The importance of recognition and aggression in the coevolution between ant social parasites and their hosts

Dissertation

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Isabelle Kleeberg

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„A truly successful parasite is commensal, living in amity with its host, or even giving it positive advantages. A parasite that regularly and inevitably kills it hosts cannot survive long, in the evolutionary sense, unless it multiplies with tremendous rapidity. It is not pro-survival.

Mr. Spock, Star Trek 2

This thesis is based on the following seven research articles, which are presented as chapters 1 – 7 and referred to in the general introduction and discussion by their respective number:

1. **Kleeberg I**, Pamminger T, Jongepier E, Papenhagen M & Foitzik S (2014) Forewarned is forearmed: aggression and information use determine fitness costs of slave raids. *Behavioral Ecology* 25: 1058 – 1063.

2. **Kleeberg I**, Jongepier E, Job S & Foitzik S (2015) Geographic variation in parasite pressure predicts intraspecific but not interspecific aggressive responses in hosts of a slavemaking ant. *Ethology* 121: 1 – 9.

3. Jongepier E, **Kleeberg I**, Job S & Foitzik S (2014) Collective defense portfolios of ant hosts shift with social parasite pressure. *Proceedings of the Royal Society of London B: Biological Sciences* 281: 20140225.

4. Jongepier E, **Kleeberg I** & Foitzik S (2015) The ecological success of a social parasite increases with manipulation of collective host behavior. *Journal of Evolutionary Biology* 28: 2152 – 2162.

5. Seifert B, **Kleeberg I**, Feldmeyer B, Pamminger T, Jongepier E & Foitzik S (2014) *Temnothorax pilagens* sp. n. - a new slavemaking species of the tribe Formicoxenini from North America (Hymenoptera, Formicidae). *ZooKeys* 368: 65 – 77.

6. **Kleeberg I** & Foitzik S (2016) The placid slavemaker: avoiding detection and conflict as an alternative, peaceful raiding strategy. *Behavioural Ecology and Sociobiology* 70: 27 – 39.

7. **Kleeberg I**, Menzel F & Foitzik S. The evolution of cuticular hydrocarbons in ants: The influence of parasitic lifestyle, caste and sex on chemical profiles. Submitted to *Evolution*.

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SUMMARY

In my dissertation I investigate host defense and parasite offense strategies in different species of *Temnothorax* slavemaking ants (social parasites) and their hosts. The thesis is mainly divided into two parts: (1) The evolution of host defense behaviors in relation to parasite pressure and (2) The evolution of slavemaker counter-adaptations. *Temnothorax* host colonies are able to recognize the slavemaker *T. americanus* as a threat and respond with high aggression towards it. During raids, having recent contact with a slavemaker worker yields fitness benefits, but only for those colonies that have a high aggressive potential. Hence, *Temnothorax* hosts are able to utilize information of an upcoming attack to better defend themselves, when being aggressive (Chapter 1). But this was only true for aggression towards conspecifics – baseline aggression - which was moreover positively related to parasite abundance in a population. This indicates that parasites select for more aggressive host colonies. The correlation was found for two host species of *T. americanus*, pointing to convergent evolution of aggression as a defense strategy (Chapter 2). Aggression towards a living and potentially manipulative parasite decreased with parasite pressure, instead host colonies switched to an alternative defense mechanism – evacuation (Chapter 3). Populations that were less aggressive towards the parasite were also less resistant to chemical manipulation and slavemakers benefitted from it, as the likelihood of parasite survival and the parasites abundance increased (Chapter 4). One of the three slavemaker species evolved a different strategy – chemical camouflage – to steal adult host ants, known as eudulosis (Chapter 5-6). Hosts of this slavemaker do recognize the two slavemaker species *T. americanus* and *T. duloticus,* but are unable to identify their sympatric slavemaker *T. pilagens*. However, if they do recognize the slavemaker, aggression is costly as most workers die during raids, and evacuation although utilized in confrontations with *T. americanus*, seems to not be employed during raids of *T. pilagens* (Chapter 6). The three slavemaker species, although of different evolutionary origin, moreover show convergent evolution of a specific chemical profile that may undermine host recognition (Chapter 7).

We detected an intricate suite of adaptations and counter-adaptations in *Temnothorax* hosts and slavemakers. I aimed to shed light on the diverse roles of recognition and aggression within this system and found evidence for convergent evolution of both, possibly due to their similar lifestyles of slavemakers and hosts, respectively. Studying different host populations with differences in reciprocal selection, based on variation in parasite abundance, allowed for the elucidation of the selective forces that shape host behavior. Comparing different slavemaker species within the same genus, each representing different origins of slavery and evolutionary age, moreover allows delineating trends in the evolution of parasitic lifestyle.

ZUSAMMENFASSUNG

Wirt-Parasit Interaktionen zählen du den stärksten Kräften der Evolution, da dabei der Parasit die Fitness des Wirtes negativ beeinflussen kann. Dies führt zu gegenseitigen evolutionären Änderungen durch natürliche Selektion – Koevolution. In meiner Dissertation untersuchte ich die Auswirkungen von Parasitismus auf das Verteidigungsverhalten von Wirten und umgekehrt die Evolution von Angriffststrategien bei Parasiten. Sklavenhaltende Ameisen sind Sozialparasiten, die Ameisenkolonien einer andern Art ausrauben, um deren Brut (zukünftige Sklaven) zu stehlen. Hier untersuchte ich zum Einen die Fähigkeit den Parasiten zu erkennen bzw. die Fähigkeit des Parasiten die Feinderkennung zu unterbinden. Zum Anderen untersuchte ich inwiefern aggressives Verhalten von Wirtskolonien Fitness Vorteile bringt, und ob dies Verhalten unter Selektion des Parasiten steht.

Temnothorax Wirtskolonien können den Sklavenhalter als Gefahr erkennen und reagieren entsprechend mit erhöhter Aggression. Wirtskolonien die kürzlich Kontakt zu einer Sklavenhalterin hatten und überdies ein hohes aggressives Potential aufweisten, konnten mehr Brut retten. Dies deutet darauf hin, dass Wirtskolonien in der Lage sind, Informationen über einen anstehenden Raubzug zu nutzen, um sich besser zu verteidigen (Kapitel 1). Außerdem lässt dies vermuten, dass Sklavenhalter Wirtskolonien mit einem hohen aggressiven Potential selektieren und in der Tat konnte dies gezeigt werden. Wirtspopulationen die einem hohen Parasitedruck ausgesetzt waren, wiesen auch ein hohes aggressive Potential aus. Dieser Zusammenhang konnte in zwei Wirtsarten gezeigt werden, was auf konvergente Evolution als Antwort auf den Parasitendruck hindeutet (Kapitel 2). Die Aggression gegenüber eines Sklavenhalters, der zudem in der Lage ist die Wirte chemisch zu seinem eigenen Vorteil zu manipulieren, war geringer in Populationen mit hohen Parasitendruck. Wirtskolonien wechselten zu einer alternativen Verteidigungsstrategie – Flucht (Kapitel 3). Wirtspopulationen die weniger Aggression gegenüber dem Sklavenhalter zeigten, waren auch anfälliger für chemische Manipulation. Sklavenhalter profitieren davon, da die Chance einen Raubzug zu überleben stieg. Zudem, waren Sklavenhalterkolonien häufiger in einer Population vorhanden, dessen Wirtskolonien chemisch manipuliert werden konnten (Kapitel 4). Einer der drei untersuchten Sklavenhalter Arten zeigt eine andere Art des Raubzuges, wobei sogar adulte Wirtsameisen versklavt werden (Eudulosis) und aufgrund von chemischer Camouflage des Sklavenhalters kaum Aggression auftritt (Kapitel 5-6). Hier scheint es als ob die Wirte bisher keine Art der Verteidigung entwickelt haben, da entweder alle Individuen, inklusive der Brut versklavt werden, oder aber, falls Wirtskolonien den Sklavenhalter doch als Gefahr erkennen, alle adulten Individuen getötet werden. Ein chemischer Vergleich aller Sklavenhalterarten zeigte, dass es ein Parasiten-spezifisches Profil gibt, mit der Möglichkeit die Erkennung durch den Wirt zu reduzieren (Kapitel 7). Da dies über

alle drei Arten hinweg gefunden wurde, deutet es auf konvergente Evolution hin, die aufgrund der ähnlichen Lebensstile hervorgerufen wird.

