this study was to investigate the selective forces shaping the host and slavemaker lifestyles, and the organisational level of convergence. The main questions we tried to answer were: Which genes are under positive selection in slavemakers or hosts? Is molecular parallelism involved in the convergent evolution of slavemaker lifestyles? Do we find convergence on the gene, functional, or pathway level?

Methods

Ant colonies were collected over the course of two years (2013 and 2014) in New York State, Ohio and Michigan (Additional file 1, supplementary information and all additional files, refer to the source publication), and brought back to the lab in Mainz. To induce raiding activity, colonies were moved during the raiding season in August to 25°C, 14-light:10-dark light cycle conditions one week prior to the onset of the raiding experiment. Raiding arenas (30 x 40 cm plastic boxes with plastered floor) were set up, in which each slavemaker species was allowed to raid colonies of its preferred host species. We waited until slavemaker scouts had returned to their mother nest and recruited additional raiders to infiltrate the host nest, and aggressive encounters between slavemaker and host workers could be observed. This was the time we sampled workers actively engaging in a raid or nest defence respectively. To obtain workers in a somewhat neutral behavioral state, we collected host individuals outside the nest before raids, as well as slavemaker workers outside the raiding season under the same external conditions. Since we were interested in the evolution of slavemaker and host genes in respect to raiding and nest defence behavior, we obtained transcriptomes of ants engaged in these respective behaviors. Six workers per species and behavior were pooled for RNA isolation in replicates of six. Libraries were constructed and sequenced paired-end on an Illumina HiSeq 2000 at GENterprise Genomics. Sequences were quality trimmed with Trimmomatic v0.32 (Bolger *et al*. 2014). *De novo* assembly of the transcriptomes was conducted using a combination of the CLC bio workbench (Qiagen) and MIRA (Chevreux *et al*. 1999) (Additional file 2; for more details see Alleman *et al*. 2018). Contigs were annotated using BlastX v.2.2.30 against the non-redundant arthropod database (November 2014). The online tool *ORFpredictor* 2.0.3 (Min *et al*. 2005) was used to predict

open reading frames and amino acid sequences for all contigs. The predicted and translated amino acid sequences were used as input for *OrthoMCL* 2.0.9 (Li *et a*l. 2003), to build ortholog sequence clusters. In total we obtained 55,521 orthologous protein clusters, out of which 6,432 clusters contained at least one sequence per species. These clusters were filtered with an in-house python script (available from GitHub: https://zenodo.org/record/60135#. V9k495h96Uk) based on pairwise Blast similarity scores, which resulted in 5,791 clusters with a single sequence per species. After trimming these sequence alignments with *Gblocks 0.91b* (Castresana 2000), 5,199 clusters remained for further analyses (NCBI Bio Project GSE95604).

Phylogenetic Analysis

We chose the myrmicine ant *Acromyrmex echinator* to include as outgroup, for which we observed the highest Blast similarity in our contig Blast searches. *A. echinator* sequences were obtained from the "Hymenoptera Genome Database" (Nygaard *et al*. 2011, aech_OGSv3.8_pep.fa). We inferred orthology between *T. curvispinosus* and *A. echinator* by applying a local *BLASTn* (Altschul *et al*. 1990). The according sequences for each cluster were obtained and aligned with *Mafft 7.0* (Katoh and Standley 2013). The alignments were trimmed using *Gblocks* 0.91b [66] with default settings. All clusters were concatenated into a single alignment and the program *ProtTest* 3.4 (Abascal *et al*. 2005) was used to calculate the appropriate evolutionary model (JTT + I + G + F). A *Maximum Likelihood* phylogenetic tree with 1000 bootstrap replicates was constructed with *RAxML 8.1.16* (Stamatakis 2006). We additionally estimated evolutionary models for each single cluster and constructed the respective *Maximum Likelihood* trees for the *codeml* analyses (see below).

Tests for Positive and Relaxed Selection

To test for signatures of positive selection the software package *PAML* 4.8 (Yang 2007) was used to apply the branch-site model A in *codeml* (model = 2, NSsites = 2). *codeml* estimates the nonsynonymous/synonymous substitution ratio (ω=dN/dS), where ω = 1 indicates neutral evolution, ω < 1 purifying selection, and ω > 1 indicates positive selection. To test for statistical significance log-likelihood ratios were calculated and FDR corrected for multiple testing (Benjamni *et al*. 1995). The cluster specific tree topology, as inferred by *RAxML* was used as input for *codeml*. To test for positively selected genes, we coded each single species as foreground branch, and additionally the set of slavemaker branches as well as the host branches respectively.

The online tool Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/) was used to visualize shared and species-specific genes. To statistically assess to what extent the observed intersection in divergent features among pairs of species would be expected by chance, we applied a randomisation procedure implemented in a custom Python script. We used 10,000 replicates to infer how often an observed intersection of size i of x and y positive draws from a base population of size z was larger or smaller than those from random draws.

Enrichment Analyses

To obtain identifiers suitable for the enrichment tool DAVID 6.7 (Huang *et al*. 2009), we inferred orthology by applying a *BLASTx* between *T. curvispinosus* contigs and *Drosophila melanogaster* protein sequences *(dmel-all-translation-r5.56.fasta)* obtained from flybase (flybase.org). The complete contig set was used as background and the according positively selected genes as test set. Furthermore, to obtain pathway information in form of KO (KEGG Orthology) assignments for the according gene sets, we utilized KAAS (Moriya *et al*. 2007), an automated annotation server. The KEGG Mapper – Reconstruct Pathway tool was used to obtain the associated pathways (http://www.genome.jp/kegg/tool/map_pathway.html).

Results

Phylogenetic Analysis

Based on the concatenated sequence of 5,199 ortholog sequence clusters the *RAxML* phylogenetic analysis resulted in a tree with well supported nodes (Figure 2.1). *T. americanus* is the most distant taxa to the other five *Temnothorax* species, as corroborated by its deviant phenotype and a previous phylogenetic analysis (Beibl *et al*. 2005). In contrast to this earlier phylogeny based on two mitochondrial loci (Beibl *et al*. 2005), our nuclear tree now supports a sister species relationship between the two younger slavemaking species *T. duloticus* and *T. pilagens* (Seifert *et al*. 2014), as well as between the two host species *T. ambiguus* and *T. longispinosus,* with *T. curvispinosus* being the next distant taxon, followed by *T. americanus*.

Positively Selected Genes

In total, we found 574 genes under positive selection; 309 on the branches leading to slavemakers, and significantly less, 161, positively selected genes on the branches to hosts

(χ^2_1 = 77.85, p < 0.0001; Additional file 3). Looking at the branches of each single species, we detected more than four times as many genes under selection in the derived slavemaker

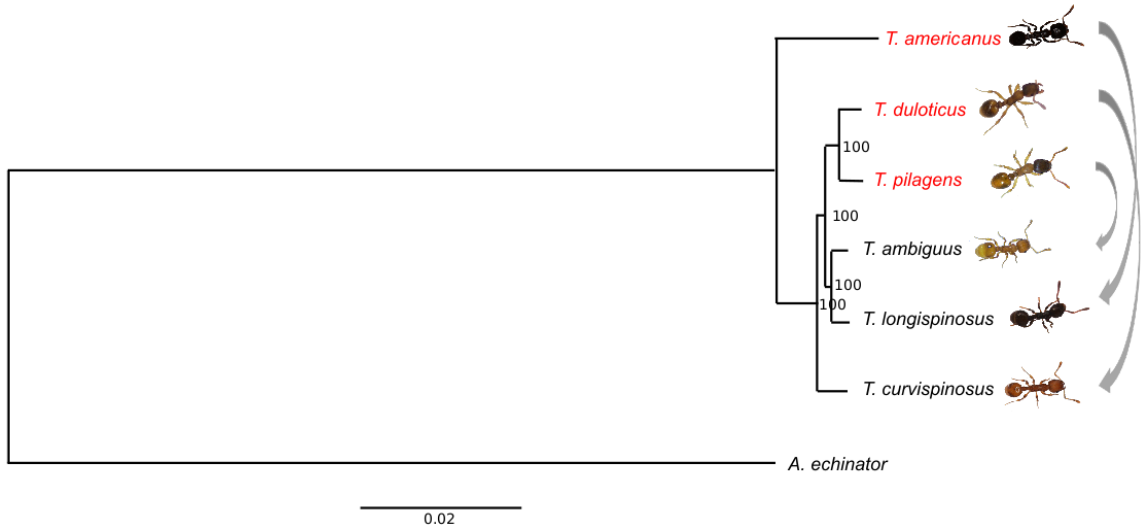

Figure 2.1: RAxML obtained Maximum Likelihood phylogenetic relationship of the six slavemaker and host species with *Acromyrmex echinatior* as outgroup, and based on 5,199 ortholog gene-clusters (ML bootstrap percentages depicted at nodes). Slavemaker species names are given in red, host species names in black. Arrows connect slavemaker-host species pairs.

T. americanus (N = 211) than in the two younger slavemaker sister species *T. pilagens* (N = 38) and *T. duloticus* (N = 54; Figure 2.2a). The host species *T. ambiguus* shares one gene under positive selection with its sister species *T. longispinosus*, and two genes with *T. curvispinosus*, whereas the latter two species do not share any positively selected genes (Figure 2.2b). In slavemakers, one positively selected gene (a hypothetical protein) is shared amongst all three species, *T. americanus* shares two genes each with the two other slavemakers, and *T. pilagens* shares only one positively selected gene with its sister *T. duloticus* (Additional file 3). In both, hosts and slavemakers, the number of shared genes under positive selection among species is less than one would expect by chance (Additional file 4 Table A). Moreover, we compared the positively selected genes to differentially expressed genes from an accompanying gene expression analysis (Alleman *et al*. 2018) based on the same host and slavemaker transcriptomes. Six genes in hosts and 36 in slavemakers appeared in both, the differential expression and the positive selection analyses (Table 2.1). None of the positively selected gene sets per species, in slavemakers or hosts, were enriched for any functional category.

Table 2.1: Genes found to possess both signatures of positive selection and significant differential expression between behavioural phenotypes in a sister study (Alleman *et al*. 2017).

T. ambiguus	*T. curvispinosus*	*T. longispinosus*
hypothetical protein G5I_08161	*Trypsin-7*	*Leukotriene A-4 hydrolase*
hypothetical protein SINV_10379	*Suppressor of tumorgenicity protein 14*	
Trypsin-7		
T. duloticus	*T. pilagens*	*T. americanus*
hypothetical protein SINV_06866	*Putative inorganic phosphate cotransporter*	*Putative inorganic phosphate cotransporter*
hypothetical protein SINV_03497	*Paired amphipathic helix protein Sin3a*	*Aminopeptidase N*
Alpha-catulin	*Zinc transporter ZIP1*	*RING finger protein 17*
Thyrotropin-releasing hormone-degrading ectoenzyme	*Uncharacterized protein*	*Sugar transporter ERD6-like 7*
hypothetical protein SINV_12600	*hypothetical protein SINV_09653*	*Matrix metalloproteinase-14*
Pleckstrin-like protein domain-containing family M member 2	*Kelch-like protein 10*	*hypothetical protein G5I_08549*
F-box/LRR-repeat protein 20	*Fatty acyl-CoA reductase 1*	*Receptor-type tyrosine-protein phosphatase beta*
hypothetical protein SINV_03929	*hypothetical protein EAI_01741*	*hypothetical protein SINV_02546*
Major facilitator superfamily domain-containing protein 6		*Cytochrome b5*
Sphingomyelin phosphodiesterase		*hypothetical protein SINV_09234*
Zinc finger protein jing-like protein		*hypothetical protein G5I_14818*
Elongation of very long chain fatty acids protein		*Putative ATP-dependent RNA helicase DDX23*
Circadian clock-controlled protein		*Trypsin-7*
hypothetical protein SINV_04023		*Circadian clock-controlled protein*

The comparison of pathways associated with selected genes indicates that selected genes between species not only belong to different functional categories, but also to many different pathways (Additional file 5). In slavemakers, we identified 128 different pathways amongst the positively selected genes, the majority (77%) of which were also species-specific ($\chi^2_1 = 73.26$, $p < 0.001$; Figure 2.2c). The genes positively selected in hosts belong to 102 different pathways, the majority (70%) of which were species-specific ($\chi^2_1 = 29.82$, $p < 0.001$; Figure 2.2d). Nevertheless, more pathways were shared than expected by chance in hosts and also in slavemakers, except between the sister species *T. pilagens* and *T. duloticus* (Additional file 4, Table B). The eight pathways shared among hosts are the metabolic pathway, biosynthesis of secondary metabolites, biosynthesis of antibiotics, p53

signalling, PI3K-Akt signalling, Wnt signalling, thyroid hormone signalling and the longevity regulating pathway. In slavemakers, four pathways were shared amongst all three species including metabolic pathways, biosynthesis of secondary metabolites, biosynthesis of antibiotics and endocytosis.

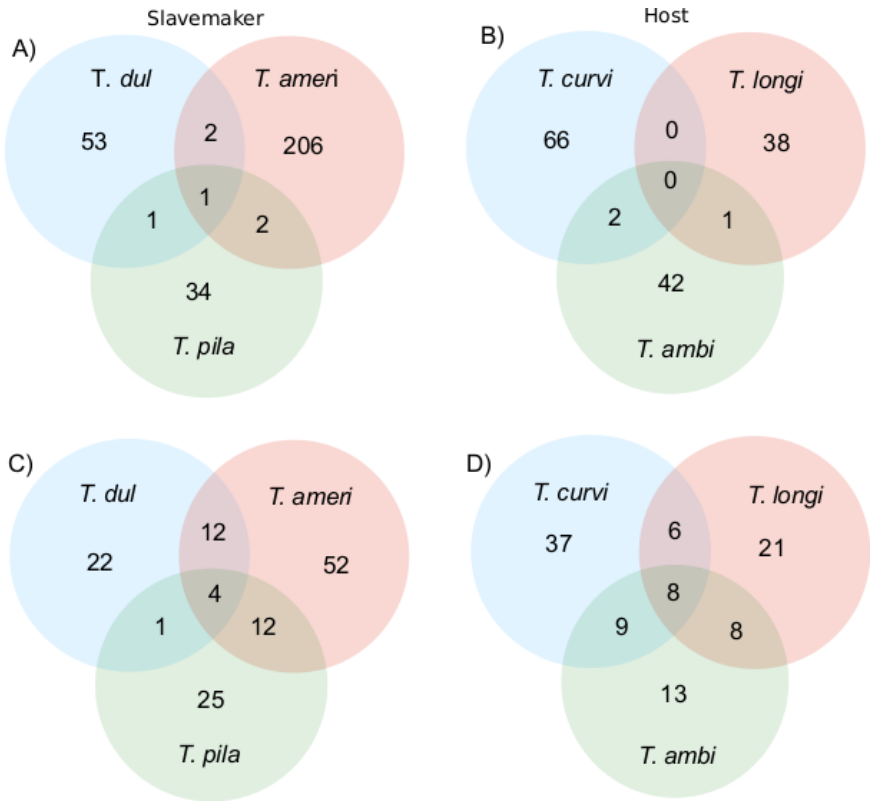

Figure 2.2: Venn diagrams depicting the number of shared and "private" positive selected genes (a + b) and corresponding pathways (c + d) in slavemakers and hosts. (*T. dul = T. duloticus, T. ameri = T. americanus, T. pila = T. pilagens, T. curvi = T. curvispinosus, T. longi = T. longispinosus, T. ambi = T. ambiguus*).

Slavemaker-Host Pairs

Genes under selection in slavemaker-host pairs should show little overlap due to their co-evolution, as different traits are under selection in slavemakers and hosts. An exception could be cuticular hydrocarbon genes, when slavemakers try to mimic host profiles (Achenbach *et al.* 2010) and utilize the same genes as their closely related hosts. A more important cause of overlap in selected genes in slavemaker and host pairs might be, that both species inhabit the same habitat and therefore adapt to the same environmental conditions. We investigated the number and functions of positively selected genes and pathways between each slavemaker-host pair in order to make inferences on local adaptation. We found between none and four shared positively selected genes (Figure 2.3a-

c; Additional file 6), and 7 - 23 shared pathways between pairs (Figure 2.3d-f). The number of shared genes and pathways was higher than expected in the pair including the most diverged parasite species *T. americanus* – *T. longispinosus*, and as expected by chance in the other two parasite-host pairs (Additional file 4: Table E+F). Moreover, we tested whether slavemakers share more genes with their preferred host in contrast to the other host species. *T. duloticus* shares more genes with *T. longispinosus* (n = 5) in comparison to its preferred host *T. curvispinosus* (n = 0) (X^2_1 = 4.811, p = 0.028). In all other cases, the number of shared genes between preferred host and the other species did not differ (results not shown).