In meiner Dissertation konnte ich eine Reihe an Verteidigungsstrategien bei Wirten aufschlüsseln, die konvergent in verschiedenen Wirtsarten aufgrund von Wirt-Parasit Koevolution entstanden sind. Der Vergleich verschieden stark parasitierter Wirtspopulationen konnte zeigen das Parasitismus eine der stärksten selektiven Kräfte ist, die das Verhalten von Wirtskolonien beeinflusst. Darüber hinaus, zeigen Sklavenhaltende Ameisen ein parasiten-typisches chemisches Profil, das über verschiedene Arten hinweg konvergent evolvierte. Dieses dient vermutlich dazu, bei Raubzügen unerkannt in die Wirtskolonie einzudringen. Die Untersuchung von verschiedenen Sklavenhalterarten einer Gattung, die alle unterschiedliche Ursprünge der Sklaverei repräsentieren, erlaubt Trends in der Evolution zum Parasitismus zu entschlüsseln.

GENERAL INTRODUCTION

The fitness of organisms is not only affected by their genetic background and their abiotic environment, but strongly by interactions with other organisms as well. In mutualistic relationships, both organisms benefit from their interaction (Boucher 1988), whereas predator-prey or parasite-host interactions are only beneficial for one interaction partner, the predator or parasite, respectively (Bush 2001; Barbosa & Castellanos 2005). Many of these interactions are selective in nature, with one organism impacting the fitness of another. These various species-interactions thus, can lead to reciprocal evolutionary changes via natural selection, namely coevolution, being the most common evolutionary process in nature, with parasitism being one of the strongest forces of evolution (Price 1980; Lafferty 2010). The parasitic lifestyle often favors extreme specialization comprising high selective pressures imposed on the reciprocally interacting organisms, leading to complex and rapidly-changing evolutionary dynamics (e.g. Altizer et al. 2003; Duffy & Sivars-Becker 2007). The underlying mechanisms, although still being under debate, can be influenced by periodic selective sweeps (*arms races*; Dawkins and Krebs 1979), negative frequency-dependent selection (*The Red Queen Hypothesis*; van Valen 1973) or a combination of both. Advantageous traits that spread throughout the population and finally got to fixation can lead to an arms race that consists of recurring and reciprocal fixation events in parasites and hosts. Under negative frequency-depend selection however, advantageous traits would be disfavored as its frequency increases in the population and parasites target the common host phenotypes, which is referred to as the *Red Queen Hypothesis.*

Host-Parasite Coevolution

Evolutionary arms races between parasites and hosts, that involve reciprocal selection pressures, have led to a wide range of defense traits in host populations (Agnew et al. 2000; Labrie et al. 2010; Schmid-Hempel 2011) as well as diverse counter-defense strategies in parasite populations (Thompson 2005; Samson et al. 2013). If parasites manage to overcome one line of defense, hosts in turn are selected to develop further defenses that can result in host defense portfolios. Thereby one strategy can increase the fitness benefits of further defenses (*strategy facilitation*), or, once an effective defense has evolved, selection on additional strategies can be relaxed (*strategy blocking*). The diversity of defense and counter-defense traits is not only apparent within populations but also between them. Species with broad geographic ranges are subdivided into populations with often only small levels of gene flow between them. They can become geographically differentiated in the degree of specialization, as coevolution acts along different evolutionary trajectories between populations. Geographically distant

communities of interacting systems can differ in their degree of reciprocal selection, depending on various biotic and abiotic factors that shape the environment. Populations with strong reciprocal selection are defined as `hot-spots´, whereas populations, in which reciprocal selection is absent, for instance if one of the two interacting species is absent, are defined as `cold-spots´. The coadaptive outcomes can hence vary among populations, depending on the intensity and type of reciprocal selection (Cushman & Whitham 1989; Thompson & Cunningham 2002). Over the entire geographic range of host species, parasites do not necessarily co-occur in uniform abundance, and thus the intensity of parasite-induced selection pressure on host species can differ between sites. Hosts are expected to lose their defenses in the absence of antagonistic interactions either through genetic drift or natural selection, if they generate lower fitness compared to individuals that lack these locally unnecessary defenses. Populations that have a "parasite past" however, might still possess specific defenses, thusly contributing to additional geographic variation. Coadaptation can hence be studied by comparing populations under divergent selection regimes, such as the prevalence of specific parasites (Dobson 1988; Soler et al. 1999; Barber et al. 2000). Understanding these factors that maintain geographic phenotypic variation can help in elucidating the causes and consequences of evolution in general.

The importance of recognition in host-parasite interactions and its function in social insects

Recognition of organisms is crucial for the maintenance of species interactions and allows appropriate responses ranging from cooperation to aggression towards the counterpart. It enables the formation of dominance hierarchies, family bonds, social groups, and allows for protection from, or evasion of predators and parasites. Biological signals that allow for recognition (e.g. via behavioral, visual, acoustic or olfactory stimuli) are under positive selection if they increase the sender´s fitness by eliciting a specific response in the receiver (Smith & Harper 1995). However, signals that elicit aggressive responses in the receiver and reduce the sender´s fitness should be selected against. If host species are able to recognize a parasite and can thereupon respond accordingly to reduce or prevent the costly effects of parasitism, the parasite should evolve strategies to reduce the costs of detection or to evade detection altogether. This can be seen in various host-parasite systems, including the recognition of parasites or pathogens by the immune system (e.g. Janeway 1992; Janeway & Medzhitov 2002; Jiggins & Hurst 2003), the recognition of infected group members or potential mates (e.g. Ugelvig & Cremer 2007; Kavaliers et al. 2005), as well as the recognition of parasitic cuckoo eggs or chicks by the avian host (Davies & Brooke 1988; Davies & Brooke 1989; Langmore et al. 2003; Lyon 2003; Sato et al. 2010; Tokue & Ueda 2010; Spottiswoode & Stevens 2011). In turn traits have evolved that allow the parasite to evade host recognition

(e.g. Brooke & Davies 1988; Moksnes 1995; Alcami 2003). For example, the socially parasitic cuckoo *Cuculus canorus* that lays its eggs in nests of its avian host to exploit their brood care behavior, evolved egg mimicry to avoid the rejection of its eggs from the host nest (Brooke & Davies 1988; Davies & Brooke 1988). Studies on recognition and detection in host-parasite systems all point to recognition as being a first step in host-parasite coevolution. It will subsequently lead to further strategies, once one line of defense - the recognition of parasites - is breached, resulting in coadaptation cycles in both parties. In hosts, this can result in defense portfolios - a set of defensive strategies to cope with their enemies (Planqué et al. 2002; Welbergen & Davies 2009).

The ability to recognize self from non-self is central to the functioning of many biological systems, but especially in societies characterized by a complex social structure. Eusociality is defined has having overlapping generations, cooperative care of brood and division of labor; typically including a reproductive division of labor (Wilson 1971) and hence, represents a complex social structure. A minority of individuals reproduce (i.e. queens and males) and the majority (workers), that are functionally sterile, carry out tasks to tend the colony. This includes not only taking care of the brood but also nest construction and nest defense. To maintain the integrity of such complex insect societies, effective communication strategies are required. Examples of communication in social insects include the honeybee waggle dance, as a form of language, describing the location and quality of food (Von Frisch 1968), or pheromone communication in ants, to e.g. signal their reproductive status and / or position in a hierarchy (Wilson 1959; Hölldobler 1995). To ensure that altruistic behaviors are directed towards related group members, individuals need to distinguish nestmates from non-nestmates. Most communication in social insects occurs through the use of chemical signals on the cuticle or to a lesser extent through the release of pheromones by glands (Blomquist & Bagnères 2010). In ants, and in many other social insects, nestmate recognition is facilitated through the comparisons of a cuticular hydrocarbon (CHC) profile of an individual, with a template that is the neural representation of the colony-odor stored in the memory. Depending on the profile´s similarity or dissimilarity, a conspecific will be accepted or rejected (Hölldobler & Michener 1980; Vander Meer & Morel 1998). The recognition system of social insects is not only utilized for nestmate discrimination but, individuals are moreover able to distinguish between differential threats (Alloway 1990; Whitehouse & Jaffe 1996; Hughes & Goulson 2001; Guerrieri & D´Ettorre 2008; Scharf et al. 2011b; Von Beeren et al. 2011). Depending on the risk - be it a competitor, predator or parasite - members of a colony often show gradual aggressive responses.

Social insects and their parasites

Compared to solitary species, social insects face a unique set of benefits and challenges generated by their lifestyle. Despite the benefits - typically including collective defense against enemies, shared brood care and improved food acquisition and provisioning - colonies of social insects are profitable targets for parasites due to their high density and similar genotypes of all individuals within a nest (Schmid-Hempel 1998). There are a large number of parasites that specialize on social insects, ranging from viruses to other parasitic insects. For parasites it may not be easy to enter the well protected nests of social insects, however once inside, the opportunities abound. Brood for example is a popular target for parasites, as the different stages until adulthood are usually immobile and depend on being fed and cared for by workers. The trophallaxis and cleaning of brood opens the possibility for nurse to brood transmission of microparasites, although grooming and other hygienic behaviors are usually beneficial for the social insect colony (Schmid-Hempel 1998). The numerous workers of social insects that exhibit a wide range of tasks, such as foraging, have many occasions to have contact with parasites. These workers will then carry parasites back and eventually spread them within the colony.