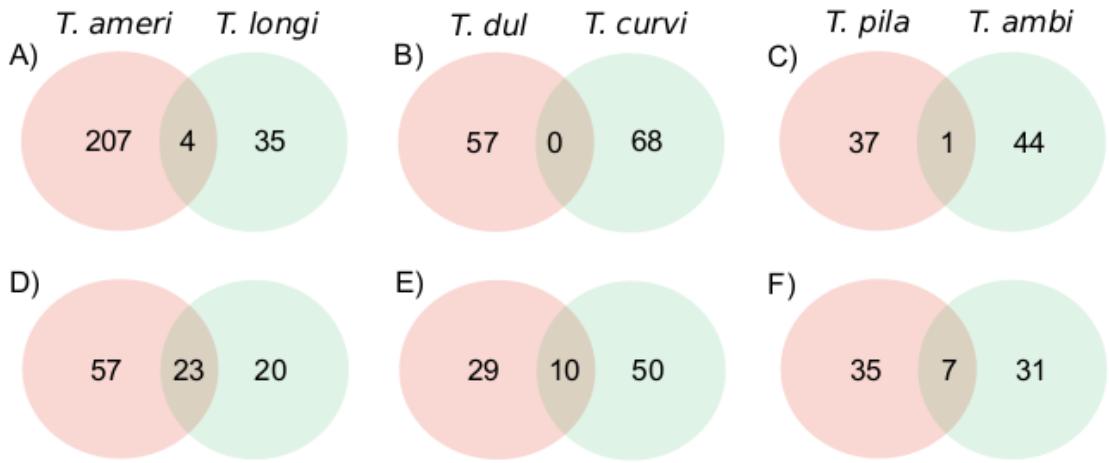

Figure 2.3: Venn diagrams depicting shared and "private" selected genes (a-c) and pathways (d-f) between host and slavemakers species pairs. For species name abbreviations please refer to Figure 2.2.

Discussion

The slavemaker lifestyle evolved several times independently in ants, with a hotspot of slavery evolution in the genus *Temnothorax* (Wallberg 2016). As slavemakers and their hosts are engaged in a constant co-evolutionary arms-race, the evolution of ant slavery is tightly linked to the evolution of behavior, physiology and morphology in their hosts (Gross 1993, Hart 1997, Sorci 2013). The focus of our study was the genomic basis of the (co-) evolution of the slavemaker and host lifestyle. We were thus interested in identifying genes with signatures of positive selection in slavemakers and hosts respectively. Furthermore, we

asked whether or not, and at which organisational level, the three slavemaker/host species show signs of genetic convergence; or whether each species follows its own specific evolutionary trajectory.

Positively Selected Genes

We identified twice as many genes under positive selection on branches leading to slavemakers as compared to host branches. This finding is in line with our expectation that the derived slavemaker mode of life should have led to the selection of more genes in comparison to the ancestral host lifestyle. However, based on the number of species-specific selected genes it becomes evident that 70% of the positively selected genes in slavemakers can be assigned to *T. americanus* only. All other slavemaker and host species have comparable and lower numbers of positively selected genes. *T. americanus* is the most distantly related species in this taxon and its behavior and morphology are most derived from the other species. *T. americanus* workers have large square heads, which make them easily distinguishable from their hosts. They do not engage in normal worker behavior, such as brood care or foraging, and are so dependent upon enslaved hosts that *T. americanus* will starve to death if not fed. During raids, they manipulate host behavior via the release of glandular secretions (Jongepier and Foitzik 2015, Alloway 1979, Brandt *et al*. 2006), but never use their stinger, which is a typical behavior for other *Temnothorax* slavemakers during aggressive interactions (Foitzik *et al*. 2001, Alloway 1979, Kleeberg and Foitzik 2016). Foraging and brood care are standard behavioral repertoires in the hosts, and in the lack of slaves, will still be performed by *T. duloticus* (Alloway 1979, Wilson 1975) and *T. pilagens* slavemakers (pers. observation) to some extent. In addition to many lifestyle differences, the longer evolutionary history with the possibility for co- and counter adaptations to *T. longispinosus* and its host ancestor might explain the large number of positively selected genes in *T. americanus* in contrast to the other species.

Within the 309 positively selected genes on the branches to slavemakers, we were able to identify several candidate genes with a possible link to their slavemaker lifestyle, three of which were different DNAJ-like protein subfamily members; heat shock protein homologs here functioning as co-chaperons. They are involved in stress response in humans (Terada and Mori 2000), and could thus play a role during stressful slave raids into fiercely defended host colonies.

Tachykinin is positively selected on the branches leading to hosts and might thus be a candidate gene for the host lifestyle. *Tachykinin* has been linked to aggressive behavior in

Drosophila (Pavlou *et al*. 2014 and authors therein), and recently also in ants (Howe *et al*. 2016). *Temnothorax* hosts need to defend their nest aggressively, not only against intra-specific intruders, but particularly against slavemakers. Thus selection might specifically act on this gene in hosts. This is further corroborated by the fact that intra-specific aggression increases with the prevalence of the slavemaker *T. americanus* in the population (Kleeberg *et al*. 2015).

In comparison to an accompanying gene expression analysis including the same six host and slavemaker sequence data (Alleman *et al*. 2018), we found six genes to be both, differentially expressed and under positive selection in hosts and 36 in slavemakers. These genes are thus prime candidates for the evolution of the slavemaker and host lifestyles. Firstly, they are directly involved in raiding behavior. Secondly, they show signs of positive selection. Among these genes *Trypsin-7* was identified in two host species and the slavemaker *T. americanus*. *Trypsin-7* is known for its function in digestion, e.g. it is blood meal induced in *Anopheles gambiae*, and may also play a role in host seeking behavior (Muller *et al*. 1995). It may thus be involved in host seeking behavior in slavemakers and slavemaker detection in hosts. Endogenous daily (circadian) and annual (circannual) rhythms serve as biological clocks that provide the major basis for timing in most organisms (Gwinner 1996). Annual timing mechanisms regulate seasonal timing of reproduction, moult, and hibernation (Wikelski *et al*. 2008, and authors therein). Positive selection on the *circadian clock controlled protein*, in slavemakers compared to hosts, suggests that this gene may regulate the aberrant activity patterns of slavemaker workers. Slavemakers are only active during raiding season in summer, and are taken care of for the rest of the year by the slaves (Herbers *et al*. 2002, Pohl and Foitzik 2011). This changed activity pattern might thus be manifested by changes in the circadian rhythm. Two more genes of interest with possible direct link to the slavemaker evolution are "*Elongation of very long chain fatty acids protein*" in *T. duloticus* and *Fatty acyl-CoA reductase 1* in *T. pilagens*. Both genes could be involved in the synthesis of cuticular hydrocarbons and thus might play a role in the avoidance of host recognition (Blomquist and Bagnères 2010). Indeed, a recent study on the cuticular hydrocarbon profiles of the same six species revealed that slavemakers show consistently different chemical profiles than the three host species (Kleeberg *et al*. 2017). A recent switch to a parasitic lifestyle could thus have led to selection on genes underlying hydrocarbon synthesis.

In slavemakers only four pathways are shared among species. Three of these (metabolic pathways, biosynthesis of secondary metabolites, and biosynthesis of antibiotics) were also identified in the hosts, and only endocytosis is slavemaker specific. In hosts, the genes with signatures of positive selection belong to 102 different pathways, eight of which are shared among the three species. The PI3K-Akt pathway is part of the mTOR pathway regulating the cell cycle, and also known for its function in longevity (Hay 2011). Furthermore the longevity regulating pathway is shared among all three host species, reinforcing the importance of longevity within these host species. Despite their small body and colony size, *Temnothorax* ants are quite long-lived with workers living up to a few years and queens over two decades (Keller and Genoud 1997). Social Hymenopterans are known for a change in the longevity-fecundity trade-off with queens being both long-lived and highly fecund compared to the short-lived sterile workers (Heinze *et al*. 2013, Oettler and Schrempf 2016, Rodrigues and Flatt 2016, Rueppell *et al*. 2016, Toth *et al*. 2016, Negroni *et al*. 2016). We identified two pathways which may play a role in morphological differences between the species and their adaptive divergence. The Wnt signalling pathway is known for its role in regulating key events during embryonic patterning and morphogenesis (Komiya and Habas 2008), and the thyroid hormone signalling pathway (in humans) is involved in the regulation of growth development and metabolism. The latter has been shown to play a role in the adaptive divergence of sticklebacks (Kitano *et al*. 2010).

Slavemaker-Host Pairs

Besides determining similarities in possible selection pressures within slavemakers and within hosts, we additionally investigated similarities in slavemaker-host pairs, because of their shared environment. We hypothesized that genes shared by both slavemakers and hosts with signatures of positive selection might give indication on local environmental selection pressures; though they could also represent genes involved in the co-evolutionary arms race. However, on the gene level there is hardly any congruence between slavemaker and host pairs (0-4 overlapping genes). On the pathway level, some of the above mentioned candidates appear, such as thyroid hormone signalling pathway in *T. ambiguus* and *T. pilagens*, PI3K-Akt and Wnt signalling pathway between *T. americanus* and *T. longispinosus*. In the *T. duloticus* – *T. curvispinosus* pair we identified circadian rhythm as well as the FoxO signalling pathway. Among others the latter coordinates the response to environmental changes, including metabolic stress (starvation) and oxidative stress (Eijkelenboom *et al*. 2013). Hence, these two pathways may give evidence for environmental selection pressures, e.g. temperature, seasonality, or food availability, experienced by *T. duloticus* and *T.*

curvispinosus which co-occur in the same environment, in comparison to the other four species which are from different locales.

Conclusions

Our positively selected gene analyses revealed several candidate genes with a possible link to the slavemaker lifestyle, which are involved in the cuticular hydrocarbon profile composition, thus species recognition, and the aberrant activity pattern of slavemaker workers. To verify the functional and phenotypic importance of these candidates will now be the next step.

Furthermore, the results show little overlap of selected genes between species. On the pathway level however, we find higher congruence between species than expected, even though the majority of selected pathways remain species specific. Furthermore, the genes under positive selection belong to a wide variety of functions, as indicated by negative results in the enrichment analyses. The same pattern was identified in social parasitic cape honeybees (Wallberg *et al*. 2016). It thus seems that the evolution of social parasites, including slavemakers is a broadly-encompassing process with species-specific evolutionary trajectories, based on selection in many genes with different functionality and pathway affiliation. Our results support the hypothesis that evolution is the unrepeatable result of stochastic events with highly contingent effects (Gould 1989).

Funding

This project was funded by a grant to Barbara Feldmeyer and Susanne Foitzik (DFG FE 1333/3-1 and FO 298/17-1) and support from the E. N. Huyck Preserve, Rensselearville NY. Collection permits were obtained at the Parks and Preserves.

Data Accessibility and Additional Information

All data analysed during this study are included in various Additional files (see original publication) and the contig ortholog clusters are available from NCBIO BioProject GSE95604 (GSE95604_2017July_TemnoClusters.fas-gb.tar.gz).

Chapter 3

The gene *Trypsin Inhibitor* affects raiding success in *Temnothorax pilagens*

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Manuscript in preparation

Abstract

Behavior is a plastic phenotypic trait, changing in response to external stimuli or rhythmically over time. Socially-parasitic slavemaking ants display characteristic slave raiding behavior for only a few weeks in summer. Recent transcriptomic studies have identified genes involved in seasonal raiding behavior of the slavemaking ant *Temnothorax pilagens*. Complex behaviors, like that of *T. pilagens'* raiding phenotype, are the amalgamation of simpler behaviors controlled by single or smaller numbers of genes. While these genes may retain aspects of their ancestral functions, their involvement in taxa-specific networks may result in novel functions. Here, we used RNAi-mediated gene knockdown to elucidate the role of *Trypsin Inhibitor* within the raiding behavior of *T. pilagens*. *Trypsin Inhibitor* knockdown affected neither the onset of raiding nor behavior during scouting. However, once raiders discovered and entered the host nest, knockdown slavemakers often remained with no attempt to steal brood or forcefully expel hosts. Consequently, as less host brood was stolen, these raiders had far lower raiding success. Thus, we present the first evidence linking a single gene – *Trypsin Inhibitor* – to raiding success in ants; strongly suggesting a re-purposing of *Trypsin Inhibitor* in *Temnothorax*, as raiding is not an ancestral function for trypsin-type genes and their inhibitors.

Keywords: Transcriptomics, gene expression, RNAi, neofunctionalization, social parasitism

Background

Elucidating the genetic mechanisms underlying the origin and diversity of novel complex characteristics is an integral component of evolutionary biology. Natural populations possess a wide array of phenotypic variation for all aspects of morphology, physiology, and behavior. The re-purposing of modular genetic components into novel molecular contexts – instead of the structural alteration of genes – is generally accepted as one of the central mechanisms for the development of novel complex phenotypes (Shubin *et al*. 2009). Many previous studies have elucidated the development of novel morphological characteristics from existing phenotypes (Erclik *et al*. 2009, Kozmik *et al*. 2008, Johanson *et al*. 2007, Emlen *et al*. 2007, Pueyo *et al*. 2008), lending credibility to the idea that neo-functionalization of existing genes and gene pathways results in the diversification of complex phenotypes. One outstanding example of the diversification of complex physical phenotypes through novel implementations of existing genes is the re-purposing of appendage patterning genes in butterflies and other insects, resulting in the rapid evolution of eyespots on the wings of many butterfly species (Brunetti *et al*. 2001, Monteiro 2015, Kijimoto and Moczek 2016). Similarly impressive is the extraordinary radiation of leaf- and treehopper “helmet” structures, which result from the recruitment of existing developmental genes into novel molecular pathways (Moczek 2011, Moczek *et al*. 2011, Prud'homme *et al*. 2011); though the precise function of genes involved in this mechanism prior to their inclusion into helmet development remains under debate (Moczek 2011, Prod'homme *et al*. 2011, Yoshizawa 2012). Thus, newly-developed gene networks or patterns comprised of existing genes can indeed contribute to both the macroevolution (e.g. development of helmets in leaf- and treehoppers) and microevolution (e.g. specific helmet structures) of complex phenotypes within species. However, studies focusing on molecular patterns associated with the evolution of complex behavioral phenotypes are far less common.

Complex behaviors represent one particularly interesting avenue for elucidating the functional impact of small numbers of genes upon otherwise highly complex phenotypes. Controlled largely through variable gene expression of an array of genes, complex behavioral phenotypes may be shaped by a range of factors, from nutritional availability to predator prevalence to environmental conditions. In many cases, the modification of molecular pathways controlling simpler, underlying behaviors can result in the evolution of highly complex behavioral phenotypes, such as social behavior in insects (Toth and Robinson 2007). While the interplay between gene expression and behavior is an ongoing field of

study, many described patterns tend to be taxa-specific (Cresco *et al*. 2004, Colosimo *et al*. 2005), and many gaps in our understanding of the relationship between gene expression and phenotype persist (Mank 2017): Can complex behavioral phenotypes be substantially influenced through differential expression of a single gene, or is the modification of more expansive gene networks necessary? If novel behaviors do arise, which genes are associated with these behavioral shifts? Indeed, it has been shown previously that gene duplication and sub-functionalization, as well as co-optation of pre-existing networks precluded the evolution of novel morphological traits (Shubin *et al*. 2009, Monteiro and Gupta 2016). Whether or not this is true for more variable behavioral traits requires in-depth manipulation experiments to link specific genes and more general gene expression patterns to specific behavioral phenotypes. A first study into novel behavioral phenotypes, such as the origin of social parasitic behavior in *Pogonomyrmex* and *Vollenhovia* ants showed that they resulted from regulatory changes within a multifunctional genome, rather than novel genomic mutations (Smith *et al*. 2015).

Raiding behavior in *T. pilagens* is a novel trait within this relatively young slavemaking species (Feldmeyer *et al*. 2017). Nonetheless, the raiding phenotype is highly complex, comprised of numerous phases and countless sub-behaviors (Kleeberg *et al*. 2016). Non-reproductive *T. pilagens* raiders must first locate a suitable host nest containing offspring in their pre-pupae and pupal stages. This scouting phase is comprised of a number of host-seeking and path-finding elements, and is characterized by elevated activity levels in scouting individuals. Once a host nest is located, slavemakers return to their own nest in order to recruit nestmates into a raiding party. The focal scout utilizes antennation and chemical cues in order to stimulate nestmates into action. Recruited individuals form a raiding party, headed by the original scout which found the host nest. Externally, slavemaker raiding parties resemble tandem-running behavior employed by non-parasitic *Temnothorax* species, where recruits learn the location of a resource by following the original scout. In such instances, leading individuals travel through a start-stop motion, which allows following individuals to keep track of leaders through direct antennation of the leader's gaster. However, this is where similarities between slavemaking and non-slavemaking raiding/tandem-running behaviors end. *Temnothorax* slavemakers utilize mixed-species raiding parties, often comprised of both parasites and enslaved hosts. Further separating raiding parties from tandem-running, slavemaker raiding parties seek out only host colonies and brood for the purpose of enslaving hosts and expanding the enslaved workforce. Raiders that do manage to find the host nest successfully then systematically drive out or kill existing

host workers and queens. *T. pilagens* raids specifically are highly variable (Kleeberg *et al*. 2016): in some cases, raiders are able to enter a host nest undetected. Through the use of chemical insignificance, chemical manipulation, and antennation, *T. pilagens* raiders are able to coerce host workers into transporting their own brood from the host nest back to the slavemaker nest. This approach allows for not only the enslavement of host brood, but host adult workers as well! However, in other cases, *T. pilagens* is quickly identified by hosts as a threat to the colony, and is openly attacked by host defenders. In such cases, *T. pilagens* raiders respond swiftly and decisively, stinging to death host workers and reproductives. Once all hosts are dead or have been driven out, *T. pilagens* raiders are able to carry out the final phase of a raid, the abduction and transportation of host brood back to the slavemaker nest.

Given the complexity and variability of behaviors comprising the *T. pilagens* raiding phenotype, it is remarkably unlikely that raiding as a whole is controlled by a single gene. To date, very little is known about the number of genes involved in raiding, or the role of specific genes within the context of the raiding phenotype. Within the entire *Temnothorax* genus, only a handful of genes have been functionally examined. One previous study has elucidated the function of *Vitellogenin-like-A*, and its role in regulating division of labor in *T. longispinosus* workers (Kohlmeier *et al*. 2017). Here, the artificially-induced knockdown of gene *Vit-like-A* revealed that it is responsible for the transition of workers from brood-caring to nestmate-caring, an intermediate step in the behavioral maturation from brood-caring to foraging. Individuals expressing high levels of *Vit-like-A* exhibit elevated responsiveness to brood-related stimuli, where individuals with low levels of *Vit-like-A* expression shift their preference towards nestmate care instead. Thus, while *Temnothorax* is a suitable model for elucidating the functional role of genes in behavior, as yet there is no investigation in *Temnothorax* into the impact of single genes on complex behavioral phenotypes such as slavemaker raiding behavior.

Here, we seek to elucidate the impact of specific genes upon the complex *T. pilagens* raiding phenotype. Utilizing individual transcriptomes compiled from RNA-Seq data from six *Temnothorax* species, a previous study compared the gene expression patterns of three slavemakers (*T. americanus, T. pilagens*, and *T. duloticus*), during two separate behavioral states; and similarly the expression patterns of three closely-related host species (*T. ambiguus, T. longispinosus*, and *T. curvispinosus*), also during two different behavioral states (Alleman *et al*. 2018). Expression data for specific genes of interest – combined with the

manual curation of functional information for strongly differentially-expressed genes – revealed a number of genes with expression patterns strongly correlating with *T. pilagens* raiding behavior. Among these, the gene *Trypsin-7* was found to be strongly negatively-associated with the raiding phenotype in all slavemaking species, suggesting that the up-regulation of *Trypsin-7* might somehow be responsible for suppressing this behavior in *Temnothorax* (Alleman *et al*. 2018). Additionally, however, only *T. pilagens* possesses a *Trypsin Inhibitor* gene that shows a strong positive correlation between expression and raiding (Alleman *et al*. 2018). Therefore, we propose that potentially the raiding phenotype itself - though more likely one or multiple sub-components of the *T. pilagens* raiding phenotype - is influenced by the level of expression of this *Temnothorax Trypsin Inhibitor;* making this gene a prime target for functional investigation through manipulative experimentation. Here, using RNAi-mediated gene knockdown based upon previous explorative RNA-Seq data, we intend to investigate the role of single genes within a complex phenotype, in this case by elucidating the functional significance of *Trypsin Inhibitor* within the context of the *T. pilagen's* raiding phenotype. Chiefly, we seek to investigate whether or not the manipulation of expression levels of a single gene can result in measurable changes to a complex phenotype. Furthermore, can the loss of a single gene result in broader fitness consequences to *T. pilagens* as a whole? And if so, are these fitness consequences the result of impaired raiding sub-behaviors, or from a general disruption of the raiding phenotype in general? Thus, the objective of this study is twofold: to simultaneously ascertain the impact of single genes within a highly complex behavioral phenotype as well as identify the functional role and fitness impact one gene - *Trypsin Inhibitor* - within the *T. pilagens* raiding phenotype.

Material and Methods

Collection and Maintenance of Colonies

T. pilagens and *T. ambiguus* colonies were collected at the Sleeping Bear Dunes National Lakeshore in Michigan during the summer of 2016 (Table 3.1), and transported in Ziploc bags within their natural nest sites. Upon arrival in our laboratory, individual colonies were transferred into their plaster-floored nesting box, containing a single slide-nest into which the colony relocated. A slide nest is an artificial nesting site comprised of a small Plexiglas cavity sandwiched between two glass microscope slides. Colonies were then kept under a constant 20°C, 14Light:10Dark light cycle and were fed twice weekly with honey and

crickets. All colonies used in raiding experiments were transferred to 25°C, 14Light:10Dark light cycle conditions one week prior to the onset of the raiding experiment to promote an increase in scouting and raiding activity in slavemakers.

Table 3.1: GPS coordinates of 2016 collection sites for slavemaker *T. pilagens* and host *T. ambiguus* colonies used throughout this experiment.

Collection sites:	*Coordinates:*
A	(44.843,-86.057)
B	(44.845,-86.058)
C	(44.844,-86.059)
D	(44.830,-86,057)
E	(44.842,-86.047)
F	(44.803,-86.058)
H	(44.831,-86.040)
I	(44.830,-86,057)

Selection of Trypsin Inhibitor as Candidate Gene for Knockdown

We utilized gene expression data produced in a previous study (Alleman *et al*. 2018) in order to create an initial list of candidate genes suitable for RNAi knockdown. *Trypsin Inhibitor* was chosen for further examination based on a number of factors: it is significantly over-expressed in the *T. pilagens* raiding phenotype (log fold change: 1.52; FDR-value: 0.0005), with a relatively high number of reads within each raiding replicate (Supplementary Table 1). These parameters ensure that *Trypsin Inhibitor* not only undergoes a radical shift in expression between phenotypes, but that this gene also displays a high level of molecular activity. We sought to avoid genes with strong changes in expression but weak levels of molecular activity even when up-regulated, as indicated by a lower number of raw reads in the up-regulated state. From this list, additional information on gene function was gathered manually. DsiRNA (Supplementary Table 2) was designed using IDT's custom DsiRNA design tool (eu.idtdna.com/site/order/designtool/index/DSIRNA_CUSTOM).

Examining T. pilagens Gene Trypsin Inhibitor

In order to examine the impact of *Trypsin Inhibitor* knockdown upon the raiding success, 58 *T. pilagens* colonies were selected and evenly distributed into 29 treatment and 29 control colonies. Overall colony composition, as well as the composition of only those colonies that carried out raids, did not differ between treatment and control groups (Supplementary Table 3). Of note is the generally small size of *Temnothorax* colonies (both slavemaker and host). Within this experiment, host colonies on average contained only 20 +/- SE 0.64 workers and 14 +/- SE 0.81 brood items (larvae and pupae), and slavemakers contained on average 4 +/- SE 0.25 slavemakers and 14 +/- SE 1.21 slaves. All colonies involved within this experiment were fed 15 µL sucrose + RNAi solution for 14 subsequent days. Treatment colonies received sucrose solution containing *Trypsin Inhibitor* dsiRNA at a concentration of 0.1 µg/µL. Control group colonies received non-functional nonsense dsiRNA at the same concentration of 0.1 µg/µL. As targets for raiding, 180 *T. ambiguous* host colonies were chosen, each containing relatively large numbers of brood items. Slavemaker colonies were given two opportunities between July and August 2016 to raid host colonies, once on the 14th day of RNAi feeding (n = 58), and again on the 24th day of RNAi feeding (n = 56). Slavemaker death constitutes to the reduction in number of suitable experimental colonies throughout the experiment. The raiding arenas are square-shaped plastic containers (l=20 cm, w=20 cm, h= 6 cm) with plastered floors and paraffin-lined edge to prevent ants from leaving the arena. Slavemaker and host nest were placed at opposite corners of the arena. As ants cannot perceive red light, transparent red plastic was placed over both slavemaker and host nests, which allowed us to observe each nest without disturbing them. All raids were conducted at 25°C with constant lighting and humidity to ensure consistency between raids. Before each raid, both slavemaker and host colonies were counted, and the number of queens, workers, larvae, prepupae, worker pupae, male pupae, and queen pupae were recorded. All slavemaker colonies were given 24 hours to raid. We observed and recorded data for the first seven hours of the raid, and let them continue the raid overnight, checking the raiding outcome the following morning. During each observation period, we recorded the time and/or number of occurrences of specific events, such as when and how often slavemaker scouts leave their home nest, and if/when a slavemaker scout discovers the host nest. Should the scout successfully relay its information and recruit nestmates into a raiding party, we recorded the size and composition of these raiding parties, and which individuals (if any) are successful in finding the host nest. If all or some members of the raiding party do enter the host nest, we recorded the strength and success of the raid by observing how many slavemakers enter the host nest and how many brood items raiders

subsequently removed from the host nest. Furthermore, we recorded the evacuation efforts of the host colony. The following morning, we ascertained the fitness outcome of the raid for both slavemaker and host. In total, 114 raiding events were observed, evenly distributed between treatment and control groups (n = 57, for each both experimental groups).

qPCR

Before carrying our qPCR, RNA extracted from *Temnothorax* whole-bodies was converted to cDNA via Qiagen Rneasy Mini Kit. Real-Time Quantitative PCR was performed using SYBR Green and a Bio Molecular Systems Mic Real Time PCR Cycler (biomolecularsystems.com/), with expression calculations made using the REST method (Pfaffl 2001, Pfaffl *et al*. 2002) within the supplied micPCR v2.6.0 software. As the micPCR software does not determine the stability of housekeeping genes, this was done externally via Wilcox Test in R.

Statistical Analyses

All statistical analyses were performed using R version 2.15.2 (R development core team 2012). Colony composition between all slavemaker treatment and control and host treatment and control colonies was compared with Mann-Whitney-U and GLMER tests. Comparison of raiding success between treatment and control groups was accomplished using GLM tests. The analysis of behavioral data between groups was carried out utilizing GLMM models. Here, Colony ID and Trial Number were entered as random factors, while experimental group (knockdown or control) was entered as an explanatory factor.

Results

Trypsin Inhibitor Knockdown in T. pilagens

T. pilagens raids can be separated into several distinct stages: scouting, raiding, and brood transport. In total, scouting activity from slavemakers was observed in 73 of 114 (64%) of all raiding experiments. Number of colonies willing to send out slavemaker scouts appeared unaffected by *Trypsin Inhibitor* knockdown, as treatment (n = 37, ~65% of treatment experiments) and control (n = 36, ~63% of control experiments) groups showed similar scouting propensity (GLMM: $X^2 = 0.08$, df = 1, p = 0.78). Similarly, the number of individual scouting events did not differ between treatment and control groups (GLMM: $X^2 = 0.76$, df = 1, p = 0.38). Interestingly, *Trypsin Inhibitor* knockdown colonies did have fewer scouting events that failed to locate the host colony (GLMM: $X^2 = 4.29$, df = 1, p = 0.038).

Once a slavemaker scout has located a host nest, slavemakers will often raid this nest. *T. pilagens* raids have four possible outcomes (Alloway 1979, Foitzik *et al*. 2001, Jongepier *et al*. 2014, Jongepier *et al*. 2015): 1] *Typical raids*, where raiders will drive out or kill adult hosts within the host nest, afterwards transporting all remaining host brood back to the slavemaker colony, 2] raider *takeover* of host nest where defending hosts are killed or driven out. Colony takeover differs from normal raids in that in the raiding slavemaker continue to reside within the host colony, assuming control of all host brood therein, 3] *failed raid*, when slavemaker raiders attack but are unable to overcome host defenses and therefore obtain no host brood, or 4] individual or multiple raiders will, if not detected by host defenders, *move into* the host nest without driving out or killing any adult hosts.

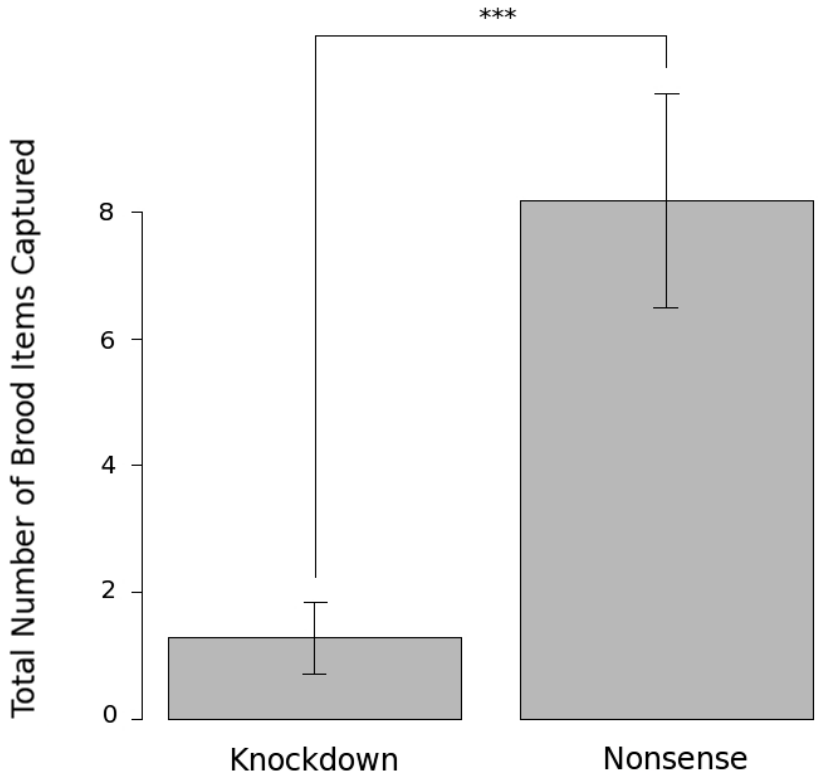

Figure 3.1: Total number of brood stolen per colony for the duration of the experiment. On average, *T. pilagens* colonies fed with nonsense RNA captured about four times as many brood items as treatment colonies fed with *Trypsin Inhibitor* dsiRNA (p-value = 0.001).

The typical raiding outcome was far more common in control experiments than treatment experiments (GLMM: $X^2 = 13.79$, df = 1, p = 0.0002). Host colony takeover by raiders was rare (n = 3, or 12% of treatment raids; n = 1, or 6% of control raids), and did not differ

between experimental groups (GLMM: $X^2 = 0.03$, df = 1, p = 0.85). While failed raids were more common in treatment experiments (n = 11, or 44% of treatment experiments) than control experiments (n = 2, or 12.5% of control experiments), this was not significantly different between these groups (GLMM: $X^2 = 2.58$, df = 1, p = 0.11). Intriguingly, within this experiment, 28% (n = 7) of treatment raids ended with slavemaker raiders moving into the host colony without first removing the existing host colony, whereas this raiding outcome was completely absent within control experiments. That treatment colonies executed fewer successful raids is reflected in the number of brood captured from host nests: control colonies obtain far more host brood items than treatment colonies (GLMM: $X^2 = 11.50$, df = 1, p = 0.001; Figure 3.1).

Discussion

Based on existing gene expression analyses (Alleman *et al*. 2018), we hypothesize that those genes with a strong positive correlation between expression and raiding behavior play an important role in the overall raiding behavior of the *Temnothorax* slavemaker *T. pilagens*. As it is unlikely that complex phenotypes are controlled chiefly by single genes, we also sought to elucidate the sub-function of differentially-expressed genes within the context of complex slavemaker raiding behavior. *Trypsin Inhibitor* was among those genes found to be highly up-regulated during raiding behavior. Subsequent knockdown of *Trypsin Inhibitor* in *T. pilagens* via dsiRNA-mediate RNA interference resulted in the appearance of intriguing behavioral aberrations during raiding experiments.

T. pilagens raiding behavior may be broken down into distinct phases, each characterized by different sub-behaviors. The first of these stages, scouting – where *T. pilagens* raiders attempt to locate and identify suitable host colonies – appears to be largely unaffected by *Trypsin Inhibitor* knockdown. Neither the number of colonies deploying *T. pilagens* scouts nor the number of specific scouting events differed between experimental groups. Previous studies have shown that scouting/raiding propensity in *Temnothorax* slavemakers is influenced largely through response thresholds; where slavemakers are more likely to perform scouting and subsequent raiding activity when their colony is subject to lowered nutritional provisioning (Pohl *et al*. 2011, Pohl *et al*. 2013). As typical foraging tasks are performed exclusively by enslaved workers, nutritional provisioning of a colony is often directly linked to the ratio of slavemakers to slaves. As such, it seems unlikely that *Trypsin*

Inhibitor is involved with the decision-making cascade associated with raid initiation. However, that *T. pilagens* scouts from treatment colonies are in fact more able to locate and/or identify host colonies is intriguing, suggesting that *Trypsin Inhibitor* might play a role in *T. pilagens* ability to detect or identify their hosts. Indeed, the involvement of Trypsin-type genes in host-seeking behavior is not unfounded: *Trypsin-7* expression is strongly associated with host-seeking behavior in *Aedes aegypti* (Müller *et al*. 1995). That *Trypsin Inhibitor* expression, and not *Trypsin-7* itself, is positively associated with raiding/host-seeking behavior in *T. pilagens* does suggest that the precise molecular patterns associated with host-seeking do differ between *T. pilagens* and *A. aegypti* while the more broad functionality is retained. However, whether or not the shifting of gene expression patterns is due the relative dissimilarity in context of host-seeking within these two species is beyond the scope of this investigation.

While *T. pilagens* scouting initiation or propensity does not appear to be affected by *Trypsin Inhibitor* knockdown, raiding behavior does differ substantially between treatment and control groups. *T. pilagens* control colonies where *Trypsin Inhibitor* was not knocked down were characterized by the execution of typical raids (Figure 3.2), where defending adult hosts were forced from their colony, and the remaining host brood transported back by *T. pilagens* to the slavemaker colony. Intriguingly, raiders from *Trypsin Inhibitor* knockdown colonies, by comparison, were far less likely to carry out typical raids; instead treatment raiders often failed to fully complete their raids and instead moved into host colonies with neither violence nor any attempt to steal host brood. This outcome was entirely absent in control colonies (Figure 3.2). While it does appear that *Trypsin Inhibitor*-knockdown individuals are still able to identify *T. ambiguus* colonies, as evidenced by the general success of knockdown individuals to locate and infiltrate these colonies, it remains unclear as to why so many of these individuals do not proceed with normal raiding behaviors after entering a host nest. However, we suspect that *Trypsin Inhibitor* plays a role in host-seeking behavior via sensory and/or olfactory perception. Indeed, *Trypsin* activity has been linked to host-seeking behavior in other insect species (Muller *et al*. 1995). In line with this, a previous study into *Temnothorax* slavemakers revealed that *Trypsin-7* is strongly down-regulated during raiding behavior in *T. pilagens*, contrary to *Trypsin Inhibitor* which is strongly up-regulated during raiding behavior (Alleman *et al*. 2018). Therefore, we speculate that *Trypsin-7* in *T. pilagens* might be regulated by the *Trypsin Inhibitor* herein examined, though the direct metabolic mechanism linking the expression of these genes to specific behaviors remains unclear.