Social insects do not only fall victim to microparasites, but also to social (macro-) parasites. A social parasite benefits in many ways from the brood care or from other socially managed resources of its host. This kind of exploitation is not restricted to social insects, but can also be found in birds (Croston & Hauber 2010). Social parasitism, especially in ants, is an exceedingly complex phenomenon. It does involve a large number of species and diverse lifestyles:

- *Xenobiosis*: Guest ants that share a nest with another species (the host), but still maintain their own nest within the host colony. The brood will be kept separately from the host brood and only the food provisioning will be exploited.
- *Cleptobiosis and Leptobiosis:* Both forms involve the stealing of brood and food of another host species. The cleptobiotic species live close to their host colony and steal the brood or food from foragers, whereas the leptobiotic species enter the host colony to steal resources.
- *Temporary parasitism*: A temporary social parasite only depends on its host species during colony founding. The parasitic queen attacks a host colony and replaces the resident host queen. Host workers accept the new parasitic queen and take care of the first batch of parasite brood. Temporal parasitic workers in contrast to dulotic workers are capable of taking care for the colony themselves. The host workers will then be gradually replaced, due to natural death, leading to a self-sustaining parasite colony.
- *Inquilinism*: A form of permanent parasitism. Inquiline species lack a worker caste. Instead the parasitic queen invades and lives inside a host colony to

produce sexual brood. Host workers will take care of her brood until emergence.

- *Dulosis (Slavery)*: A form of permanent parasitism, with slavery. Dulotic workers have lost the ability to take care for their colony; instead they raid close-by host colonies of another species to steal their brood. This brood will then be raised, by already established so called host slaves, to replenish the slave work force. This form of social parasitism is the focus of this thesis and will further be discussed in detail.

Socially parasitic slavemaking ants

Social parasitism has evolved multiple times independently in the eusocial insects, including three origins in wasps (Choudhary et al. 1994; Cervo 2006; Carpenter & Perera 2006), at least 12 in bees (Alford 1975; Cameron et al. 2007; Hines & Cameron 2010), and certainly more than that in ants (Huang & Dornhaus 2008; Buschinger 2009). Slavery, a special form of social parasitism, has been described in a number of ant species worldwide (Seifert 2007; D´Ettorre & Heinze 2001) and is thought to have evolved independently more than ten times, with six origins among the ant tribe Formicoxenini alone (Beibl et al. 2005). Slavemaker – host systems are tractable systems for studying coevolutionary processes in nature and provide several features that make them ideal models. Slavemakers and hosts have similar evolutionary potentials in contrast to parasites with often shorter generation times and hence, larger population sizes than their hosts. Fast evolving parasites are often able to rapidly respond to host adaptations compared to slavemaking ants. The slavemaker-host systems is thus very convenient to study the coevolutionary process, as the arms race proceeds through stepwise reciprocal adaptations and can hence be traced through space and time.

During their evolution, slavemakers have completely lost the ability to take care of their own brood and themselves, which was already discovered by Darwin (Darwin 1859; Alloway & Del Rio Pesado 1983). Instead they are contingent on ant workers from another colony and species - the slaves - that incur all necessary tasks performed within an ant colony. This includes brood care, foraging and feeding the slavemaker workers and the queen via trophallaxis. Thus, obligate slavemakers are strongly dependent on their host species and need to replenish their slave work force regularly through the raiding of nearby ant host colonies (details on raiding behavior in the next section). These often destructive raids exert strong selection pressure on host species to develop effective defenses. However, once enslaved, host workers cannot gain any direct fitness benefits, as they do not reproduce (Gladstone 1981). Slave rebellion though can lead to indirect fitness benefits. By killing the parasitic brood, slavemaker colony sizes can be reduced, which leads to fewer and less destructive raids on related host colonies in the vicinity (Achenbach

& Foitzik 2009; Pamminger et al. 2013; Pamminger et al. 2014; Metzler et al. 2016). To gain direct fitness benefits, host species need to evolve defenses at the frontline, e.g. avoiding the raid or reducing the costs of a raid, which will mainly be investigated in this thesis.

Temnothorax slavemakers and hosts

The North American genus *Temnothorax* includes three slavemaker and three host species, in which slavery evolved at least twice independently (Beibl et al. 2005; Feldmeyer et al. *unpublished data*). All three slavemaker species are potential parasites of all three host species, however, their preferences differ. *Temnothorax americanus* (formerly known as *Protomognathus americanus*, Ward et al. 2015), has been investigated intensively over the last decades (e.g. Alloway 1979; Alloway 1990; Hare & Alloway 2001; Herbers & Foitzik 2002; Brandt et al. 2007; Pohl & Foitzik 2011), can be found with all three host species, *T. longispinosus*, *T. curvispinosus* and *T. ambiguus*. It does however prefer *T. longispinosus* as its host (Herbers & Foitzik 2002; Brandt & Foitzik 2004; Foitzik et al. 2009) over *T. curvispinosus* and is rarely found with *T. ambiguus*. *Temnothorax duloticus*, a second slavemaker species within this genus, can be found with *T. curvispinosus* and *T. longispinosus* and seems to prefer the former species. The recently described third slavemaker species, *T. pilagens* (Seifert et al. 2014, Chapter 5), was until now only found in three locations in the United States (personal communication Susanne Foitzik) and has its highest density in the North of Michigan. It was found with *T. longispinosus* and *T. ambiguus*, but was shown to prefer *T. ambiguus* as its main host species (Kleeberg et al. 2016, Chapter 6).

 All three slavemaker species raid several host colonies during a couple of weeks in summer ("raiding season") every year to replenish their slave work force. These raids are performed by slavemaker workers but also already established slaves. During the raiding season, slavemaker workers firstly scout within the vicinity of their nest to find a target host colony (*Scouting Phase*, Fig. 1A), after which, they often enter the host colony to acquire information about the colony status (Pohl & Foitzik 2011). Scouts need to decide whether or not a particular host colony is worth being raided. This depends on the number of host workers in the colony, and subsequently the number of brood items likely available for abduction. Larger colonies might be more risky, as more host workers are involved in defending their colony during raids, though, fewer raids by slavemakers are required, if larger colonies are targeted, decreasing the total risk during one raiding season (Pohl & Foitzik 2011). After evaluating the host colony, scouts return to their own colony to recruit additional slavemakers and slaves. The scout guides this newly recruited raiding party back to the target host colony (*Recruitment*, Fig. 1A). Slavemakers and slaves then need to infiltrate the host colony and are confronted

with defending host workers (*Raiding Phase*, Fig. 1A and B). As slavemaker species are usually recognized as a threat, host workers respond with various defending techniques, which are investigated in more detail in this thesis (*Evacuation // Fighting*, Fig. 1C). Host ants respond with high aggression towards the slavemaker, possibly a way to fight off the intruder altogether (Alloway 1990; Pamminger et al. 2011). Colony aggression was moreover shown to be consistent over time and generations (Modlmeier et al. 2012), which would imply a genetic basis and allows to assume that colony aggression can be under selection. After breaching this first line of defense, slavemaker workers and their slaves start stealing the brood, preferentially worker pupae, as these do not need to be fed anymore. An attacked host colony, if not able to defend itself, will eventually die and its fitness is reduced to zero. Young slavemaker queens go on nuptial flights and after mating they invade host colonies to take over their brood. The first batch of slaves will then take care of the slavemaker queen´s brood (*Mating*, Fig. 1D). Once developed, slavemaker workers will then continue raiding.
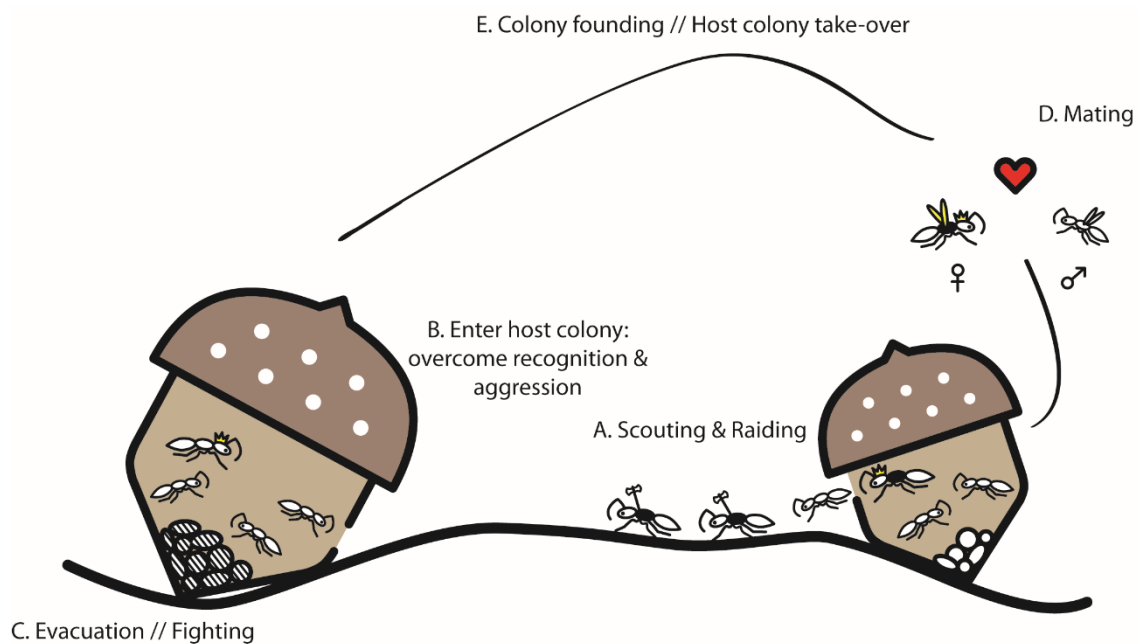
Figure 1. The lifecycle of *Temnothorax* slavemaking ants. Ants with black thorax represent slavemaking ants, and ants with white thorax host ants. (A) Scouts leave the nest to find target host colonies. Eventually they will recruit additional slavemakers and slaves to raid the host colony. (B) Slavemakers need to overcome recognition and hosts respond with fierce attacks. (C) Possible defense strategies include attacking the invading slavemakers and / or evacuating part of the colony. (D) Nuptial flight of slavemaker queens and males. After mating, males die and queens invade host colonies to found a new slavemaker nest.