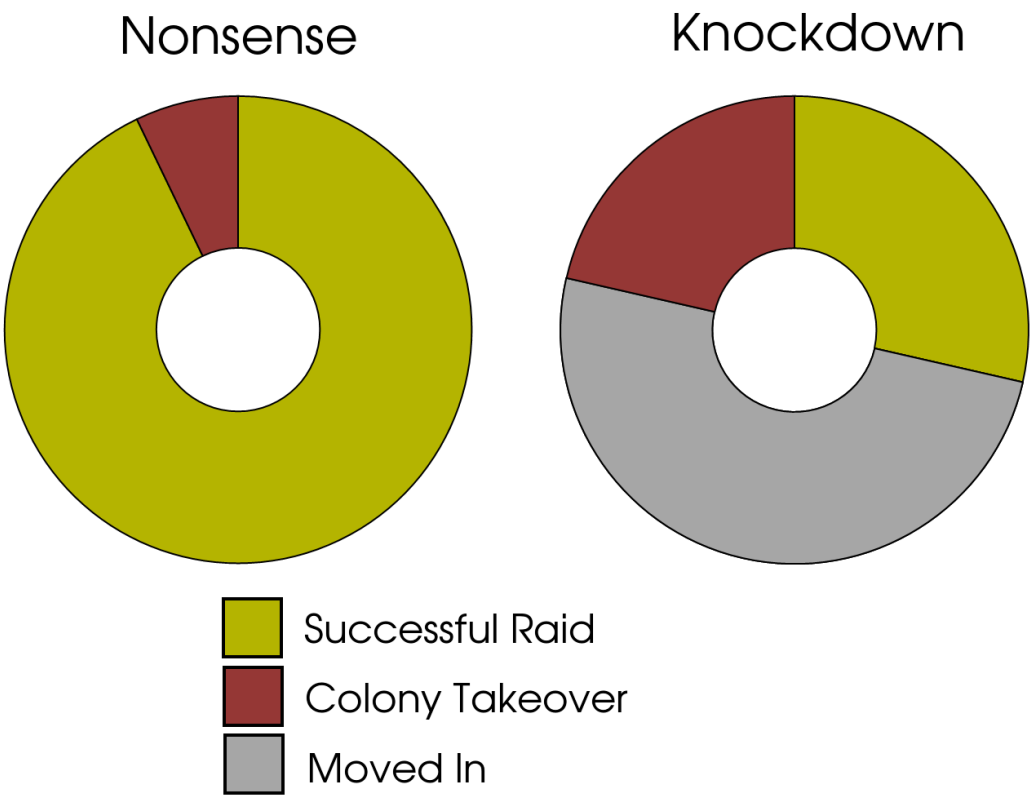

Figure 3.2: Proportion of raiding outcomes for those a) control-nonsense and b) treatment-knockdown *T. pilagens* colonies that performed raids. Colonies fed with nonsense RNAi showed a much greater propensity to carry out typical raids, with only a small subset of total raids resulting in the takeover of the host *T. ambiguus* colony by *T. pilagens* raiders. No raiders from nonsense RNA colonies were observed moving into the host nest. By contrast, moving into host *T. ambiguous* colonies by *T. pilagens* raiders from *Trypsin Inhibitor*-knockdown colonies was the most common raiding outcome in this experimental group; diverging substantially from the more ordinary pattern of raiding outcomes displayed by nonsense RNA-fed individuals.

It is already known that *T. pilagens* slavemakers and their primary host *T. ambiguus* possess highly similar cuticular hydrocarbon profiles (Kleeberg *et al*. 2015). Therefore, reliably distinguishing between nestmate and non-nestmate is crucially important for both slavemaker and host: slavemakers for distinguishing between host species as well as parasitized and un-parasitized host colonies, and hosts for identifying intruding slavemakers (Lenoir *et al*. 2001). However, simple involvement in sensory perception does not adequately explain why *Trypsin Inhibitor*-impaired individuals are still able to locate *T. ambiguus* nests, but subsequently do not proceed with normal raiding behavior. As such, it is possible that *Trypsin Inhibitor* is intertwined with *T. pilagens* capacity to differentiate between *T. ambiguus* workers and brood, or ability to identify host brood items altogether. Thus, it is not unlikely that slavemakers where *Trypsin Inhibitor* has been artificially down-regulated

retain the ability to identify *T. ambiguus* colonies, but lack the mechanisms necessary to trigger proper raiding activity.

Interestingly, *Trypsin Inhibitor* knockdown in *T. pilagens* has a direct, negative impact on the fitness of affected slavemaker colonies. Indeed, in our experiments, knockdown colonies controlled only a quarter as many host brood items after raiding as nonsense-fed colonies (Figure 3.1). Unsurprisingly, moving in is non-adaptive for *T. pilagens*, as no new slaves are captured and thus the slavemakers' workforce is not replenished. As such, we may conclude that *Trypsin Inhibitor* does play an important role in the *T. pilagens* raiding phenotype, where artificial down-regulation of this gene results in a direct, negative impact upon raiding success and subsequent colony-level fitness. As *T. pilagens* workers are ordinarily not reproductive, this inability to obtain additional slaves directly results in a weakened workforce in the following months.

Taken together, the results of this experiment shed some light onto single genes in complex behavioral phenotypes. Characterized by raiding behaviors during summer months, slavemaking ants in the *Temnothorax* genus are good models for investigating the genetic underpinnings of behavior. While it has been shown previously that single genes can control complex behavioral phenotypes (Ben-Shahar *et al*. 2002), in most cases complex behaviors are driven by multiple genes; for example hygienic behavior in the honeybee *Alpis melifera* (Lapidge *et al*. 2002, Oxley *et al*. 2010). In this regard, *T. pilagens* certainly seems to follow general patterns, where a single gene is involved in but not completely controlling the raiding phenotype. In *T. pilagens, Trypsin Inhibitor* is strongly up-regulated during the raiding phenotype, and knockdown of this gene via RNAi during raiding season results in atypical raiding outcomes and a severe decrease in the number of brood stolen by slavemaker raiders. Thus, while our results suggest that *Trypsin Inhibitor* is unlikely to be involved in raiding initiation or scouting behavior in *T. pilagens*, it is strongly associated with raiding outcome and enslavement of host brood. Taken together, *Trypsin Inhibitor* has a strong influence on overall colony fitness in *T. pilagens*. However, given that the raiding phenotype In *Temnothorax* is highly complex and largely species specific (Alleman *et al*. 2018), additional functional studies are necessary to fully elucidate the genetic and regulatory mechanisms underlying raiding behavior within this genus.

Conclusions

While evidence from previous transcriptomic studies suggests that a large number of genes are differentially expressed during slavemaker raiding behavior, our knockdown of a single gene resulted in a clear deviation from the normal raiding phenotype. As originally posited, not all aspects of the raiding phenotype were affected by the RNAi knockdown of *Trypsin Inhibitor*; for example scouting initiation and motivation was unchanged. However, later stages of raiding were clearly disrupted after RNAi-knockdown. The outcome of slavemaker raiding, and the subsequent number of host brood taken by raiders, showed strong changes between experimental groups; having an intimately negative impact on slavemaker fitness. Thus, this indicates that a single gene can have dramatic effects on complex traits, and furthermore is the first evidence of one gene playing a substantial role in raiding behavior in ants.

Acknowledgements

Thanks go to S. Beros and M. Kever for performing qPCR verification. This project was funded by a grant to Barbara Feldmeyer and Susanne Foitzik (DFG FE 1333/3-1 and FO 298/17-1) and support from the E. N. Huyck Preserve, Rensselearville NY. Collection permit was obtained at the Sleeping Bear National Sand Dunes (Permit Number: SLBE-2016-SCI-0005). These collections adhered to local guidelines and no ethical approval or license was required.

Data Accessibiliy

Raw reads for transcriptome analysis (Alleman *et al*. 2018) deposited into NCBI's GEO database under Accession Number GSE95604.

Supplementary Data

Supplementary Table 1: Annotation and gene expression data of the contigs identified as *Trypsin-7* and *Trypsin Inhibitor* within *Temnothorax pilagens*.

Annotation Information	
Contig	*Blast Hit / Alignment Title*
Sample_683_PF_w10_smadr_Tpila_(paired)_contig_13954	gi|307184874|gb|EFN71152.1| *Trypsin-7* [*Camponotus floridanus*]
Sample_683_PF_w10_smadr_Tpila_(paired)_contig_6537	gi|307184874|gb|EFN71152.1| *Trypsin-7* [*Camponotus floridanus*]
tpila-same-mixed-beh.fa_rep_c15176	gi|332019407|gb|EGI59891.1| *Trypsin inhibitor* [*Acromyrmex echinatior*]

Gene Expression Statistics			
Contig	*logFC*	*PValue*	*FDR*
Sample_683_PF_w10_smadr_Tpila_(paired)_contig_13954	-6.846551648	1.66276E-05	0.001393721
Sample_683_PF_w10_smadr_Tpila_(paired)_contig_6537	-6.386196647	0.001408874	0.044170372
tpila-same-mixed-beh.fa_rep_c15176	1.519875806	5.53589E-06	0.000547784

Normalized Read Counts							
Contig	*BR_680*	*BR_681*	*BR_682*	*BR_683*	*DR_684*	*DR_685*	*DR_686*
Sample_683_PF_w10_smadr_Tpila_(paired)_contig_13954	2	14	6	322	0	1	1
Sample_683_PF_w10_smadr_Tpila_(paired)_contig_6537	7	0	0	464	1	0	3
tpila-same-mixed-beh.fa_rep_c15176	1974	1733	937	3392	7790	4896	6018

Supplementary Table 2: dsiRNA sequences used for the artificial knockdown of *Trypsin Inhibitor* in *T. pilagens*.

T. pilagens Trypsin Inhibitor	*Sequence*
Sequence 1 Sense	5' rArGrArUrCrArUrArGrCrUrGrArUrCrArArCrCrGrUrCrAGT 3'
Sequence 1 Antisense	5' rArCrUrGrArCrGrGrUrUrGrArUrCrArGrCrUrArUrGrArUrCrUrCrA 3'
Sequence 2 Sense	5' rGrCrArArArCrGrCrArArArCrArArUrUrUrCrArArArArGTA 3'
Sequence 2 Antisense	5' rUrArCrUrUrUrUrGrArArArUrUrGrUrUrUrGrCrGrUrUrUrGrCrArU 3'

Supplementary Table 3: Statistics demonstrating overall colony composition similarity between nonsense dsiRNA and *Trypsin Inhibitor*-targeting dsiRNA treatment groups.

Results (Day 1, before raid)	*Wilcox Test*	
Queens	W = 391.5	p-value = 0.5499
Slavemakers	W = 446	p-value = 0.6951
Slaves	W = 398.5	p-value = 0.7376
Total slavemaker brood	W = 455	p-value = 0.568

Results (Day 1, after raid)	*ChiSquare Test*		
	ChiSq	Df	Pr(>ChiSq)
Queens	0.6797	1	0.4097
Slavemakers	0.4033	1	0.5254
Slaves	7.8851	1	0.004984 **
Total slavemaker brood	16.454	1	4.983e-05 ***

Chapter 4

Tandem-Running and Scouting Behavior is Characterized by Up-Regulation of Learning genes within the Ant Brain

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Manuscript under revision, Molecular Ecology

Abstract

Tandem-running in ants has been described as a form of teaching, where spatial information possessed by a leader is conveyed to a following nestmate. Within *Temnothorax* ants, tandem-running is used within a variety of contexts, from foraging and nest relocation to – in the case of slavemaking species – raiding parties. Here, we elucidate the transcriptomic basis of scouting, tandem-leading, and tandem-following behavior across two species with opposing lifestyles: the slavemaking *Temnothorax americanus* and its primary, non-parasitic host *T. longispinosus*. Analysis of gene expression data from brains revealed that only a small number of unique differentially-expressed genes are responsible for scouting and tandem-running behaviors. Comparison of orthologous genes between *T. americanus* and *T. longispinosus* suggests that tandem-running is characterized by species-specific patterns of gene usage. However, within both species, tandem-leaders showed gene expression patterns median to those of scouts and tandem-followers. This is not unexpected, as leaders can be recruited from either of the other two behavioral states. Most importantly, a number of differentially-expressed behavioral genes were found, with functions relating to learning and memory formation in other social and non-social insect species; including glutamate and tyramine receptor genes. Learning and memory genes were specifically up-regulated within scouts and tandem-followers, which use spatial learning extensively to navigate novel environments. As such, results of our analyses suggest that tandem-running in *Temnothorax* ants involves learning of novel information by following individuals and possibly even teaching by leading individuals.

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

Background

Individuals of a social group acquire information through interactions with their conspecifics (Gould 1982, Caro and Hauser 1992, Caro 1995, Franks and Richardson 2006), often occurring through social learning where the behaviors of one individual are observed and imitated by other individuals of the society. In most cases, the transmission of information is of mutual interest for sender and receiver. The widespread ecological success of social insects can be in part attributed to their efficient sharing of information (Hölldobler and Wilson 1990). Information about resources or predators is often conveyed to nestmates and subsequently proliferated throughout the entire colony. For example, the waggle dance performed by honeybee workers benefits not only the individuals receiving the information - who are then able to more easily locate quality food sources - but also the colony as a whole for which the food is obtained (von Frisch 1967). Mediums for intra-species social learning within insects vary greatly, from the head-drumming by termites in response to invaders (Howse 1964) to the laying of pheromone trails to resources of interest in many ant species (Buschinger and Winter 1977). Social information is not always preferred, however, as honeybees can use both private information - which they obtained by exploring themselves - as well as the social information provided by a nestmate's waggle dance in order to locate resources (Seeley 1995, Menzel *et al*. 2011).

Despite their comparatively unsophisticated brains, many insects are good learners and have impressive long-term memory; the formation of which occurs in the mushroom bodies of the brain (Pascual and Preat 2001). Learning in insects has primarily been examined on both the behavioral and molecular levels using *Drosophila* flies and honeybees. In *Drosophila melanogaster*, learning behavior seems to be mediated by *dance*, *rutabaga*, *linotte*, *latheo*, *turnip*, and *protein kinase A-deficient* genes (Dudai *et al*. 1976, Livingstone *et al*. 1984, Dura *et al*. 1993, Rohrbough *et al*. 1999, Choi *et al*. 1991, Li *et al*. 1996, Scheunemann *et al*. 2018). Honeybees and other social Hymenoptera are of particular interest for learning studies as many species exhibit social learning, which can promote the spread of novel behaviors (Duskas 2008). In *Apis mellifera*, *protein kinase A*, *adenylyl cyclase*, *AmCREB*, *AmGluRA* and *AmTYR1* – are known to be involved in learning (Fiala *et al*. 1999, Wachten *et al*. 2006, Eisenhardt *et al*. 2003, Kucharski *et al*. 2007, Blenau *et al*. 2000).

Ants in particular have evolved an array of mechanisms for intra-colonial

communication, ranging from the usage of pheromones to sounds and touch (Jackson and Ratnieks 2006); though genetic information about these behaviors is relatively scarce. Nestmate recruitment plays a large role in the functional ant colony, from informing nestmates to the location of resources and nesting sites to alerting nestmates the presence of invaders or predators. By far the most common mechanism in ants for recruitment to resources is the laying of pheromone trails, constantly renewed and reinforced by subsequent individuals (Jackson and Ratnieks 2006). However, a less common form of nestmate recruitment – tandem-running - is often employed in species with small colony sizes, and is frequently cited as a more primitive form of transferring spatial information between nestmates than the pheromone trails employed by most ant species. Tandem-running behavior likely evolved convergently in Ponerine and Formicoxenine ants, and has been extensively studied in ants of the genus *Temnothorax*, which use it in order to convey information between nestmates (Wilson 1959, Hölldobler 1974, Möglich *et al*. 1974, Franks and Richardson 2006, Franklin and Franks 2012, Franklin 2014). Here, individual scouts with information about a resource of interest recruit a small number – typically one but occasionally more - nestmates, which are then physically led to the resource. Intriguingly, previous experience as a tandem-leader or follower appears to influence the propensity for tandem-running more so than age in *Temnothorax* (Franklin *et al*. 2011); contrary to the more common caste- or age-specific tasks observed across Hymenopterans (Kohlmeier *et al*. 2018). Tandem-running in *Temnothorax* is characterized by three distinct behavioral components: a scouting component, a leading component, and a following component (Möglich, *et al*. 1974). **Scouts** perform the highly risky task of searching for and reporting back the location of resources of interest such as food or nesting sites. Like in other social Hymenopterans, scouts are often the oldest and thus most expendable individuals of the colony (Seeley, 1983; Dreller, 1998), which face many dangers when exploring novel environments. Additionally, prior experience is invaluable for exploration (Seeley 1983, Dreller 1998). Scouts that do locate a resource of interest then return to their home nest to recruit additional workers to the resource, effectively becoming **Leaders** of the ensuing tandem-runs. Tandem-run leaders physically guide one to a handful of nestmates - **Followers** - to the new resource (Wilson 1959). While leading individuals tend to have first been scouts, those followers that reached the resource of interest via tandem-run can return to the home nest and become leaders by recruiting additional nestmates. Indeed, there is some evidence from *Temnothorax albipennis* that the follower orients in a way that best facilitates the learning of the path taken to the destination, and afterwards is then able to effectively guide new nestmates (Franks and Richardson 2006, Franklin and Franks 2012).