Raiding strategies however differ between the three slavemaker species investigated in this thesis. Raids of *T. americanus* usually proceeds as described above. Hosts of this slavemaker are able to recognize the parasite as a threat (Alloway 1990; Pamminger et al. 2011) and respond with high aggression during raid encounters. Slavemaker workers are able to disrupt the colony functioning by eliciting a propaganda substance through the Dufour´s gland (Brandt et al. 2006; Jongepier et al. 2015, Chapter 4). Host workers that are marked with the Dufour´s gland secretion will be attacked by its own nestmates, thereby disrupting the collective defense ability of the colony. Raids of *T. duloticus* are very similar; however, workers of this slavemaker species make use of their stinger in contrast to *T. americanus*. Raids of *T. duloticus* are more virulent as it can kill most of the host ants and even emigrates to the raided host colony (Alloway 1979). *Temnothorax americanus* raiding parties rarely kill the whole host colony and hosts are often able to evacuate the queen into a new nest site. Hence, *T. americanus* has a less detrimental impact on its host populations and can achieve local population densities much higher than those for *T. duloticus* (Alloway 1979; Frank 1996; Hare & Alloway 2001). *Temnothorax pilagens* raids are very different. Although the principle is the same, *T. pilagens* is able to abduct adult host workers, next to the brood, during raids (Seifert et al. 2015, Chapter 5; Kleeberg et al. 2016, Chapter 6) – a strategy called eudulosis and only known for three more slavemaker species, *Strongylognathus afer*, *Formica naefi* and *Polyergus rufescens* (Kutter 1957; D´Ettorre and Heinze 2001; Sanetra & Güsten 2001). During such raids, almost no aggression occurs, supposedly because hosts are not able to recognize the threat. If hosts however manage to recognize the parasite and react aggressively, *T. pilagens* workers kill up to 100% of the host workers using its stinger (Kleeberg & Foitzik 2016, Chapter 6). Despite their differences, the principle of raiding is very similar, which evolved convergently in these species. These systems provide a great opportunity to draw general conclusions about the evolution of slavery and its impact on the host defense strategies.

This thesis

In my dissertation I aimed to investigate host defense and slavemaker offense strategies in different slavemaker and host species of the genus *Temnothorax.* This thesis is divided into two parts: the first part investigates host defense adaptations in response to parasite pressure (Chapter 1-4) whereas the second part concentrates on counter-adaptations in parasites (Chapter (4)5-7).

One major aspect of my dissertation work is the geographic variation of different but codependent host adaptations in relation to parasite pressure. Aggression and recognition, both of them being fundamental in this system (Alloway 1990; Pamminger et al. 2011), are of particular interest and are addressed

in each chapter. In the first chapter, we focused on information use and aggression in the host *T. longispinosus* during raids of *T. americanus*. The second chapter continues to investigate whether or not the aggressive potential of hosts is linked to, or the direct result of, parasite pressure. In the third chapter we expanded the effect of parasite pressure on collective fight and flight strategies and in the fourth the resistance to chemical manipulation by the parasite and its relation to the slavemaker´s success. During my studies we discovered the third slavemaker species, *T. pilagens*, which we described taxonomically in the fifth chapter. *Temnothorax pilagens* was further investigated to compare raiding strategies between the three slavemaker species and the ability of hosts to recognize their different slavemakers. Additionally we tested for parasite counter-adaptations to inhibit recognition (Chapter 6 and 7). The seventh study moreover investigates the influence of parasitic lifestyle and caste on the chemical profile, and the expression of general chemical strategies to reduce the costs of raiding for slavemakers.

We firstly investigated the benefits of aggressive behavior in the host *T. longispinosus* during raids of its slavemaker *T. americanus*. Pamminger et al. (2011) found, that host colonies increase their aggression level towards conspecific non-nestmates after having contact with a slavemaker in their colony. Hence, we hypothesized that *T. longispinosus* colonies are able to gain information on an upcoming slavemaker attack, and make use of this information to better defend their colony. Thus we used raiding experiments to reveal whether informed host colonies were better able to defend themselves, and whether this was dependent on their aggressive potential assessed previously.

In our second and third study we further tested whether aggressive potentials of host colonies (Chapter 2) that were shown to be beneficial during raids (Chapter 1) and whether flight vs. fight strategies (Chapter 3) are shaped by parasite pressure. To this end we collected host colonies of two species, *T. longispinosus* and *T. curvispinosus*, both of which are host species to *T. americanus*, from 17 populations with varying parasite pressure. A set of colonies per species and population was firstly tested in aggression essays towards frozen conspecifics, heterospecifics and slavemaker workers to assess which behavioral responses are under selection by parasite pressure. Secondly, the same set of colonies were tested in evacuation experiments, to investigate the response towards a living slavemaker worker. Possible outcomes here were that host colonies either evacuate their colony to a new nest-site escaping parasitism altogether or respond with collective attacks to hinder the slavemaker from recruiting nestmates. As both studies revealed that host colonies with a high aggressive potential do not necessarily show high aggression towards intruding slavemakers, we next investigated the role of host manipulation through the use of the slavemaker´s Dufour´s gland (Chapter 4). We predicted that the degree of manipulation differs between populations, impacting the success and prevalence of parasites. For this purpose we tested variation in host resistance to manipulation of the two host species of all

populations. We aimed to investigate its consequences for the efficacy and intensity of host colony aggression as well as the effect on host preference and prevalence of the slavemaker.

In the fifth chapter, we taxonomically described a new slavemaker species, *T. pilagens* in comparison to *T. duloticus*, a morphologically very similar species. We continued to investigate, based on previous observations of "peaceful raids" (Seifert et al. 2014, Chapter 5) in *T. pilagens*, the role of recognition and aggression in this slavemaker-host system. At the same time, we compare raiding strategies between the three *Temnothorax* slavemakers and host responses between the three *Temnothorax* host species. Finally, in the seventh chapter, we focus on the evolution of cuticular hydrocarbons, substances known to be important in communication in ants and the influence of parasitic lifestyle on the chemical profile. We predicted, that the parasitic lifestyle selects for similar chemical traits in all three slavemakers, despite their independent origins of slavery. Next to workers of all species, we additionally analyzed males and virgin queens, to investigate how the diverse selection pressures on different castes, sexes and lifestyles (parasitic vs. non-parasitic) are reflected in their cuticular hydrocarbon profiles.

Chapter **1**

Forewarned is forearmed: aggression and information use determine fitness costs of slave raids

Isabelle Kleeberg*, Tobias Pamminger*, Evelien Jongepier, Mirjam Papenhagen and Susanne Foitzik

ABSTRACT

Many animals use reliable indicators of upcoming events such as antagonistic interactions to prepare themselves. In group-living animals, not only the cue perceiving individuals are involved in mobilization, but the entire group can use this information. In this study, we analyze whether social insects, which perceive reliable information on an upcoming social parasite attack, can use this knowledge to better defend their colony. We focus on the interaction between the ant *Temnothorax longispinosus* and the slave-making ant *Protomognathus americanus*, which conducts destructive raids on host colonies to steal their brood. As a behavioral defense, host colonies show aggression, which has a constitutive and inducible component: earlier studies demonstrated both behavioral consistency and an increase in aggression after slavemaker contact. Here, we analyze the fitness benefits of aggression in the context of slave raids. Aggression only facilitated host colony defense if the hosts were informed about a pending slave raid: slavemaker scouts were not only less likely to enter forewarned host colonies, but more aggressive colonies who previously encountered a slavemaker saved a higher fraction of their brood. We thus demonstrate that the fitness costs of raids depend on an interaction between the collective defense abilities and reliable information on impending dangers.

Keywords: aggression, information use, social parasite, defense, colony personality, induced defenses

INTRODUCTION

Enemies such as parasites and predators can select for behavioral, physiological and morphological defense traits (Nesbitt et al. 1996; Janeway et al. 1999; Sell 2000), which can be either constitutive or inducible. Constitutive defenses are constantly expressed and have the advantage that they can be directly employed when an attack occurs, but they often carry costs in the absence of enemies (Harvell 1990). In contrast, inducible defenses are plastic and triggered only after enemy detection, thereby avoiding potential costs of constitutive trait expression. However, time-delayed expression provides no benefit if immediate defense is needed (Svennungsen et al. 2011). Hence, the evolution of inducible defense traits is favored if cues are available that can give reliable information on enemy presence in the local habitat (Harvell 1990; Dall et al. 2005). For example, *Daphnia pulex* develops spines as a morphological defense only after detecting chemical cues of its invertebrate predator *Chaoborus* (Sell 2000).

Many animals use specific behavioral traits to avoid or fend off parasites or predators. Defensive behaviors often involve aggression – a risky, but widespread behavioral trait employed by many animals during competition for resources (Stamps and Krishnan 1997; Garcia and Arroyo 2002), mating partners (Heinze et al. 1992; Nelson-Flower et al. 2013) or in predator-prey interactions (Redondo and Carranza 1980). Under food or habitat limitation, animals often use aggression to gain and maintain access to limited resources (Innocent et al. 2011). However, aggressive behaviors can carry not only energetic costs, as fights can also lead to injury or death (Georgiev et al. 2013). The fitness costs and benefits of aggression are consequently context dependent, favoring the coexistence of individuals with a wide range of different aggression levels in a population (Humfeld et al. 2009; Archard and Braithwaite 2011).

In social insects, aggression is an important colony personality trait (Barth et al. 2010; Chapman et al. 2011; Modlmeier and Foitzik 2011; Pinter-Wollman 2012; Jandt et al. 2014) exhibited during territorial fights (Newey et al. 2010; Scharf et al. 2012), nest defense (Modlmeier et al. 2012) and foraging (Biesmeijer and Slaa 2004). Aggression helps ants to protect their societies from competitors and social parasites (Pamminger et al. 2011; Fürst et al. 2012). For example, more aggressive *Temnothorax longispinosus* host colonies were able to rescue more brood under attack of young queens of the slavemaking ant species *Protomognathus americanus* (Pamminger et al. 2012). Colonies of this slavemaking ant conduct frequent and destructive attacks on surrounding host colonies to steal the host brood (Foitzik and Herbers 2001). These slave raids can be divided into two phases, possibly selecting for different phase-specific host responses: during the scouting phase, single slavemakers search for suitable host colonies, sometimes entering to evaluate them as raiding targets (Pohl and Foitzik 2011). The raiding phase starts with the recruitment of a raiding party by the scout consisting of additional

slavemaker workers and slaves, which proceeds to attack the host nest. *Protomognathus americanus* slave raids have detrimental effects both on the attacked host colonies and the entire host population (Foitzik and Herbers 2001; Foitzik et al. 2009; Scharf et al. 2011a). The fitness of attacked host colonies is reduced due to the loss of brood and the death of part of the workforce and possibly the queen(s) (Foitzik et al. 2001). As a response, host colonies have developed various defense strategies to circumvent exploitation, most of which involve aggression (Alloway 1990; Foitzik et al. 2001; Brandt et al. 2005a). However, during raids, not all host workers fight against their attackers, some start to evacuate the nest and flee with the brood (Foitzik et al. 2001).

In the ant *Temnothorax longispinosus*, the aggression level of a colony was found to be consistent over several worker generations (Modlmeier et al. 2012) potentially indicating a genetic component of aggression. Although colony aggression leads to fitness benefits during slavemaker queen attacks (Pamminger et al. 2012), the aggression level between ant colonies varies greatly in the field (Modlmeier and Foitzik 2011; Crosland 1990). This could indicate that spatial variation in ecological or social conditions affects the costs and benefits of aggression. Consequently, different aggression levels could be selected for. For example, host population density appears to influence the selection regime for colony aggression in *T. longispinosus*, as aggression increases with nest density (Modlmeier and Foitzik 2011). Aggression in *T. longispinosus* colonies was found to be in part inducible. After slavemaker contact, host colonies increased their aggression level for several days, revealing an inducible behavioral defense (Pamminger et al. 2011).

Here, we analyze whether this increased aggression level yields fitness benefits for host colonies under attack by *P. americanus* slavemaker colonies. We investigate benefits of modulated host aggression during antagonistic interactions with its social parasite *P. americanus*. In particular, we are interested whether host colonies can use information on an upcoming social parasite attack to forearm if group members had preceding contact with slavemaker scouts.

MATERIALS AND METHODS

Ant collection and colony maintenance

In summer 2011, we collected colonies of *P. americanus* (N = 78) and *T. longispinosus* (N = 200) for the raiding experiments at the E. N. Huyck Preserve, Albany County, NY (42°31`353``N, 74°89`301``N) and colonies of a second host species, *T. curvispinosus* (N = 19), at Alum Creek State Park, Delaware County, OH (40°13`337``N, 82°58`543``W). Ant collection permits were obtained at both sites, import and export licenses are not required. Following discovery, we moved the ant

colonies with their nests (acorns or little sticks) and a hand full of leaf litter into resealable bags and provisioned them with tuna fish and cookie crumbs. They were stored at 15°C in an incubator, until being transported to our laboratory where the ant colonies were counted, and housed in artificial nests (5.0 x 1.1 x 0.3 cm) within three-chambered plastic boxes (9.5 x 9.5 x 2.7 cm). The plastic boxes were covered with a lid and paraffin oil was applied to the walls to prevent ants from escaping. Artificial nests were made out of glass to allow observations during experiments. Ants were stored in a climate chamber at 20°C : 15°C in a 12 L : 12 D cycle and fed weekly with honey and crickets.

Fitness benefits of constitutive aggression and information on upcoming attacks

We determined the constitutive aggression level ("baseline colony aggression") of *T. longispinosus* host colonies 24hrs prior to the raiding experiment against a dead non-nestmate conspecific ant, following the protocol of Pamminger et al. (2011). All opponents were killed by freezing in -20°C at the day of the aggression experiment and carefully placed into the focal host colony 1 cm from the entrance using a featherweight forceps. All interactions between host ants that discovered the dead ant and the opponent were recorded for 5 minutes. Dead ants were used to eliminate the behavioral variation between the stimuli. We thus focused on the focal colony´s response to the chemical stimulus. We recorded six different interactions: antennation was scored as non-aggressive; mandible spreading, biting, holding, dragging and stinging as aggressive interactions. An aggression index was calculated for each host colony, expressing the total number of aggressive interactions as a percentage of all interactions with the opponent.

As we were interested in the influence of host colony baseline aggression and enemy contact on raiding outcomes, we included two types of prior slavemaker contact: experimental introduction of a slavemaker 12hrs before the raiding attack and contact to a slavemaker scout during the initial raiding phase. For the manipulation, 100 *T. longispinosus* colonies were confronted for 1h with a living *P. americanus* slavemaker worker (Treatment-group) and 100 colonies with a *T. curvispinosus* worker (Control-group) 12hrs before the raiding trials. Opponents were allowed to enter the host colony and the nest was closed for an hour. We did not test for an induced aggression increase after slavemaker contact, as this was shown before (Pamminger et al. 2011). We chose contact to a non-parasitic congener as a control as we were interested in species-specific responses to slavemaker contact. Our study design allowed for both treatment and control colonies to experience aggressive interactions with a closely related heterospecific worker (Beibl et al. 2005). *Temnothorax longispinosus* has overlapping ranges with both ant species (i.e. Brandt and Foitzik 2004), but the focal population in Upstate New York is sympatric only with *P. americanus*. As the closest *T. curvispinosus* population that we know of is about 200 km away (in Stamford CT, unpublished data), *T. longispinosus* colonies from our study population were naïve to this

congener. Nevertheless, *T. longispinosus* colonies from our NY site show species-specific responses during encounters with both species (Scharf et al. 2011b). Yet, whereas the aggression level of *T. longispinosus* colonies increases following slavemaker contact, this is not the case after encountering *T. curvispinosus* (Pamminger et al. 2011).