Tandem-running behavior has previously been described as a form of teaching, as it involves bi-directional feedback between leader and follower, where leaders must modify their behavior in order to allow followers to keep up and thus learn the route to the new resource (Franks and Richardson 2006, Richardson *et al*. 2007). During a tandem-run, followers closely track the leader via physical contact: when in close proximity followers tap their antenna on the gaster of their leader. When leader and follower are separated, the leader will stop and wait for the follower to re-establish contact, or even go searching on their own for their follower/s. While tandem-running in *Temnothorax* is much slower than the nestmate carrying seen in other taxa, tandem-followers gain experience about the environment and do learn the path to the target resource. Thus, costs in the form of a slower recruitment rate are associated with learning in tandem-running systems. Ultimately, both scouts and leaders possess information that may be transferred to nestmates; though the method of acquisition of this information can differ substantially depending upon whether the leader became so after scouting or following (Möglich 1978). Within both lifestyles, some overlap does occur between behaviors: both scouts and followers are acquiring new information, whereas both leaders and followers are engaged in social interactions.

However, within certain lifestyles, tandem-running behavior appears to have undergone contextual modification. The genus *Temnothorax* possesses a number of parasitic slavemaking species in addition to free-living species. The slavemaking lifestyle has evolved multiple times independently within *Temnothorax*, with slavemakers closely-related to their hosts (Emery 1909, Beibl *et al*. 2005, Prebus 2007, Feldmeyer *et al*. 2017). These social parasites perform slave raids, whereby slavemaker workers invade a closely-related host species' nest in order to steal host brood (Wilson 1971, Hölldobler and Wilson 1990). These stolen individuals are incorporated into the slavemaker nest and effectively bolster the slavemaker workforce. While enslaved workers perform all tasks inside the nest - including brood care - the slavemaking workers specialized primarily in a single task: To find and raid new host colonies (Alloway 1979, Pohl *et al*. 2011). To recruit nestmates for slavemaking raids, *Temnothorax* slavemakers employ leading strategies that are, externally, highly similar to non-slavemaker tandem-running (Buschinger and Winter 1977). However, the extent to which tandem-running behaviors are molecularly similar between contexts and lifestyles remains poorly understood.

T. longispinosus is the preferred host of *T. americanus*, and both are closely related species (Beibl *et al*. 2005, Feldmeyer *et al*. 2017). Since they are not direct sister species,

they only loosely match Emery's rule, which states that parasite and host are closely related (Emery 1909, Le Masne 1956, Ward 1996). The workers of both species are monomorphic and do not show morphological differentiation (Modlmeier and Foitzik 2011), but instead show temporal division of labor (Robinson *et al*. 2009). Like other *Temnothorax* species, *T. americanus* also utilizes tandem-running to recruit nestmates in this case for raiding parties (Pohl and Foitzik 2013). Workers of *T. americanus* do not show normal worker behavior, as they neither forage nor take care of the queen or her brood (Wesson 1939). As such, *T. americanus* workers are entirely dependent upon enslaved workers. In contrast to the slave-making ants, workers of *T. longispinosus* show a clear division of labor for tasks like nursing and foraging, and they continue performing these tasks even if enslaved by *T. americanus*. *T. longispinosus* workers extensively utilize tandem-running behavior in order to recruit nestmates to new resources (Hölldobler and Wilson 1990).

Here, we seek to elucidate the molecular patterns underlying the contextually-distinct tandem-running behaviors of the slavemaking ant *Temnothorax americanus* and its non-parasitic host *T. longispinosus,* and evaluate whether or not the tandem-running behavior of *T. americanus* during raiding and *T. longispinosus* during nest relocation share a common evolutionary - or molecular - basis. As a non-slave-making lifestyle is the ancestral state for *Temnothorax*, a shared molecular pattern between slave-maker and host during scouting and tandem-running behaviors would indicate that slavemakers have conserved and even re-functionalized existing genes responsible for ancestral, non-parasitic tandem-running behavior. As gene expression within the brain can strongly influence behavior in social insects (Whitfield *et al*. 2003, Zayed and Robinson 2012, Liang *et al*. 2014), we first sought to determine the gene expression patterns within the brains of *T. americanus* and *T. longispinosus* during the tandem-running behaviors of each species. While scouting behavior is relatively well studied, comparatively few studies explore the differences in brain gene expression between the behaviors of scouts and other workers in ants (Ingram *et al*. 2005). Moreover, we hypothesize that in scouts and followers genes associated with learning in other social insects are over-expressed - like *protein kinase A* - since it is involved in learning in both honeybees and fruit flies. Additionally, we expect to see genes over-expressed in *T. americanus* and *T. longispinosus* scouts that are associated with scouting or foraging in other social insects, like biogenic amines, their receptors, or glutamate signaling genes (Liang *et al*. 2012). As tandem-running also requires extensive interaction and communication between nestmates, genes involved in information transmission and usage might also show behavior-specific patterns of expression, as is the case in a number of other social

Hymenoptera. Indeed, the two biogenic amines octopamine and dopamine seem to play a key role in the use of information, since treatment of bees with octopamine increased use of private information, while treatment with dopamine increased the use of social information. Additionally, the foraging gene *AmFor*, which influences foraging activity in the honeybee, is known to also affect use of social information in *Drosophila* (Thamm and Scheiner 2014, Foucaud *et al*. 2013). As such, these genes are likely candidates for up-regulation during both the leading and following behaviors of *T. americanus* and *T. longispinosus*.

Methods

Collection and Maintenance of Ant Colonies

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

Behavioral Experiments and Sampling

T. americanus and *T. longispinosus* behavioral experiments were carried out from the 29th of August to the 9th September, 2016. Both slavemaker and host colonies were provided with a plaster-floored arena 43cm x 28cm x 10cm in dimensions. Arena floor was kept damp for all colonies throughout the course of each experiment. Slavemaker and host colonies were tested under highly similar though lifestyle-specific conditions, selected specifically for this experiment to induce scouting and tandem-running. Slavemaker and host workers were sampled during three separate behavioral states: a scouting state, a tandem-leading state, and a tandem-following state.

In order to sample individuals displaying these behaviors of interest in *T. americanus* slavemakers, one slide nest containing the slavemaker colony was placed at one corner of the arena and one slide nest containing a *T. longispinosus* host colony, with queen and brood, was placed at the opposite end of the arena. This setup affords slavemakers the

chance to scout out and find the host colony, recruit additional individuals, and perform coordinated slave raids against that host colony. Slavemaker workers were sampled during scouting (a single *T. americanus* worker searching at least 10cm from own nest entrance), leading a raiding tandem-run (a slavemaker worker leading one or more slavemakers at least 10cm from the home nest entrance), or following a raiding tandem-run (following a slavemaker worker in a raiding party).

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

In both slavemaker and host experiments, workers observed performing a behavior of interest were quickly placed into a 1.5mL centrifuge tube and then placed into liquid nitrogen for freezing. Sample tubes were then transferred from liquid nitrogen to a -80°C freezer for short-term storage. In total, 42 *Temnothorax* individuals were sampled: 21 *T. americanus* and 21 *T. longispinosus*, of which 30 samples were sequenced (15 *T. americanus*, five samples per behavioral state; and 15 *T. longispinosus*, also five samples per behavioral state).

Subsequent dissection of *T. americanus* and *T. longispinosus* brains and extraction of RNA using the RNeasy Mini Extraction kit (Qiagen), was carried out before sending RNA samples to Beijing Genomics Institute (BGI) for library construction and sequencing 100 base pair (bp) paired-end reads on an Illumina HiSeq 4000.

De novo Transcriptome Assembly

RNA-Seq resulted in approximately 20 million 100 bp paired end reads per sample. Removal of *Illumina* adapters and trimming of paired-end reads was carried out using *Trimmomatic v0.32* (Bolger *et al.* 2014). Afterwards the trimmed reads were quality checked using *FastQC v0.11.5* and sequences flagged as poor quality were removed (Andrews 2010). After trimming 99.5% (328,117,222 in total) remained for *T. americanus* and 99.5%

(330,634,890 in total) for *T. longispinosus* (see Table S1, sheets 1 and 2, in supplementary materials).

With the trimmed paired reads, we created one *de novo* transcriptome per species using the data of all states with *Trinity v2.4.0* (Grabherr *et al*. 2011) and the following non-default parameters: --SS_lib_type FR --min_contig_length 300 --full_cleanup --no_bowtie --bflyHeapSpaceMax 20G --bflyGCThreads 1. Annotation of the transcriptomes was carried out using *BLAST v2.6.0* (Altschul *et al*. 1990) against the NCBI non-redundant protein invertebrate database (November 2016). Non-default parameters used were: --evalue 1e-5 --outfmt 5 --max_target_seqs 20. The quality of the resulting transcriptomes was determined using the script TrinityStats.pl included alongside *Trinity* (Grabherr *et al*. 2011).

Analysis of Gene Expression

Reads were aligned to their respective transcriptome using *Bowtie* (v2.2.6, Langmead and Salzberg 2012). Read count tables for both the *T. americanus* and *T. longispinosus* transcriptomes were produced with RSEM v1.3.0 (Li and Dewey 2011) as implemented in the *align_and_estimate_abundance.pl* script provided with *Trinity* and the following non-default parameters: --est_method RSEM --prep_reference --aln_method bowtie2.

For both *T. americanus* and *T. longispinosus*, we used the package *DESeq2 v1.16.1* in order to obtain the differentially expressed contigs (Love *et al*. 2014) (Table 4.1, Supplementary Tables S2 and S3). In both species, six pairwise-comparisons between each of the behavioral groups were made: “Scout vs Leader”, “Scout vs Follower”, “Leader vs Scout”, “Leader vs Follower”, “Follower vs Scout” and “Follower vs Leader”. The resulting p-values were adjusted for multiple-testing using Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) and only contigs with adjusted p-value of 0.05 or less were taken as differentially expressed for further analysis. Privately over-expressed contigs as well as over-expressed contigs shared between two behavioral groups were visualized as a Venn-diagram using the *package VennDiagram v.1.6.17* provided in *R* (Chen and Boutros 2011). Separation of differentially-expressed contigs by behavioral state was visualized by carrying out a Principle Component Analysis (PCA) on count data of only differentially expressed contigs using the function *plotPCA* provided by *DESeq2*. Finally, one dendrogram per species was produced (Figure 4.2), linking overall gene expression to individual behaviors and samples as an alternate method of visualizing gene expression similarities and

differences within each species using the function flashClust as implemented in the R package WGCNA (Zhang and Horvath 2005).

Weighted Gene Co-Expression Network Analysis

We used Weighted Gene Co-expression Network Analysis (WGCNA) in order to track contigs with similar patterns of expression between behaviors within a species (Zhang and Horvath 2005). Two separate WGCNA were performed, each in a block-wise fashion using all contigs from one of our two transcriptomes. WGCNA for both *T. americanus* and *T. longispinosus* contig subgroups were performed with default parameters, with the exception of soft threshold (power = 7 for both species), – which was chosen according to the approximated scale-free topology - minimum module size (50), and dissimilarity threshold (0.3) (Zhang and Horvath 2005). Eigengene values of the resulting modules were extracted and a Kruskal-Wallis test used to determine whether or not individual modules were associated with specific behaviors.

GO Enrichment and Pathway Analysis

Nucleotide contigs of both *T. americanus* and *T. longispinosus* transcriptomes were first transcribed into protein sequences with *TransDecoder* v3.0.1 (Haas *et al*. 2013) before further Gene Ontology (GO) analyses. *InterProScan* v5.25-64.0 was run on the protein sequences of both transcriptomes in order to obtain GO terms and KEGG annotations for each contig (Ogata *et al*. 1999, Jones *et al*. 2014, Ashburner *et al*. 2000). Finally, GO enrichment was performed using the package *TopGO v2.28.0,* executing a Fishers exact test on the lists of “Biological Process” GO annotations of the differentially expressed contigs / module contigs compared to the complete list of GO annotations of each respective transcriptome (Alexa and Rahnenfuhrer 2016) (Supplementary Table S4). We used the KEGG-ID output of *Interproscan* to obtain the associated pathway information from the *KEGG* database. (Ogata *et al*. 1999, http://www.genome.jp/kegg/tool/map_pathway1.html). Relative representation of pathways was compared between those contigs over-expressed in the three behavioral states of each species (Supplementary Table S5).

Construction of Orthologue Sequence Clusters

Finally, we constructed orthologue sequence clusters using *OrthoFinder* v1.1.8 (Emms and Kelly 2015) with translated amino acid sequences of transcriptome contigs of both species in order to determine if orthologous genes were similarly expressed in like behaviors between *T. americanus* and *T. longispinosus*. We used two approaches, since the

first approach only yielded a low number of orthologous clusters: a) all contigs of the two transcriptomes in their entirety, and b) only the DEGs of one species against the transcriptome of the other species. For our analysis we were only interested in orthologue clusters containing one sequence of each species. Clusters which contained one sequence per species and more than a single sequence in the other species were reduced to single-copy orthologous clusters based upon the highest BLAST score using an in-house R script (Supplementary Materials). Orthologue sequence clusters were used to (a) determine whether or not gene expression patterns are influenced more by behavior than by species by conducting a WGCNA on all orthologues, and (b) cross-compare DEGs from *T. longispinosus* and *T. americanus*. Finally, a GLMM (package lme4 v1.1, Bates *et al*. 2015) using read counts and a combination of contig ID and sample ID as random effect, plus a Friedman test on mean expression per gene, were used to test whether or not gene expression patterns could be explained by behavioral group.

Results

De novo Assembly and Annotation

De novo assembly of the trimmed paired forward and reverse reads using *Trinity* resulted in 294,117 transcripts for *T. americanus* and 256,533 for *T. longispinosus* (see Table S1 in supplementary materials). Mapping the trimmed reads back to the transcriptomes showed almost a similarly high back-mapping rate for both transcriptomes of 92.6% for *T. americanus* and 92.8% for *T. longispinosus*. The BLAST search against the non-redundant invertebrate database from November 2016 resulted in annotations for 57.74% (169,810 in total) of the contigs of the transcriptome from *T. americanus* and 59% (151,366 in total) of the contigs of the transcriptome from *T. longispinosus*. For both species the top ten species in the BLAST annotations were from ants of the subfamily Myrmicinae, to which the focal species also belonged.

Analysis of Gene Expression

PCA plots of transformed contig read counts from all samples revealed high similarity between behaviors within both species (Figure 4.1). Sample dendrograms confirmed this high level of intra-species similarity, showing no clear pattern of sample clustering by behavior or by home colony for both *T. americanus* and *T. longispinosus* (Figure 4.2). Gene expression analyses between the three different behavioral states revealed a total of 102

differentially expressed genes (DEGs; FDR-p < 0.05) for *T. americanus* and 68 DEGs for *T. longispinosus* (Figure 4.3). In *T. americanus*, the highest number of up-regulated genes occurred in scouts (50), followed by leaders (45). *T. longispinosus* leaders showed the highest number of up-regulated genes (35), followed by scouts (30). The lowest number of up-regulated genes was found in *T. americanus* followers (41) and *T. longispinosus* followers (29). Within both species, more over-expressed contigs were shared between scouts and leaders and followers and leaders than followers and scouts, and a higher number of over- to coeval over-expressed in the other two behaviors was observed within leaders compared to followers (χ^2=4.707, p=0.03) (Figure 4.3). Additionally, scouts of *T. longispinosus* showed a higher number of over-expressed contigs shared with leaders than with followers (χ^2=6.429, p=0.011).

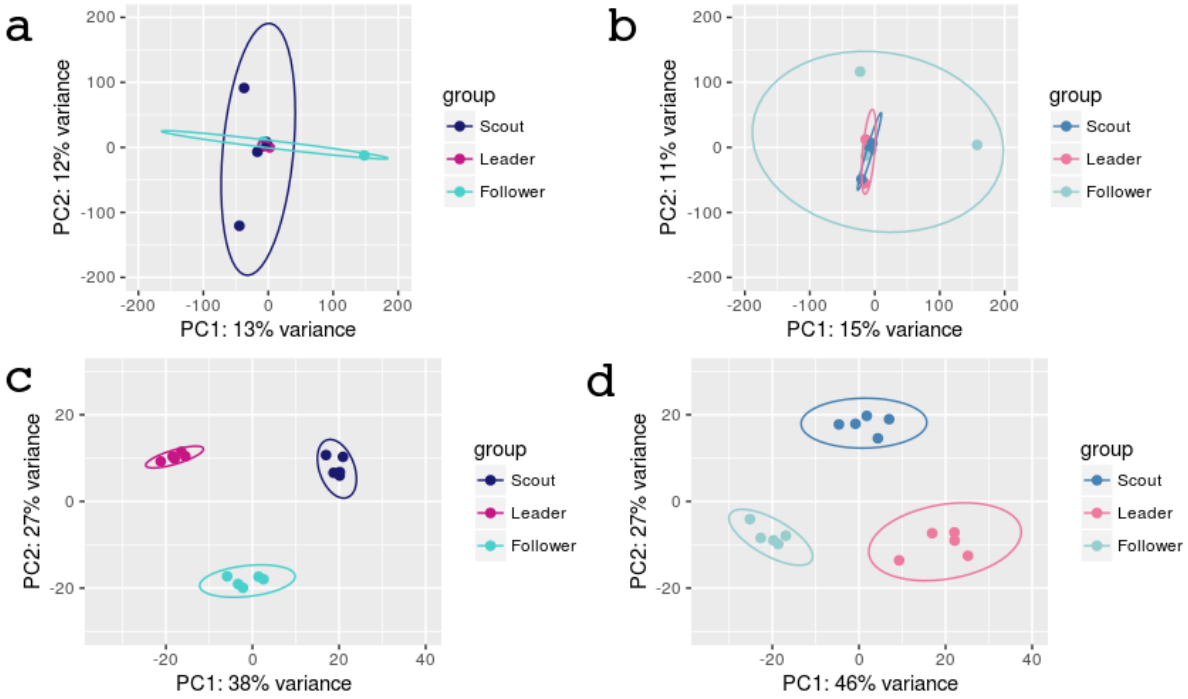

Figure 4.1: PCA plots outlining read-count variance between sample individuals of each tandem-running behavior in **a.** similarity between all reads of each *T. americanus sample*; **b**. similarity between all reads of each *T. longispinosus* sample; **c.** similarity between only DEGs of *T. americanus*; and **d.** similarity between only DEGs of *T. longispinosus*. Within **a** and **b** we see no consistent grouping of samples according to behavior, suggesting high gene expression similarity between the tandem-running behaviors of a species. However, differentially-expressed genes (**c** and **d**) do cluster according to behavior.