A second measure of how slavemaker contact influences raiding outcomes was based on the fact that some slavemaker scouts enter a host colony, whereas others do not (Pohl and Foitzik 2011). Hence, we included as a predictor in our analyses whether or not a scout entered the host nest during the scouting phase of the raid. The rationale was that if a host colony has reliable information on an upcoming raid because it encounters a slavemaker inside its nest shortly before the raiding party arrives, it could prepare for the upcoming fight.

We chose experimental host and slavemaker colonies of about the same size, regarding the number of host or slave workers, as worker numbers influence raid outcomes (Foitzik and Herbers 2001). Host colony sizes did not differ between the two treatments ($F_{1,62} = 0.283$, $P = 0.596$). On the day of the raiding trial, treatment and control colonies were placed in their artificial nest sites at one corner of a plastered experimental arena (36 x 30 cm). The slavemaker colony was placed at a distance of 22 cm from the host nest entrance. An empty nest was placed 10 cm from the host nest, as a potential escape for the fleeing hosts.

During the observations of the raiding experiments we documented the number of larvae and pupae evacuated by host workers or stolen by slavemakers or slaves. We evaluated the success of host nest defense based on the proportion of brood the host colony was able to save. The raiding experiment was terminated 90 min after the last brood item was removed from the host nest. Additionally we recorded for most of the raids the time (in minutes) between nest evacuation start (first brood removed by the host) and arrival of the raiding party ($N = 23$) and between nest evacuation start and start of the raiding party (the time when the raiding party leaves the slavemaker colony, $N = 61$).

We successfully staged raids on 66 host colonies, for 64 of which we determined the baseline colony aggression level previously. Each host colony participated in a single trial. Thirty-five host colonies had previous contact to a slavemaker (Treatment-group), whereas thirty-one colonies (Control-group) encountered a heterospecific competitor. A raid was recorded as successful, when the scout recruited a raiding party to the host colony. Thirty-five slavemaking colonies participated in the raiding experiments, seventeen of which were involved in a single raid, eleven in two raids, four in three raids and three in five raids.

We assessed whether host colony baseline aggression and previous slavemaker encounter was related to a host colony's ability to salvage brood during a raid. Hereto, we analyzed the proportion of brood saved (i.e. brood saved vs.

brood lost) using a set of generalized linear models with quasi-binomial error distribution and logit link function. We included baseline aggression level, treatment and scout contact (i.e. whether or not a scout entered a host colony during the scouting phase) as predictors. The full model also included all interactions. For model selection we used a backwards stepwise selection procedure based on the F statistic ($\alpha = 0.05$). We conducted a Wilcoxon Signed Rank Test to compare the evacuation speed (the time between the start of the evacuation and the start or arrival of the raiding party) of host colonies that did or did not encounter a scout inside their nest during the scouting phase. All analyses were performed using R version 3.0.0. (R Core Team 2013).

RESULTS

Host colonies fended off slavemaker attacks in 9 (13.6%) of 66 successfully staged raids, either by directly attacking the scout, which was then unable to recruit more slavemakers, or by preventing the raiding party to enter the host colony. On average host colonies saved 47.45% (SE ± 3.79) of their brood, whereas *P. americanus* colonies captured on average 52.54% (SE ± 3.79) of the host brood. The comparison of the treatment revealed that host colonies exposed to a *P. americanus* worker 12hrs before saved on average 48.73% (SE ± 5.17) of their brood, whereas control colonies saved 45.99% (SE ± 5.65). Thus experimental manipulation of slavemaker contact neither affected the proportion of saved brood (treatment: $F_{1,59} = 0.92$, $P = 0.343$), nor did it interact with colony baseline aggression ($F_{1,58} = 0.60$, $P = 0.443$), scout contact ($F_{1,57} < 0.01$, $P = 0.927$) or both (3-way interaction: $F_{1,56} = 3.13$, $P = 0.082$).

However, raiding outcomes were associated with the level of baseline colony aggression assessed prior to the raids when combined with information on an upcoming slavemaker attack received during the experiment (Fig. 1.1; colony aggression – scout contact interaction: $F_{1,60} = 21.97$, $P < 0.0001$). Host colonies with higher baseline aggression, that encountered a slavemaker scout in their nest during the scouting phase, saved a higher fraction of their brood (estimate ± SE = 3.71 ± 0.81, $t = 4.59$, $P < 0.001$). Contrastingly, baseline aggression was unrelated to the fraction of brood saved for colonies without scout contact ($t = -1.01$, $P = 0.315$). Slavemaker scouts entered host colonies at a median of 42 min (interquartile range 22 – 85.5) prior to the attack of the raiding party.
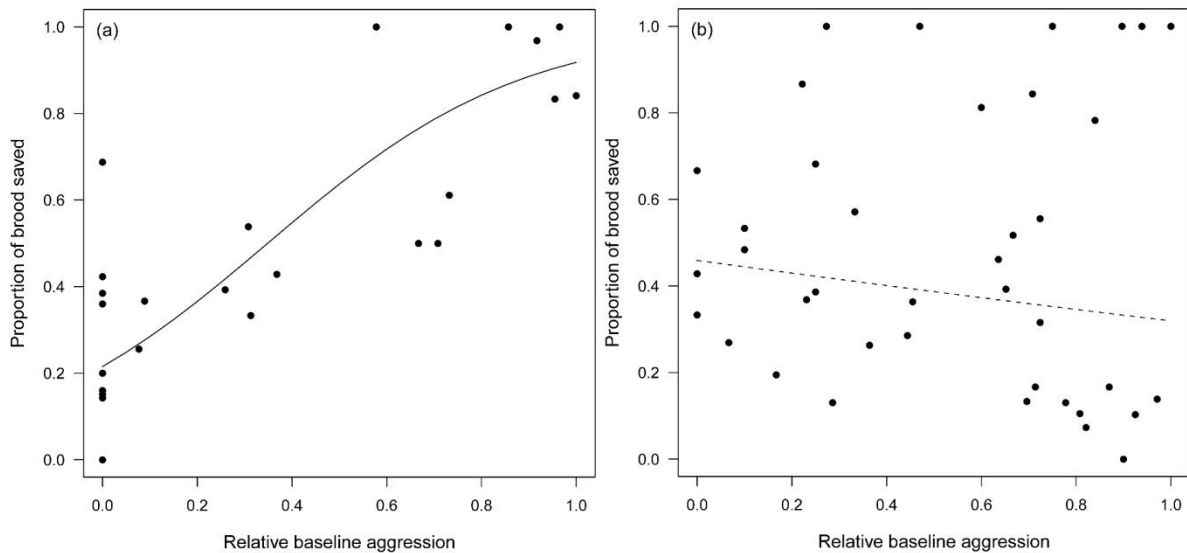
Figure 1.1 Association between the proportion of brood saved by the host colony and the aggression level of the colony. More aggressive host colonies saved more brood if they encountered a scout during the raid (GLM: 3.711 ± 0.809, t = 4.588, P < 0.0001) (a), but percentage of host brood saved was independent of host aggression when the host colony had no contact to the slavemaker scout before (-0.591 ± 0.583, t = -1.014, P = 0.315) (b).

However, we detected a treatment effect (contact to slavemaker vs. contact to heterospecific worker 12 hrs prior to the experiment) when focusing on the scouting phase: slavemaker scouts were less likely to enter host colonies that encountered a slavemaker 12hrs before (22.9% of 35 raids), compared to nests with no previous slavemaker contact (51.6% of 31 raids; X^2 = 4.70, P = 0.030).

There was no difference in evacuation speed between host colonies that did or did not encounter a scout inside their nest during the experiment (time lag between evacuation start and raiding party arrival: W = 65.5, P = 1.00, N = 23; time lag between evacuation start and start of raiding party: W = 346, P = 0.177, N = 61).

DISCUSSION

In this study, we demonstrate that ant societies can use information obtained during enemy contact to better defend themselves during a subsequent attack. Host colonies that encountered a slavemaker worker were more often able to keep a slavemaker scout outside their nest and to rescue a larger fraction of their brood when a slavemaker raiding party attacked their nest. The latter effect increased with colony baseline aggression, revealing an information-dependent fitness benefit of a constitutive behavioral defense as a colony personality trait. How exactly the ant colonies managed to keep the scout outside their nest and how they prepare themselves for an upcoming fight is unresolved. Most likely induced

defense mechanisms including physiological and behavioral changes could explain our findings as host colonies were shown to become more aggressive for several days after slavemaker contact (Pamminger et al. 2011). Alternatively, the experimentally introduced slavemaker worker might have left chemical cues at the host nest entrance, which could influence the scout´s decision to enter the host colony as the cues could signal that the host nest has already been discovered.

Our study design allowed for two types of pre-raid slavemaker contact information: a) we experimentally introduced a slavemaker 12hrs before the raiding trials and b) some slavemaker scouts entered host nests during the scouting phase, whereas others did not. The latter information was more recent as scout entry happened less than an hour before the attacks of the raiding party. We found an information-dependent fitness effect only for the 2nd contact type, whereas the first one only influenced the likelihood with which a slavemaker scout entered the host nest. This difference might be due to the differential reliability of the signal, as the likelihood of a raid will decrease over time. It has been argued that outdated information is costly to use (Dall et al. 2005), and as mobilization might disrupt colony functioning, it should only be maintained as long as the benefits of fight preparation outweigh the costs.

Temnothorax host colonies react more aggressive towards the slavemaker *P. americanus* – a virulent social parasite – than to conspecifics or heterospecific competitors (Alloway 1990). Differences in aggression levels between colonies are consistent over these different contexts (Kleeberg I, Jongepier E, Job SA, Foitzik S, unpublished data). The ants presumably recognize their enemies by their cuticular hydrocarbons as chemical profiles are species-specific (Brandt et al. 2005b) and the strength of their response varies with the potential threat (Scharf et al. 2012). The fact that *Temnothorax* ants modulate their aggressive responses depending on the type of enemy – competitor versus parasite – can be interpreted as an "evolutionary" risk assessment. Similar adjustments in aggression to external factors have been reported for other animals, which vary their fighting behavior in reaction to the competitive ability of the opponent (Maynard Smith 1979; Robinson 1985; Riechert 1998; Arnott and Elwood 2009), the threat level (Scharf et al. 2011b) or food and resource availability (Nicieza and Metcalfe 1997; Kim 2000; Hoogenboom et al. 2013). To be able to adjust the behavior to specific situations, animals have to continuously gather information about the environment, including the social one, to reduce uncertainty (Dall and Johnstone 2002).

Indeed, we can show that the fitness benefits of baseline aggression depend on the information on impending dangers. Information on the presence and strength of enemies is crucial for an individual's and a group's decision to fight or to avoid antagonistic encounters (Lima and Dill 1990). If some members of a social group encounter an enemy, they can change their behavior according to the given situation and / or alarm others to elicit collective threat responses (Caro 2005;

Brechbuhl et al. 2013). In our study system, only part of the workforce had direct physical slavemaker contact. Therefore, the shown fitness benefits could be due to behavioral changes in those workers with immediate slavemaker contact. They could possibly fend off intruding slavemakers, facilitating brood evacuation by their nestmates (Foitzik et al. 2001) or take over brood evacuation themselves. Alternatively, workers encountering slavemakers might communicate this information to their nestmates, either by behavioral interactions or by using alarm pheromones (Vander Meer and Alonso 1998). Indeed, we observed that host workers reacted to slavemaker contact by rapidly drumming their antenna on the head of their nestmates, which has been interpreted as an aggressive or excitatory behavior and might alarm others (Blatrix and Jaisson 2000; Heinze and Weber 2011). The informed individuals might then start evacuating the brood while the rest of the colony tries to fight off the raiding party. An alternative hypothesis would have been that warned colonies respond faster by evacuation; however we can exclude this possibility. We recorded for most of the raids the time between nest evacuation start and arrival, and start of raiding party and did not detect any difference between warned and non-warned host colonies.

Recently, evidence is accumulating that consistent variation in behavior, so-called animal personalities, has measurable fitness consequences (Smith and Blumstein 2008). This raises the question how consistent behavioral variation is maintained in a population. The concept of animal personality has been successfully applied to a variety of taxa, including insect societies (Cote et al. 2010; Chapman et al. 2011; Modlmeier and Foitzik 2011; Pruitt et al. 2011; Modlmeier et al. 2012; Pinter-Wollman et al. 2012). Bolder individuals of freshwater killifish, for example, have a higher growth rate and disperse further than shy ones (Fraser et al. 2001). Under the risk of predation, selection favored bold ewes (Réale and Bianchet 2002) suggesting predator-induced selection for bold personalities. In social insects, including our focal species, consistent variation in aggression was found to be associated with differences in colony productivity (Modlmeier et al. 2012; Wray et al. 2011). Our results provide insights into the maintenance of colony personalities as we demonstrate information-dependent benefits of colony baseline aggression. Under parasite attack, high baseline aggression is beneficial for host colonies, but these risky behaviors might be costly in the absence of parasites. In ants and many other animals, aggressive behavior does not only carry the risk of death or injury (Dimarco et al. 2010; Georgiev et al. 2013), but also high metabolic costs (Gobin et al. 2003; Seebacher et al. 2013). These context-dependent costs and benefits could explain why heritable behavioral variation persists in a population. In our focal species, variation in host colony baseline aggression was found to be consistent over time and to co-vary with nest density (Modlmeier and Foitzik 2011). As host and parasite density is patchily distributed on a small spatial scale (Foitzik et al. 2009), different aggression levels might be selected for in different locales depending on the local importance of competition and social parasitism.

In conclusion, our study reveals fitness benefits of a constitutive behavioral defense trait - colony baseline aggression - but also flexible information-dependent responses. Indeed, parasite-induced changes that result in fitness benefits of forewarned colonies can be compared to the priming of the immune system by micro-parasites (Moret and Siva-Jothy 2003). Although the underlying mechanisms and the effectiveness clearly differ, similarities can be seen in the specific priming during a first parasite contact, which lead to physiological or behavioral changes in the host that result in lower success rates during consequent parasite attacks. Further studies should explore the physiological and behavioral mechanisms underlying the abilities of forewarned colonies to forearm themselves against a parasite attack.

Chapter **2**

Geographic variation in parasite pressure predicts intraspecific but not interspecific aggressive responses in hosts of a slavemaking ant

Isabelle Kleeberg, Evelien Jongepier, Sylwester Job and Susanne Foitzik

Author contributions: IK, EJ and SF designed the experiment. IK, EJ and SJ collected the data. EJ and IK analysed the data. IK wrote the first draft and IK, EJ and SF revised it until completion and responded to reviewer comments.

ABSTRACT

Variation in community composition over a species' geographic range leads to divergent selection pressures, resulting in inter-population variation in trait expression. One of the most pervasive selective forces stems from antagonists such as parasites. Whereas hosts of micro-parasites developed sophisticated immune systems, social parasites select for behavioural host defences. Here, we investigated the link between parasite pressure exerted by the socially parasitic slavemaking ant *Protomognathus americanus* and colony-level aggression in *Temnothorax* ants from 17 populations. We studied almost the entire geographic range of two host species, including unparasitized populations. As previous studies have demonstrated that host colonies responding highly aggressively towards conspecifics fare better during slavemaker attacks, we predicted higher aggression levels in severely parasitized populations. Indeed, we demonstrate an increase in aggression towards conspecifics with parasite pressure, a pattern that was consistent over the two host species. In contrast to other studies, aggression against the parasite itself did not shift with parasite pressure. This may be explained by an absence of costs of parasite-specific aggression in parasite-free populations. The preferred host species *T. longispinosus* was generally more aggressive; however, the association between parasite pressure and aggression was found for both species, suggesting convergent co-adaptation. Two potentially confounding factors, colony density and the co-occurrence of a competing *Temnothorax* species in the community, could not explain the level of colony aggression in intra- and interspecific interactions. Instead, our study points to social parasite pressure as the determining factor shaping antagonistic interactions within, but not between, host species.

Keywords: colony aggression, parasite pressure, co-evolution, collective defence, antagonistic interactions

INTRODUCTION

Species interactions are among the most powerful evolutionary forces (Thompson 1994; Vermeij 1994; Paterson et al. 2010). Hosts and parasites can form long-lasting (Combes 2001), highly specialized associations, characterized by strong reciprocal selection (Price 1980) resulting in co-adaptation cycles (Thompson 1982). The strength of reciprocal selection often varies among communities. The presence of parasites in a population may alter the host´s biotic environment and shapes the evolution of behavioural traits (Dobson 1988; Barber et al. 2000). Hence, co-adaptation can be studied by comparing populations under divergent selection regimes, like the presence and absence of parasite pressure. To avoid or limit the adverse effects of parasitism, hosts develop physiological, morphological or behavioural adaptations that increase their resistance or tolerance (Wakelin 1984; Hart 1997; Gross 1993; Sorci 2013). If parasites are common and their impact severe, hosts are expected to evolve effective defences, which in turn select for counter-adaptations in the parasite. If trait expression is costly, more effective defensive traits are expected only in severely parasitized host populations (co-evolutionary 'hot spots'; Thompson 1999). The local co-evolutionary process is furthermore affected by the co-occurrence of alternative hosts in a community (Brandt and Foitzik 2004). Parasites often favour a single host species and this preference is related to local host densities and defensiveness. As a consequence, the overexploited host might suffer from a density reduction or develop better defences, which in turn can influence host competition and the parasites' preferences (Price et al. 1986; Hatcher et al. 2006). The spatial variation in the outcome of species interactions, in other words, the analyses of these natural laboratories, can contribute to our understanding of the adaptive process itself (Holt and Keitt 2005).