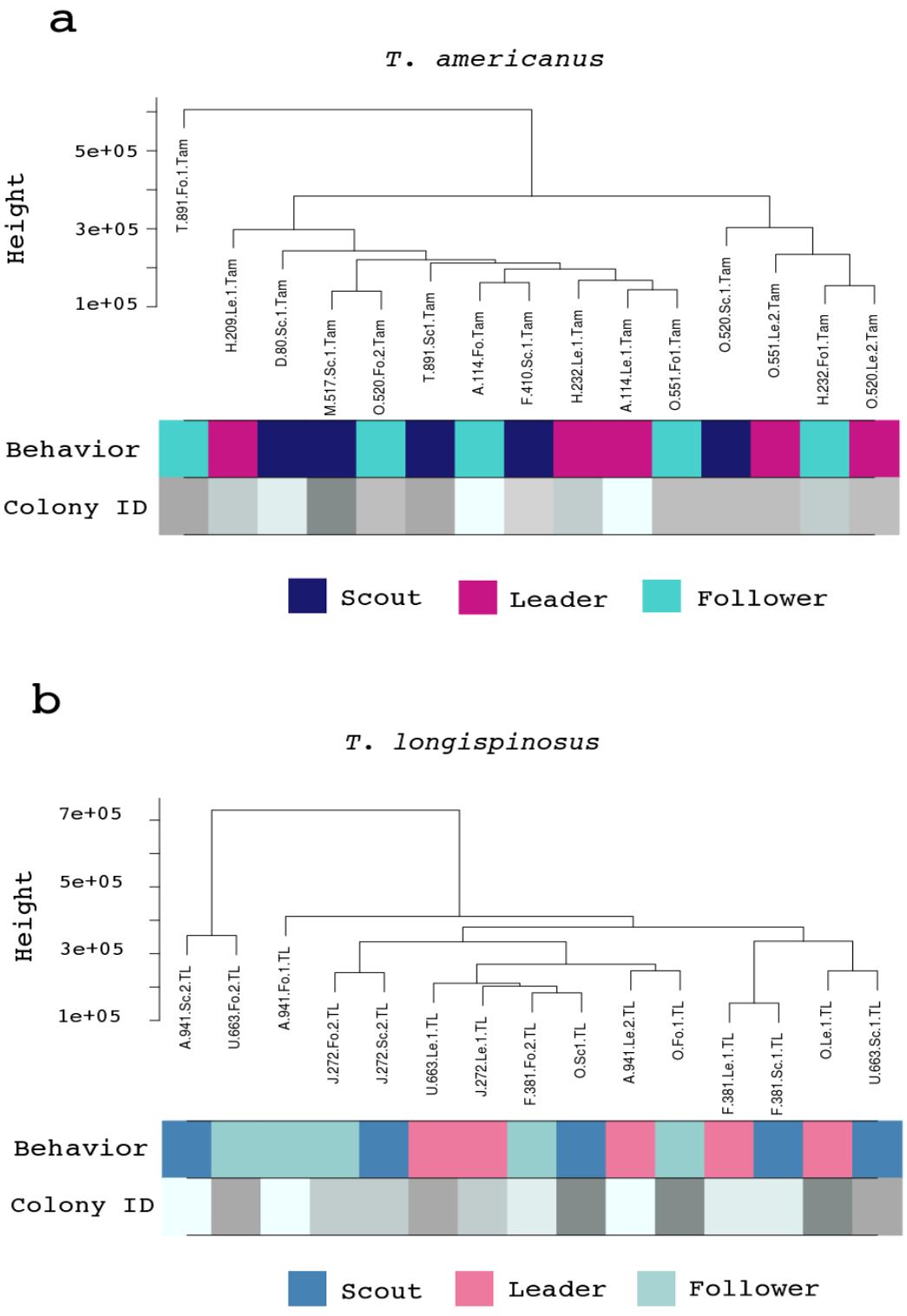

Figure 4.2: Dendrograms of gene expression similarity between samples. Identifiers at the ends of branches contain individual and colony IDs. **a**. *T. americanus*; **b**. *T. longispinosus*. Both species show high levels of intraspecific similarity between tandem-running behaviors. Neither behavior nor colony of origin strongly influences sample similarity.

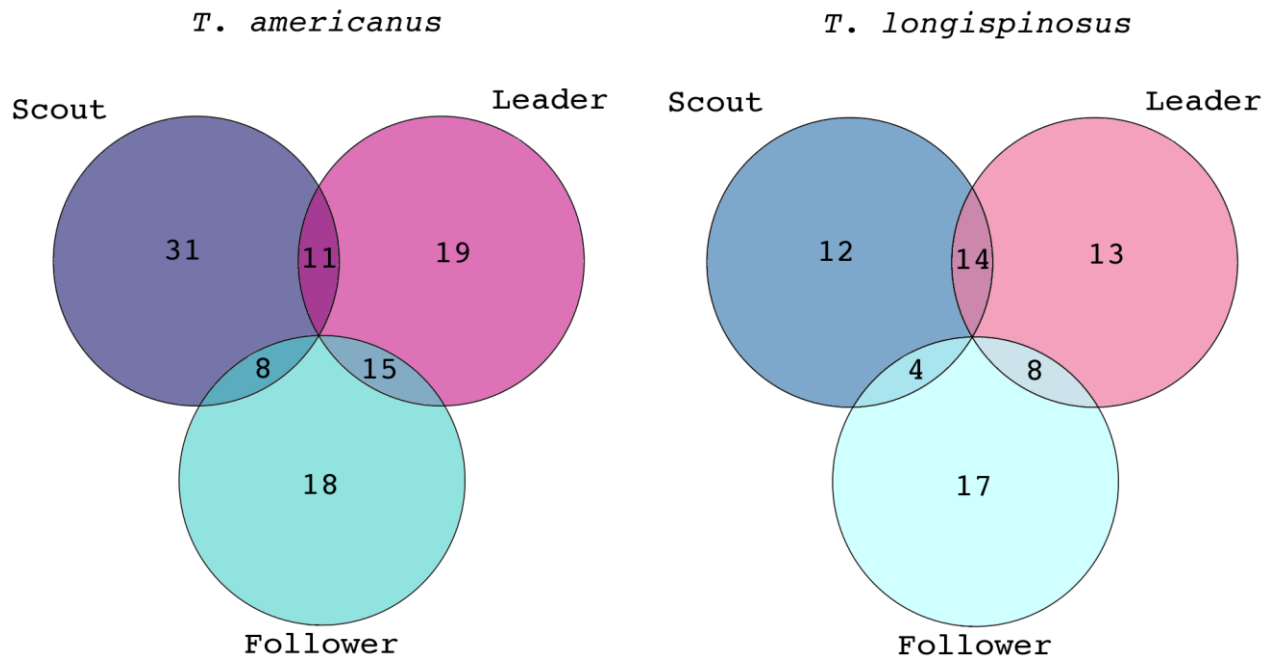

Figure 4.3: Venn Diagrams outlining shared and private DEGs between the tandem-running behaviors of **a**. *T. americanus* and **b**. *T. longispinosus* depicting numbers of up-regulated contigs of specific behaviors.

Weighted Gene Co-Expression Network Analysis

Block-wise WGCNA yielded a relatively large number of modules for both *T. americanus* and *T. longispinosus*; 465 and 392, respectively. Of these modules, 11 of *T. americanus* and 11 of *T. longispinosus* were significantly associated with behavior. However, after correction for multiple testing we found that no modules remained significantly associated with behavior in either species. This finding is likely due to high gene expression similarity between behavioral types within both *T. americanus* and *T. longispinosus.*

GO Enrichment Analysis

For both *T. americanus* and *T. longispinosus*, *GO* enrichment analyses were performed on a number of datasets (Figure 4.3, Supplementary Table S4): all contigs over-expressed during a focal behavior, over-expressed contigs unique to only the focal behavior, and contigs over-expressed coeval in both other behaviors compared to the focal behavior (here termed as “under-expressed”). In *T. americanus*, the terms *acetyl-CoA biosynthetic process*

from acetate and *negative regulation of transcription from RNA polymerase II promoter* were found to be enriched in over-expressed DEGs in followers and leaders, and under-expressed DEGs in scouts (Table 4.2). Additionally, *regulation of response to reactive oxygen species* was enriched in the over-expressed contigs of followers and scouts and in the under-expressed contigs of leaders. Some *GO* functions only occurred in a single behavioral state, for example: *DNA replication, synthesis of RNA primer* in followers, *regulation of cell cycle* in leaders (also found to be enriched in under-expressed contigs in scouts), and *G-protein coupled receptor signaling pathway*, enriched in scouts. Generally, up-regulated genes pertaining to signaling were found primarily in scouting individuals, while oxidative stress functionality was enriched in both scouts and followers.

In *T. longispinosus* the function *actomyosin structure organization* was enriched in the over-expressed DEGs of followers and leaders, and in under-expressed DEGs of scouts (Table 4.2). The function *translational initiation* was enriched within the over-expressed DEGs of leaders and scouts and the under-expressed DEGs of followers. One term found only to be enriched among the DEGs of *T. longispinosus* followers was *regulation of cyclin-dependent protein serine/threonine kinase activity;* while the term *transforming growth factor beta receptor* was specific for the DEGs over-expressed in leaders. The functions specific for the scout were generally broad: *biosynthetic process*, *ion transport*, *proteolysis* and *transmembrane transport*.

Pathway Analysis

Searching for pathways uniquely present in the over-expressed contigs of followers, leaders, and scouts in *T. americanus* and *T. longispinosus* yielded very few results (Supplementary Table S5). Only the *Porphyrin and chlorophyll metabolism* and *RNA* degradation pathways were uniquely present in *T. americanus* followers. The latter was also the only unique pathway in *T. longispinosus*, this time within leaders.

Gene Expression comparison based on Orthologous Clusters

In order to compare gene expression patterns between species, we performed an ortholog/homolog analysis upon the complete transcriptomes of both species. Using *Orthofinder*, we identified a total of 181,685 orthogroups which comprised 49% of all contigs used as input. Only 12,149 of these orthogroups were single-copy with one contig per species. An additional analysis using a custom *R* script (Supplementary Materials) – designed to obtain additional single-copy orthogroups from non-single-copy orthogroups

originally created by *Orthofinder* – resulted in an additional 13,950 single-copy orthogroups, bringing the final number of single-copy orthogroups up to 26,099. However, after inter-species comparison, none of the genes differentially expressed within one species were found to also be differentially expressed in the same behavior of the other species. Additional, strength of expression (measured by normalized read counts) did not differ between species. Neither single-copy orthogroups of *T. americanus* DEGs (Orthologues privately over-expressed in *T. americanus* scouts, also over-expressed in *T. longispinosus* scouts: Chi-square = 1.81, p-value = 0.41; Orthologues privately over-expressed in *T. americanus* leaders, also over-expressed in *T. longispinosus* leaders: Chi-square = 5.91, p-value = 0.052; Orthologues privately over-expressed in *T. americanus* followers, also over-expressed in *T. longispinosus* followers: Chi-square = 0.65, p-value = 0.72) nor orthogroups of *T. longispinosus* DEGs (Orthologues privately over-expressed in *T. longispinosus* scouts, also over-expressed in *T. americanus* scouts: Chi-square = 2.89, p-value = 0.24; Orthologues privately over-expressed in *T. longispinosus* leaders, also over-expressed in *T. americanus* leaders: Chi-square = 1.56, p-value = 0.46; Orthologues privately over-expressed in *T. longispinosus* followers, also over-expressed in *T. americanus* followers: Chi-square = 0.52, p-value = 0.77) were found to possess significantly different patterns of expression between these two species; indicating overall no similar gene expression patterns between homologous genes between these two species.

Discussion

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

Genes Revealed to be involved in Scouting and Tandem-Running

Unlike in other social insect species, tandem-running behavior in *Temnothorax* ants is exhibited by workers of the same behavioral caste, which likely accounts for the relatively low number of behavior-specific DEGs found (Seeley 1983, Dreller 1998). Scouts, followers, and leaders are likely the oldest and most experienced workers of a colony, as they engage in riskier outside tasks (Negroni *et al*. 2016, Kohlmeier *et al*. 2018). Therefore, it was likely that the expression patterns in the brain would vary only slightly between workers exhibiting these different behaviors. Indeed, data gathered here does support this. As outlined previously, tandem-run leaders could have been either scouts or followers prior to becoming a leader, whereas scouts will rarely become followers and likewise followers will rarely become scouts. As such, we initially expected leaders to exhibit the lowest number of uniquely expressed genes, and our findings support this expectation. Moreover, scouts and followers should share fewer genes than the other behavioral types, a pattern that again is apparent in both species. Gene expression analysis was thus most distinct between the most differentiated behavioral groups.

Among DEGs found to be associated with specific behaviors within this study were firstly those known to be involved in learning in other social insects. That scouts up-regulate genes involved in insect learning is expected, as scouts more so than the other two behavioral groups have to gather spatial information to be able to navigate back to their host nests. If a scout is lost, she might never find the way back to the home colony. *T. longispinosus* scouts up-regulated *glutamate receptor ionotropic, NMDA 2B isoform X3* (Table 4.1). This is in line with previous investigations in honeybees, where it was found that genes related to glutamate signaling were over-expressed in scouting individuals (Liang *et al*. 2012). Additionally - and also within honeybees - glutamate signaling genes were found to be differentially expressed between both food source- and nesting site-scouts compared to their recruits (Liang *et al*. 2014). Moreover, inhibition of expression of a subunit of the NMDA receptor in the honeybee brain resulted in impairment of memory formation (Müßig *et al*. 2010). Similarly, genes associated with learning were also found to be over-expressed within *T. americanus* scouts; the most notable of which is *Tyramine receptor 1-like* (Table 4.1). Tyramine – together with octopamine – is a member of the biogenic amides found within insects. Tyramine does play a role in honeybees, where the binding of tyramine to the receptor *AmTYR1* inhibits cAMP production; a pathway important for learning in *Drosophila* (Blenau *et al*. 2000, McGuire *et al*. 2005). Furthermore, a higher expression of *AmTYR1* in the mushroom bodies of honeybee foragers compared to the developing brain of pupae

suggests a correlation to age (Mustard *et al*. 2005). It has been previously shown that older ant workers (Kohlmeier *et al*. 2018) - even those of slavemaking species (Pohl *et al*. 2011) - are more commonly the workers to undertake the more dangerous scouting behavior, and as such it is fitting that *AmTYR1* is also over-expressed within *Temnothorax* scouts. Thus, as expected, we find multiple genes involved in learning that appear to be important for successful scouting in *Temnothorax* ants, independent of lifestyle.

Oftentimes, scouts will become leaders for subsequent tandem-runs in order to teach nestmates the location of resources of interest. We identified *tyramine beta-hydroxylase* as being over-expressed in *T. americanus* leaders. This gene likely plays a role in octopamine biosynthesis, and thus directly or indirectly influences information usage and by extension foraging behavior of leaders (Scheiner *et al*. 2002, Lehman *et al*. 2006, Behrends and Scheiner 2012). In *Drosophila* it was shown that mutations of the *tyramine beta-hydroxylase* gene block the biosynthesis of octopamine (Monastirioti *et al*. 1996). This indicates that - in leaders - more octopamine is produced through hydroxylation, which might result in a higher foraging activity (Schulz and Robinson 2001); though whether or not foraging behavior is synonymous with recalling a previously-explored area is unclear. Furthermore, *Allatostatin-A receptor-like isoform X1* was over-expressed in scouts and leaders of *T. americanus* and under-expressed in followers. In the honey bee brain allatostatin-A receptors are highly expressed in the antennal lobes and mushroom bodies, areas that are crucial for learning and memory retrieval (Kreissl *et al*. 2010, Menzel 2014). Additionally, it has been shown that increase of allatostatin decreases olfactory learning success in the honeybee (Urlacher *et al*. 2016). That this gene is under-expressed in *T. americanus* followers is congruent with their learning information from leaders, though that this gene is not also under-expressed in scouts is surprising; as effective scouting also requires that the individual learn the surrounding environment.

Functional Analyses and Molecular Pathways

A number of functional Gene Ontology (GO) terms were also identified as associated with specific behavioral states of *T. americanus* and *T. longispinosus* (Table 4.2). *T. americanus* scouts show a disproportional over-expression of genes associated with the *G-protein coupled receptor signaling pathway,* which is in line with contig annotations of *T. americanus* scouts with one contig encoding a tyramine receptor and another an allatostatin receptor - both broadly classified as G-protein coupled receptors. In a study examining the molecular basis of foraging experience in honeybees, this function was also found to be

enriched in more experienced foragers (Lutz *et al*. 2012). This finding further strengthens our previous expectation that primarily older individuals engage in scouting tasks. Similar functionality was not enriched within *T. longispinosus* scouts, however. Here, genes involved in immune response were enriched, possibly because scouts are more frequently exposed to extrinsic mortality factors and therefore require heightened immune defenses.

One term occurring in contigs over-expressed in both *T. longispinosus* followers and leaders was *regulation of cyclin-dependent protein serine/threonine kinase activity* (Table 4.2), suggesting a causal relationship between the kinase and G-protein coupled receptors, which – as mentioned above – seem to play a role in foraging behavior. That followers also show functions enriched which appear to be connected to foraging experience could be an indication that followers are more experienced foragers (Schultheiss *et al*. 2015). There is also strong evidence that protein kinase C activity is involved in learning, as the *Drosophila* learning mutant *turnip* shows a reduced protein kinase C activity (Choi *et al*. 1991). Protein kinase C activity in *T. longispinosus* followers is in keeping with our expectations that genes linked to learning would be positively-associated with these behaviors.

Orthology between Slavemaker and Host

While *T. americanus* and *T. longispinosus* do show similar tandem-running behaviors during raiding and nest emigration, respectively, homology analysis between these two species reveals that they do not use the same genes to carry out these behaviors. We find a similar pattern in a recent study comparing the transcriptomes of three slavemaker species during raiding behavior and outside raiding season with three host species during nest defense and idle (Alleman *et al.* 2018). Most of the DEGs found here exhibit species-specific expression patterns with some convergence on the functional level, which was also the case when looking into genes under selection between the species (Feldmeyer *et al*. 2017). Moreover, even within a single species - the honeybee - scouting occurs in two different contexts: the search for food sources and nest emigration, and brain gene expression patterns differed to a large extent between these two contexts (Liang *et al.* 2014). Thus, while there is some evidence to suggest that brain gene expression can differ radically between contexts with seemingly minor behavioral alteration, within this study we examine two separate species with two contextually-unique tandem-running behaviors. Thus any differences in gene usage between these two species could be due to one or both of these factors. Furthermore, the genetic toolkit hypothesis states that the similarities between different lineages of social insects like ants and honeybees - and within comparable castes -

is due to the differential expression of common, highly conserved, and structurally similar toolkit genes (Toth and Robinson 2007). A more recent study on the different castes of honeybees, social wasps, and ants showed that - across these distinct lineages of social insects - functions and pathways rather than genes are conserved (Berens *et al*. 2014). However, in the paper wasp it has been shown that regulation of genes associated with foraging and provisioning behavior are also conserved across lineages (Toth *et al*. 2010). Thus, the extent to which genes or functions are conserved across lineages does appear to be unclear and potentially lineage-specific. Framed by these previous findings, we neither find gene overlap nor functional similarity between the raiding behavior of the slavemaker *T. americanus* and tandem-running associated with colony relocation in the non-slavemaking *T. longispinosus*. Since the common ancestor of all *Temnothorax* was non-parasitic (Beibl *et al*. 2005), this would seem to suggest that modern *Temnothorax* slavemakers such as *T. americanus* utilize a genetically-distinct behavior from traditional non-slavemaking tandem-running. Whether or not this is the result of heavy modification of existing tandem-running pathways induced by selective pressures resulting from a parasitic lifestyle or an entirely new behavioral phenotype remains unclear; however as *Temnothorax* ants also do not lay pheromone trails, any mechanism for leading nestmates to important resources would likely be under strong selective pressures for fixation within the species.

Conclusion

Here we elucidate which genes underlie scouting, leading, and following behavior within the tandem-running-like behaviors of two *Temnothorax* ant species: the slavemaking *T. americanus* and its primary, non-slavemaking host *T. longispinosus*. A number of genes were identified within these behaviors that have previously been linked to functions such as learning, memory, or foraging in other insect species like *Apis mellifera* and *Drosophila melanogaster*. Especially intriguing is the tyramine-receptor expressed in scouts of *T. americanus*, since its function – compared to the octopamine-receptor – is generally unknown. Our findings in part support previous behavioral studies that demonstrate that tandem-running is a process of learning and subsequently passing that learned information on to nestmates (Richardson *et al*. 2007, Caro and Hauser 1992). Further RNAi knockdown of genes involved in learning/teaching and found to be differentially-expressed between the behaviors herein examined would be a logical next step for understanding the role genes and pathways play in slavemaking and non-slavemaking tandem-running behavior.

Relatively low numbers of DEGs within each tandem-running behavior indicates that

the differential expression of only a few genes is required to alter *Temnothorax* behavior. However, we were not able to identify DEG orthologs between the comparable tandem-running behaviors of *T. americanus* and *T. longispinosus*. Whether or not this lack of orthology in externally-similar behaviors is due to the context under which the behaviors are utilized, or due to species-specific dissimilarities, is unclear. At present, however, the raiding behavior of the slavemaker *T. americanus* and the nest relocation tandem-running behavior of *T. longispinosus* do appear to have very little overlap in gene expression, suggesting lifestyle-driven divergent evolution between these two *Temnothorax* species.

Acknowledgements

The authors would like to thank M. Negroni for his assistance in extracting brain tissue and M. Kever for performing RNA isolation prior to sequencing. This project was funded by a grant to Barbara Feldmeyer and Susanne Foitzik (DFG FE 1333/3-1 and FO 298/17-1) and a student research grant from the Edmund Niles Huyck Preserve to A. Alleman. Collection permits were obtained prior to field collection of specimens at the Huyck Preserve Rensselearville, New York.

Data Accessibility

See source publication.

Synthesis

Molecular Underpinnings of Behavior Revisited

Austin Alleman

Synthesis

While social parasitism is not uncommon among Hyminopterans and Myrmicidae, *Temnothorax* represents an evolutionary hotspot for the slavemaking lifestyle (Beibl et al. 2005, Feldmeyer *et al*. 2017). As with other antagonistic parasite-host relationships, *Temnothorax* slavemakers and their hosts are engaged in an ongoing evolutionary arms-race (Foitzik *et al*. 2009, Pennings *et al*. 2011, Jongepier *et al*. 2014, Cini *et al*. 2015, Jongepier *et al*. 2015): the behavior, physiology, and morphology of both slavemaker and host are tightly linked, evolving together through reciprocal adaptation and counter-adaptation (Gross 1993, Hart 1997, Sorci 2013). This co-evolutionary relationship has resulted in the varied and often species-specific implementation of raiding strategies between *Temnothorax* slavemakers; though host defensive strategies tend to vary more by population and parasite pressure rather than by species (Foitzik *et al*. 2001, Foitzik *et al*. 2009, Jongepier *et al*. 2014, Kleeberg *et al*. 2015). This thesis seeks to elucidate whether or not there is molecular parity with the existing behavioral observations – that species-specificity characterizes slavemaking phenotypes – or if common molecular elements and patterns underlie behaviorally dissimilar raiding phenotypes in *Temnothorax* slavemakers.

While each chapter in this thesis is framed by different questions and objectives, the central theme of investigating the genes and molecular patterns that influence behavior and, ultimately, the co-evolutionary dynamics of *Temnothorax* slavemakers and hosts, runs throughout each. As such, the purpose of this synthesis is not to summarize the findings presented in each chapter, but instead to provide perspective on how the collective findings presented in this thesis contribute to our understanding of the molecular underpinnings of parasite-host interactions. However, much of these analyses are explorative by nature, utilizing homology and additional information from other species as a starting point for identification and functional classification of molecular elements. As such, conclusions drawn in chapters relying on *de novo* transcriptome assembly and annotation (i.e. Chapters One and Four) are largely speculative by necessity, and serve to inform future investigations rather than confirm current co-evolutionary theory. Indeed, Chapter Three – for example – functions as an explanatory exploration into the precise functions of genes hypothesized in Chapter One to have a strong impact on complex behaviors.

Molecular Patterns of Evolution

T. americanus, *T. pilagens*, and *T. duloticus* all share the slavemaking lifestyle, but demonstrate some degree of specialization in that each species prefers and raids

preferentially a different *Temnothorax* host. Indeed, resource partitioning is not unheard of in ecosystems where multiple *Temnothorax* species occur side-by-side (Prather *et al*. 2018). This specificity in host preference has potentially set each slavemaker on a different evolutionary trajectory, co-evolving more closely with each species' respective preferred host. There is at least some additional evidence for this, as *T. americanus, T. pilagens*, and *T. duloticus* do display species-specific raiding behavior strategies within the more broadly similar slavemaking context.

Molecular patterns associated with the raiding phenotypes of these three species seem to confirm that species-specific raiding strategies are similarly driven by species-specific molecular mechanisms. This is strongly evidenced in both Chapter One – where a comparatively high number of orthologous contigs were determined to possess species-specific patterns of expression during the raiding phenotype with that pattern also being reflected on the pathway level – as well as in Chapter Two, with the finding that very few homologous contigs share patterns of positive selection between the three slavemaking species; though there was unexpected functional overlap between species despite the majority of pathways still being species-specific. Combined, these findings do suggests that the species-specific divergence of regulatory and functional molecular mechanisms characterizes the *Temnothorax* raiding phenotype.

Furthermore, in Chapter Two it was revealed that twice as many genes with signatures of positive selection were identified on branches leading to slavemaking species when compared to branches leading to host species. This finding illustrates the elevated rates of selection acting upon *Temnothorax* slavemakers. Taken together with the above findings that unique molecular patterns underlie the raiding phenotype, we can reason that socially-parasitic *Temnothorax* species are experiencing more rapid molecular evolution than their non-slavemaking counterparts, with slavemakers as a whole largely evolving away from one another; as previously expected by their partitioning of host species and use of species-specific raiding strategies.

In light of the above, that the slavemaker lifestyle in *Temnothorax* is defined by such specificity, it is unsurprising that hosts species also possess largely species-specific molecular responses to their slavemakers. However, unlike their antagonists, the defensive phenotypes of hosts are characterized by much smaller changes in regulatory mechanisms (Chapter One), suggesting a far less dramatic shift into their defensive phenotypes. Indeed,

there appears to be almost no overlap in the molecular patterns of slavemaker and host behaviors; even in those behaviors that appear externally similar. This is perhaps illustrated best by the findings of Chapter Four, where no overlap of homologous differentially-expressed contigs could be identified between the tandem-running behaviors of the slavemaker *T. americanus* and its host *T. longispinosus*. However, whether or not this lack of molecular orthology is due to the context within which the behaviors are carried out, or due to lineage-specific repurposing of existing genes remains unclear; though examined in the light of other results presented here, it is likely that the latter is the case.

Functions and Mechanisms Driving Co-Evolution within Temnnothorax

While there is largely no overlap in gene homology or pathway utilization between *Temnothorax* slavemakers during their raiding phenotypes, annotations relating to broad functionalities were found within those groups of differentially expressed contigs. As functional determination was accomplished through the use of homologous annotations, the precise functions of each of these genes within *Temnothorax* slavemakers during their raiding phenotypes is - by necessity, as explained above - unknown. Still, any insight into the functions of differentially-expressed contigs, or contigs possessing signatures of positive selection, is invaluable as a starting place for both interpretation of results as well as for informing future follow-up studies.

Behavior and Aggression: Gleaning insight into the role of genes in behavior through the use of homology and annotation is particularly dubious. The protein product of a gene, its homology to previously-described genes as measured by amino acid sequence similarity – and to a lesser extent the molecular role of that protein and its place in metabolic networks – may all be determined to one degree or another through proper respective analyses of the original nucleotide sequence. Higher-level understanding of gene function, such as the roles played in morphology or behavior, require species-specific manipulation experiments. Consequently, results are also often (though not always) species-specific; though even species-specific findings can inform studies in non-related taxa. As such, any gene annotations from unrelated species pertaining to behavioral functionality are almost certainly not directly applicable to behavior – and especially the relatively uncommon and highly specific raiding behavior – in *Temnothorax*. Despite this, a few genes identified throughout the course of this thesis with annotations from other species relating to behavior were found to possess interesting molecular patterns worthy of further investigation.

Perhaps the most intriguing of these genes is *Trypsin-7*, which was found to be universally down-regulated in slavemaking species during the raiding phenotype, while being universally up-regulated in host species during nest defense against slavemakers (Chapter One). Furthermore, *Trypsin-7* was found to possess signatures of selection in two of three host species - *T. ambiguus* and *T. curvispinosus* - suggesting elevated importance of this gene within *Temnothorax* slavemaker-host evolutionary dynamics. However, the exact role that *Trypsin-7* plays in *Temnothorax* remains unknown, despite the investigation of function through homology and annotation as well as extensive RNAi+behavioral experimentation. Investigations into the function of *Trypsin-7* in the malaria mosquito *Anopheles gambiae* provided the first clue into the role of *Trypsin-7:* in this species, it is involved in the digestion process after a blood-meal and, potentially, as a driver for host-seeking behavior (Muller *et al*. 1995). It is unlikely that this functionality is retained in its entirety within *Temnothorax* slavemakers, however, as host-seeking is more directly influenced by digestion (i.e. the drive to feed) within mosquitos than in slavemakers; though some evidence exists to suggest that *Temnothorax* slavemakers are more likely to engage in raiding when the number of slaves is low and the slavemakers are receiving less care (Pohl 2011). As such, it could be that "hunger" is one aspect driving the raiding phenotype. This might additionally explain the inverse relationship between *Trypsin-7* expression and the onset of the raiding phenotype in *Temnothorax*: as digestive function decreases, so does the need for *Trypsin-7*, and thus *Trypsin-7* expression falls accordingly. Determining whether or not *Trypsin-7* expression itself is the trigger for the initiation of the raiding phenotype or merely a proximate pattern, however, falls beyond the scope of this thesis.

Attempts were made to further elucidate the role of this gene in *Temnothorax* slavemakers, however. Raiding experiments following the artificial knockdown of *Trypsin-7* via RNAi were performed, though very few behaviors were found to differ significantly between treatment and control groups, and even those that were found were of little relevance (unpublished data). Indeed, the uninformative results of this experiment likely stem from the knockdown of *Trypsin-7* during the raiding phenotype: a period characterized by the natural down-regulation of *Trypsin-7*. A curious and potentially exciting follow-up to this experiment could also be the experimental knockdown of *Trypsin-7* in *Temnthorax* slavemakers who are out of raiding season, to see if the raiding phenotype can be artificially induced.

Of course, the relationship between *Trypsin-7* and host-seeking behavior is unnecessary within the context of our non-slavemaking host species, where *Trypsin-7* is universally up-regulated during nest defense and is also experiencing positive selection (in *T. ambiguus* and *T. curvispinosus*). In this context, then, it is perhaps more useful to examine only *Trypsin-7*'s role in digestion.

Complicating *Trypsin-7*'s role in *Temnothorax* is the finding that *Trypsin Inhibitor* was strongly up-regulated within *T. pilagens* during its raiding phenotype. As such, an alternate explanation to the down-regulation of *Trypsin-7* during the raiding phenotype is that *Trypsin Inhibitor* either directly or indirectly suppresses *Trypsin-7*, thereby triggering the raiding phenotype. Subsequent RNAi-mediated knockdown of *Trypsin Inhibitor* in *T. pilagens*, in conjunction with experiments to detect behavioral alterations after knockdown, if any, were performed in order to shed some light onto the function of this gene within the raiding phenotype of *Temnothorax* slavemakers. Where the experimental knockdown of *Trypsin-7* yielded little additional understanding, knockdown of *Trypsin Inhibitor* proved to be much more insightful. *T. pilagens* individuals in whom *Trypsin Inhibitor* was knocked down were less likely to successfully complete their raids, instead often peacefully staying within the host colony they originally set out to raid (Chapter Three). These findings seem to suggest a sensory deficiency is the result of *Trypsin Inhibitor* knockdown – knockdown *T. pilagens* are no longer able to identify that they are in a host colony, or are unable to effectively differentiate between their own colony and a host colony – rather than a generally decreased propensity for raiding initiation. Indeed, if lowered *Trypsin-7* levels trigger the onset of raiding, and the knockdown of *Trypsin Inhibitor* prevents the decrease of *Trypsin-7* from occurring, then we would expect no initiation of the raiding phenotype whatsoever; which was not found to be the case.

Rhythms and Response to Stress: Genes annotated with circadian rhythm functionality were found to be both up-regulated during the raiding phenotype and possessing signatures of positive selection in *Temnothorax* slavemakers. Circadian rhythms are the internal biological clocks for organisms, and represent the daily, seasonal, and annual timing for events such as reproduction, hibernation, etc. (Gwinner 1996, Wikelski *et al.* 2008). *T. americanus*, *T. duloticus*, and *T. pilagens* all show seasonal activity patterns, only raiding in summer months and exhibiting very low levels of activity throughout the rest of the year. Likely, slavemakers raid during this specific time of the year to avoid harsh winter weather, but more importantly to acquire host brood as they are pupating. Stealing host brood while they are still in their pupal stages represents the minimum investment into brood-rearing for

raiding slavemakers. These individuals will, in a matter of days, develop into functional adult slaves with almost no care required from slavemakers or existing slaves. As such, the timing of slavemakers' shift into the raiding phenotype is of crucial importance.

Additionally, genes involved in stress response were also shown to be both up-regulated in slavemakers during the raiding phenotype, as well as found to possess signatures of positive selection. The raiding phenotype is inherently risky, with slavemakers regularly exposed to predators, pathogens, and parasites outside of the nest. Aggression from host species is common. Continual utilization of stress response processes, as evidenced in our gene expression analysis, could certainly place the involved genes under the elevated selective pressures that we observed during selection detection.

Cuticular Hydrocarbons / Biochemical Synthesis: A number of contigs with annotations relating to the biosynthesis of fatty acids and other biochemicals were continuously recurring within our datasets; primarily represented here by *Acyl-CoA-Delta (11) desaturase* (up-regulated during the raiding phenotype in all slavemakers), *Elongation of very long chain fatty acids protein* (signatures of positive selection in *T. duloticus*) and *Fatty acyl-CoA reductase 1* (signatures of positive selection in *T. pilagens*). That genes relating to these functions are both differentially-expressed and possess signs of positive selection indicate that this functionality is important to the slavemaking lifestyle. Indeed, there is a large body of existing work illustrating that slavemakers possess consistently different CHC profiles than their three respective host species (Kleeberg et al. 2017), and that they utilize numerous chemical modifications and weapons when raiding host nests (Blomquist and Bagnères 2010). The modification of CHC profiles among slavemakers can serve a number of beneficial functions: ranging from the potential for more difficult detection or identification by host defenders when raiding, to increased desiccation resistance. Additionally, the diversification of chemical strategies by slavemakers is clearly important for an effective slavemaking lifestyle, as much of the success of *Temnothorax* slavemakers relies upon delayed or avoided detection by host defenders. Thus, while previous studies have shown the utilization and importance of chemical mechanisms on a behavioral level, we can confirm that these characteristics do indeed appear to be involved in the molecular evolution of *Temnothorax* slavemakers as well.

Teaching and Learning: In Chapter Four of this thesis, we explored more closely the molecular patterns associated with raiding parties and tandem-running in *T. americanus* and *T. longispinosus*, respectively. These strategies represent two of the most important vectors

for information transfer between nestmates in *Temnothorax*. Tandem-running and raiding party behaviors rely extensively upon learning (in scouting and following individuals) and teaching (in leading individuals) functionality; and because of this we would expect genes involved in these functions to be differentially-expressed. It is quite curious that we find no overlap between genes differentially expressed during raiding parties/tandem-running, and genes possessing signs of positive selection in *T. americanus* and *T. longispionsus*. The simplest explanation is that the environment, conditions, and challenges within which raiding party and tandem-running behaviors are currently utilized are not changing quickly or substantially enough to place these behaviors under significant selective pressure. In this context, alternate explanations seem unnecessary: evidence suggests that co-evolution of parasite and host is facilitated most strongly through interactions and the adaptations influencing those interactions, and in the case of raiding parties and tandem-runs themselves, there is ordinarily no interaction between slavemaker and host.

Temnothorax and Other Insect Social Parasites

Slavemaking and social parasitism are not rare modes of life, even (or especially) among insects (Kupper and Schwammberger 1995, Bolton 2003, Carpenter and Perera 2006, Buschinger 2009, Hines and Cameron 2010, Smith *et al*. 2013, Cini *et al*. 2015, Miller *et al*. 2015, Geffre *et al*. 2017, Aumer *et al*. 2018). Hymenopterans in particular, with their complex social societies and broad division of labor, are especially susceptible, with exploitation being common within ants (Seifert 2007, Buschinger 2009), bees (Alford 1975, Kupper and Schwammberger 1995, Cameron *et al*. 2007, Hines and Cameron 2010, Tierney *et al*. 2008, Smith *et al*. 2013, Gibbs *et al*. 2012), and wasps (Choudhary *et al*. 1994, Cervo 2006, Carpenter and Perera 2006); though social parasitism also arises in other, less social insect taxa such as aphids (Miller *et al*. 2015). This prevalence of social exploitation within *Temnothorax* and other insects raises the obvious question as to whether or not the genes found to be involved with the socially parasitic behaviors of *Temnothorax* ants are similarly involved in the parasitic strategies of other insect social parasites. Given that we find relatively little overlap in gene usage among closely-related *Temnothorax* slavemakers during their raiding behavior, the default assumption should be that there will be very little overlap in genes important to social parasitism in other insect species; and indeed this does appear to be the case (Syn. Table 1, Syn. Table 2).

In previous chapters, we ascertained the importance of genes to the slavemaking *Temnothorax* lifestyle through either differential expression during raiding behaviors (Chapter

One) or signatures of positive selection in the slavemakers *T. americanus*, *T. duloticus*, and *T. pilagens* (Chapter Two). Firstly, taking those genes found to be differentially-expressed in all *Temnothorax* slavemakers, and comparing them to genes found to be important to the social behavior of other parasitized insects reveals some interesting patterns (Syn. Table 1). A number of genes involved in fatty acid synthesis and maintenance appear important to *Temnothorax* slavemakers, wasps of the genus *Polistes* that have been infected with the endoparasite *Xenos vesparum* which alters their social behavior (*fatty acyl-CoA reductase*; Geffre *et al*. 2017), and the socially parasitic aphid *Tamalia inquilinus* and its host *T. coweni* (*major facilitator superfamily domain-containing protein*, *fatty acyl-CoA reductases*, *glucose dehydrogenase*; Miller *et al*. 2015). As the identification of conspecifics in these species relies heavily on olfactory cues, it stands to reason that genes involved in the production of these cues – whether as cuticular hydrocarbons or otherwise – would be important in socially parasitic systems where blending in with, misleading, or avoiding heterospecific individuals is paramount. Additionally, genes involved in circadian rhythms appear to be of recurring importance in social parasites (protein *takeout Temnothorax* as well as parasitized *P. dominula,* Geffre *et al*. 2017), as do genes involved in locomotion (*voltage-dependent calcium channel* proteins, *Temnothorax* and *T. inquilinus*, Miller *et al*. 2015).

We can additionally take a more conservative approach to classifying genes crucial to social parasitism in *Temnothorax* slavemakers by identifying the overlap between only those genes found to be simultaneously differentially-expressed during raiding activity *and* displaying signatures of positive selection, and genes important to social parasitism in other insect species (Syn. Table 2). Perhaps most strikingly, that *Trypsin-7* is found to be an important gene not only for *Temnothorax* slavemakers, for the socially parasitic pseudoqueens of *Apis melifera capensis* as well is intriguing (Aumer *et al*. 2018). As discussed above (Functions and Mechanisms Driving Co-Evolution in *Temnothorax*: Behavior and Aggression), *Trypsin-7*s role in the raiding behavior of *Temnothorax* is suggested to be linked with nutrition levels and individual slavemaker hunger. However, nourishment levels do not appear to influence the behavior of socially parasitic *A.m. capensis* pseudoqueens, suggesting that *Trypsin-7* might play an as-yet unknown role in social – or socially-parasitic – behavior. Additionally, *matrix metalloproteinase* is found to be involved in a number of important processes, one of which is the development of fat bodies. As many fatty acids and hydrocarbons are produced within the insect fat body, this gene could be involved in the functional modification of chemical signals by social parasites. Lastly, the

Synthesis Table 1: Genes important to other insect social parasites and parasites that influence social behavior in insects, compared to genes found to be differentially expressed within all examined *Temnothorax* slavemakers (Chapter One, Alleman *et al*. 2018). Species under examination in other studies: Geffre *et al*. 2017, genes differentially expressed between both stylopized and worker individuals, and worker and gyne individuals of the paper wasp *Polistes dominula*; Wallburg *et al*. 2016, genes found to be under selection in the cape honeybee *Apis melifera capensis*; Aumer *et al*. 2018, genes found to be up-regulated in parasitic pseudoqueens of the cape honeybee *A.m. capensis*; Miller *et al*. 2015, genes found to be differentially expressed between the socially parasitic aphid *Tamalia inquilinus* and its host *T. coweni*.

Publication	*Protein Name*	*Publication*	*Protein Name*
Alleman *et al.* 2018	Fatty acyl-CoA reductase 1	**Geffre *et al.* 2017**	putative fatty acyl-CoA reductase
	Putative fatty acyl-CoA reductase		
	Tetratricopeptide repeat protein 39B		tetratricopeptide repeat protein 30A
	Protein takeout		takeout-like
	Retinol dehydrogenase 11	**Wallburg *et al.* 2016**	Epidermal retinol dehydrogenase 2-like
	Trypsin-7	**Aumer *et al.* 2018**	trypsin-7
	Alpha-tocopherol transfer protein		alpha-tocopherol transfer protein-like
	Cytochrome P450 6k1		cytochrome P450 6k1
	Solute carrier family 41 member 2		solute carrier family 25 member 38-like
	Sodium-independent sulfate anion transporter		sodium channel protein Nach-like
	Sodium-coupled monocarboxylate transporter 1		
	Major facilitator superfamily domain-containing protein 6	**Miller *et al.* 2015**	major facilitator superfamily mfs_1
			major facilitator superfamily protein
			major facilitator superfamily mfs_1
	Fatty acyl-CoA reductase 1		fatty acid synthase-like
	Putative fatty acyl-CoA reductase		fatty acid synthase-like
	Elongation of very long chain fatty acids protein		
	Solute carrier family 41 member 2		solute carrier family 25 member 45-like
	Glucose dehydrogenase		glucose dehydrogenase
	Tetratricopeptide repeat protein 39B		tetratricopeptide repeat tpr
			tetratricopeptide repeat tpr
	Glutamate receptor, ionotropic kainate 2		glutamate receptor 4- partial
	Voltage-dependent calcium channel subunit alpha-2		calcium voltage- alpha2 delta subunit partial
			calcium voltage- alpha2 delta subunit partial

Synthesis Table 2: Genes important to other insect social parasites and parasites that influence social behavior in insects, compared to genes found to be differentially expressed and under positive selection within at least one *Temnothorax* slavemaker (Chapter 1, Alleman *et al*. 2018; Chapter 2, Feldmeyer *et al*. 2017). Species under examination in other studies: Aumer *et al*. 2018, genes found to be up-regulated in parasitic pseudoqueens of the cape honeybee *A.m. capensis*; Miller *et al*. 2015, genes found to be differentially expressed between the socially parasitic aphid *T. inquilinus* and its host *T. coweni*.

Publication	*Protein Name*	*Publication*	*Protein Name*
Alleman *et al.* 2018	Cytochrome b5	**Aumer *et al.* 2018**	cytochrome P450 6k1
	Trypsin-7		trypsin-7
	Matrix metalloproteinase-14	**Miller *et al.* 2015**	matrix metalloproteinase
	Putative ATP-dependent RNA helicase DDX23		atp-dependent dna helicase pif1 pre-mrna-splicing factor atp-dependent rna helicase dhx16-like
	Major facilitator superfamily domain-containing protein 6 Major facilitator superfamily domain-containing protein 6		major facilitator superfamily mfs_1 major facilitator superfamily protein major facilitator superfamily mfs_1

appearance of *major facilitator superfamily* proteins is curious, as one primary function of this gene is response to starvation; a conspicuously similar function to that of *Trypsin-7*. It does appear that nutritional deprivation in some species might trigger host-seeking behavior, making this gene and others of similar function prime candidates for further functional investigation in *Temnothorax* slavemakers and/or *T. inquilinus*.

Conclusions and Future Directions

With evidence from both gene expression and selection detection analyses, it is becoming increasingly apparent that the species within this group are experiencing independent evolutionary trajectories. Most conspicuously, even within lifestyles we note relatively little overlap of molecular patterns. That each slavemaker preferentially raids a different host species likely accounts for some of this dissimilarity. Additionally, primary parasite-host pairs also demonstrate little overlap. This broad lack of similarity is curious, as we would expect primary parasite-host pairs to at least experience identical environmental conditions within their natural habitat. However, that the behavioral experiments and subsequent collection of RNA was carried out in the laboratory under constant environmental conditions, paradoxically, likely accounts for at least some of this measured lack of similarity.

As environmental conditions were the same both at the time of RNA collection during the raiding/defensive phenotypes and the non-raiding/non-defensive phenotypes, those genes that respond to changing environmental conditions will have broadly uniform expression patterns, as the environment was similarly uniform across this time period. Therefore, these genes would not have been detected in our differential expression analysis. Generally, however, the combined findings of the studies within this thesis do suggest that evolution is acting uniquely upon each of the six species herein examined, with individual genes adapted to fill novel roles within each separate species. Furthermore, there is even less overlap in gene usage between *Temnothorax* slavemakers and other insect social parasites, though a number of curious similarities are present. Genes involved in lipid synthesis and transport, as well as hunger and nutritional deprivation, appear across a number of socially-parasitic taxa. While both of these functions are important to social parasites – in the form of chemical mimicry/avoidance and trigger for host-seeking, respectively – additional functional studies would shed some much-needed light on the role of specific genes within the context of social parasitism within individual species.

Thus, to fully understand the role that individual genes play within the context of *Temnothorax* behavior, artificial manipulation of the expression of these genes becomes necessary. Unlike some model organisms, inference of function from homology and annotation is of limited usefulness within the context of *Temnothorax* raiding behavior: not only, is *Temnothorax* not closely related enough to model organisms for all but the most conserved gene annotations to be reliable, but raiding behavior itself is absent in model organisms and likely utilizes novel and “non-standard” patterns of expression for otherwise conserved genes. Additionally, that the slavemaking species display species-specific usage of most raiding-associated genes suggests that, even within *Temnothorax*, functional determination through simple gene homology is risky at best. Understanding this, a number of additional RNAi-mediated gene knockdown experiments could be performed to further elucidate the role of specific genes in the *Temnothorax* slavemaker-host co-evolutionary system. *Trypsin-7* is undeniably an important gene for both slavemaker and host. Future studies could further explore the interplay between *Trypsin-7* expression, need for nestmate care (primarily in slavemaker workers), aggression (primarily in hosts), and raid initiation. As *Trypsin-7* was universally down-regulated during the raiding phenotype, this reciprocally means that it was up-regulated in all slavemakers during the non-raiding phenotype. A simple experiment could be designed to knock down *Trypsin-7* via dsiRNA in any or all of the three *Temnothorax* slavemakers examined here while they are out of the raiding

season/phenotype, and observe whether or not affected individuals resume raiding activity. In this way, one could verify whether or not *Trypsin-7* expression levels do indeed trigger the onset of the raiding phenotype in *Temnothorax*. Similarly, *Trypsin-7* could also be artificially down-regulated in host species, after which they could be challenged by a slavemaker antagonist. Defensive performance of treatment host individuals could be measures and analyzed to determine if their proficiency in nest defense is affected by *Trypsin-7* knockdown.

However, modification of gene expression presents its own share of complexities. Chapter Three of this thesis, for example, sought to experimentally down-regulate the *Trypsin Inhibitor* gene within the slavemaker *T. pilagens* during the raiding phenotype, with partial success. While it was possible to demonstrate significant behavioral alteration with the application of *Trypsin Inhibitor*-targeting RNAi, the actual knockdown of this gene was never able to be externally verified with qPCR. Indeed, it was unexpected that preliminary qPCR verification of knockdown within experimental individuals tentatively suggests that *Trypsin Inhibitor* experienced *up-regulation* within RNAi-treated individuals. While it is possible that *T. pilagens* can somehow compensate for the sudden drop in *Trypsin Inhibitor* RNA, one would expect that the continued application of *Trypsin Inhibitor*-specific RNAi would eliminate these sequences as well; unless one or more splice variants – which are not targeted by our RNAi – are used to compensate. Tissue specificity could also account for this discrepancy, with *T. pilagens* compensating for the loss of *Trypsin Inhibitor* within tissues that are not fully reached by our RNAi sequences. Without further molecular investigations, however, it is unknown exactly why *T. pilagens* seems to exhibit an up-regulation of *Trypsin Inhibitor* RNA after the application of RNAi. Future studies utilizing RNAi-knockdown as a mechanism for investigating the role of individual genes within a broader phenotype would likely benefit from tissue-specific qPCR verification of knockdown, as whole body verification as yet has not been met with much success. Some work has been done into investigating the tissue(s) in which *Trypsin Inhibitor* expression is down-regulated most strongly following knockdown with dsiRNA, with (very) preliminary results suggesting that the fat bodies of *T. pilagens* showing the greatest amount of activity. Alternatively, however, recent studies have been published successfully with no qPCR verification of RNAi knockdown at all (Cameron *et al*. 2013), bringing into question altogether the need for qPCR verification if substantial phenotypic alterations are observed.

One additional line of investigation could be to further tease apart the gene expression patterns associated with raiding-party and tandem-running behaviors, following

up on the findings presented here in Chapter Four. We found very little overlap in gene expression patterns between these two behaviors in a slavemaking and host species, respectively. However, enslaved hosts will also occasionally engage in raiding activity. This provides us with an intriguing opportunity to investigate raiding-party activity within the same species, albeit not a slavemaking one. This should allow for the effective elucidation of whether or not species-specificity or behavioral context underlies the differences in gene expression between raiding-party and tandem-running behavior.

Furthermore, an epigenetic approach might provide additional insight into the mechanism and conditions under which the raiding phenotype is initiated. An appropriate starting point for this investigation could come in the form of a pair of behavioral observation experiments, following the experimental inhibition of *histone acetyltransferase* (HAT). The first of these two experiments would focus on one or multiple slavemakers previously examined here, with the application of a HAT inhibitor immediately prior to the onset of raiding season. Subsequently observing full raiding experiments should indicate whether or not the onset of the raiding phenotype can be delayed through the artificial modification of epigenetic mechanisms. Perhaps more insightful, however, would be the second proposed experiment, where a HAT inhibitor is given to slavemakers already at the peak of their raiding activity. If the timing and length of the raiding phenotype is controlled through epigenetic mechanisms, we might expect to see an elongation of the raiding phenotype past its seasonal limits (though the continued application of HAT inhibitor of course).

As touched upon earlier in this thesis, many of the studies performed as part of this project are explorative by nature. Therefore, this thesis should provide the first insights into the molecular underpinnings of slavemaking behavior in *Temnothorax* ants, and, more broadly, behavior in general. With adequate experimental and functional investigations, it is my hope that this thesis will serve as a crucial stepping-stone to a greatly enlightened understanding of insect behavior.

[Acknowledgements removed for privacy purposes]

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