An important behavioural trait in species interactions is aggression, which is not only displayed during competition for resources (Stamps 1977), but also used against predators and parasites (Huntingford 1976; Robertson and Norman 1977; Gottfried 1979). Aggression carries the risk of injury and death (Georgiev et al. 2013) in addition to energetic expenses; thus organisms face a trade-off between these costs and the benefits of antagonistic behaviour. The aggression level should therefore correlate with ecological factors (e.g. parasite or predator abundance or competition) that influence this trade-off (Archard and Braithwaite 2011).

Here, we investigate the association between parasite pressure and aggression in two *Temnothorax* ant species parasitized by the obligate social parasite *Protomognathus americanus* (*Temnothorax americanus* according to Ward et al. 2014), a slavemaking ant. The frequent raids of this social parasite severely reduce the fitness of host colonies (Foitzik and Herbers 2001; Foitzik et al. 2009). Slave raids are initiated by slavemaking workers, but during the raiding phase enslaved host workers participate in pillaging attacked nests and are responsible

for most of the losses among the defending host workers. Hosts have developed defensive strategies to circumvent parasitism (Alloway 1990; Foitzik et al. 2001; Brandt and Foitzik 2004); among the most effective of these strategies is collective aggression (Jongepier et al. 2014, Chapter 3). Indeed, more aggressive host colonies save a larger fraction of their brood during slavemaker queen invasions (Pamminger et al. 2012) and during raids (Kleeberg et al. 2014, Chapter 1). Colony-level aggression presumably has a genetic basis, as it is consistent over different worker generations (Modlmeier et al. 2012). Moreover, aggression varies spatially both within and between populations (Foitzik et al. 2001; Brandt and Foitzik 2004; Modlmeier and Foitzik 2011; Jongepier et al. 2014, Chapter 3), which suggests that different ecological conditions favour different levels of trait expression. *Temnothorax* hosts show fine-tuned aggression behaviour, where the intensity and type depend on opponent species and the perceived threat that they pose (Scharf et al. 2011b): social parasites and heterospecific competitors elicit more aggressive responses than alien conspecifics, yet aggression against the latter has been shown to be predictive for the outcome of social parasite interactions (Pamminger et al. 2012; Kleeberg et al. 2014, Chapter 1). Nonetheless, little is known on how parasite pressure affects aggressive responses across different populations.

As recent coevolution theory emphasizes the importance of studying the entire geographic range of hosts, including populations where parasites are absent (Gomulkiewicz et al. 2007), we do so for the host species, *Temnothorax longispinosus* and *T. curvispinosus*. To gain insights into the generality of the patterns observed, we studied behavioural adaptations in two of the three host species of *P. americanus*. We investigated whether parasite pressure co-varies with aggressive colony responses towards conspecific hosts, social parasites and heterospecific hosts. We compared 17 host populations with differential parasite pressure to examine whether differences in colony aggression can be explained by the absence or presence of parasites. We hypothesize that aggression against conspecifics will be positively related to parasite pressure as more aggressive host colonies fare better during slavemaker attacks (Pamminger et al. 2012; Kleeberg et al. 2014, Chapter 1). The same positive association could be expected for aggression against the social parasite itself, though, in the absence of costs, all host populations might exhibit highly aggressive responses towards this enemy. Finally, we tested for aggression between *Temnothorax* host species. We expected that if competition is an important selective force, host populations occurring in sympatry respond more aggressively towards the other species. We not only studied the covariance between different aggression contexts and parasite pressure in the two hosts, but we also compared the associations across species, where we predicted higher aggression levels towards the slavemaker in the preferred and more severely exploited host species, *T. longispinosus* (Brandt and Foitzik 2004).

MATERIAL AND METHODS

Collection and colony maintenance

We collected non-parasitized ant colonies of the host species *Temnothorax longispinosus* and *T. curvispinosus* from mid-May to mid-July 2012 at 14 study sites in the Eastern United States and Southern Canada (Fig. 2.1; Table S2.1; Jongepier et al. 2014, Chapter 3). Ants of these species harbour hollow sticks, acorns or hickory nuts and colonies contain on average 24 workers and are facultative polygynous and seasonal polydomous (Alloway et al. 1982; Alloway Del and Rio Pesado 1983). Ant colonies were taken from the field before the annual raiding season from late July to end of August. The experiments were conducted during five weeks from the beginning of September until mid-October 2012. The same ant colonies were used in a different trial series to test for evacuation responses towards a live slavemaker worker (Jongepier et al. 2014, Chapter 3); however, there is no behavioural data overlap between the two studies. As parasite-induced short-term behavioural changes were shown to level-off within a few days (Pamminger et al. 2011), any differences between populations are likely due to adaptation rather than recent experience.

In total, we sampled 17 populations of the two host species at 14 study sites: eight populations of *T. curvispinosus* and nine of *T. longispinosus*, including communities in which both hosts co-occur (Fig. 2.1; Table S2.1). From each population, we collected approximately 100 colonies in order to estimate parasite pressure by the slavemaking ant *Protomognathus americanus*, with the exception of *T. longispinosus* colonies from Kentucky (66 colonies) due to the low colony density at this site. Parasite pressure was defined as the proportion of slavemaker colonies (with slaves of this host species) per host colony (Jongepier et al. 2014, Chapter 3). Long-term parasite pressure estimates were available for five of our *Temnothorax* study populations at the locales in New York, West Virginia and Ohio North (Herbers and Foitzik 2002; Brandt and Foitzik 2004; Foitzik et al. 2009), which we included in our parasite pressure estimate, as host populations are expected to respond over decades to changes in parasite pressure due to their long generation times of several years (Keller and Genoud 1997).

To account for the potentially confounding effects of host population density, which can as well influence colony aggression (Modlmeier and Foitzik 2011) we estimated population density as the number of host colonies collected per hour (Table S2.2). This crude measure was a compromise between obtaining density information and covering most of the geographic range of our study species. We further accounted for the potentially confounding effects of sympatry of different hosts, which was defined as whether or not a second *Temnothorax* host species was present in the local community (Table S2.3).

Collected ant colonies were transferred into Ziploc bags, stored at 7°C during the remainder of the trip, and transported back to our laboratory in Germany. The colonies were housed in artificial glass nest sites and kept in plastered three-chambered boxes. We kept the ant colonies at constant 25°C in a climate chamber and fed honey and crickets twice a week.

Experimental set-up

For the standardized aggression experiments, we chose from each population 21-36 (mean ± SD: 30.17 ± 3.29; Tab. S2.1) similar-sized host colonies. The colony sizes did not differ between 16 out of 17 populations (mean no. of workers ± SD: 30.64 ± 12.86; quasi-poisson GLM: χ^2_{12} = 16.969, P = 0.151) with the exception of New Hampshire, which had smaller colony sizes (mean ± SD: 20.53 ± 11.32; all P < 0.01). However, by excluding New Hampshire from the following analyses the results remained qualitatively the same. Moreover, colony sizes did not differ between host species (poisson GLMM with colony identity, nested in population identity as random factor: χ^2_1 = 1.700, P = 0.192).

To test for aggressive responses towards different opponents we conducted three aggression trials: (1) aggression against a non-nestmate conspecific worker from a non-parasitized host colony, (2) aggression against a *P. americanus* social parasite worker, and (3) aggression against a competing worker from the other *Temnothorax* host species (heterospecific competitor). To rule out carry-over effects (Pamminger et al. 2011), we chose a three day time interval between test (1) and (2) and, on average, a 18 day interval in-between trial (2) and (3). In total, we conducted 1227 aggression experiments (Table S2.1). For trial rounds (1) and (2) we used approximately 30 colonies (mean ± SD: round 1: 29.82 ± 3.13; round 2: 29.76 ± 3.12), whereas the third trial against the competing *Temnothorax* species was conducted only for a subset of colonies per population (mean ± S.D. = 9.0 ± 1.6). We randomly selected an equal number of host colonies from each population per test day. Testing order of colonies was randomized within these days in order to rule out potential test date and time-of-day effects.

We used dead, freshly frozen opponents in order to exclude variance generated by the opponents' behaviour (Crosland 1990; Roulston et al. 2003). Aggression trials with live and dead opponents have been shown to be highly correlated (Modlmeier and Foitzik 2011). The opponent was carefully placed inside the colony, 1 cm from the entrance and all interactions with the opponent were observed every 30 seconds for 5 min. We recorded the number of interacting ants and the following aggressive interactions with the opponent: Stinging, biting, holding, mandible spreading and dragging. Antennation was included as a non-

aggressive interaction. Relative aggression is defined as the proportion of aggressive interactions of all interactions with the opponent.
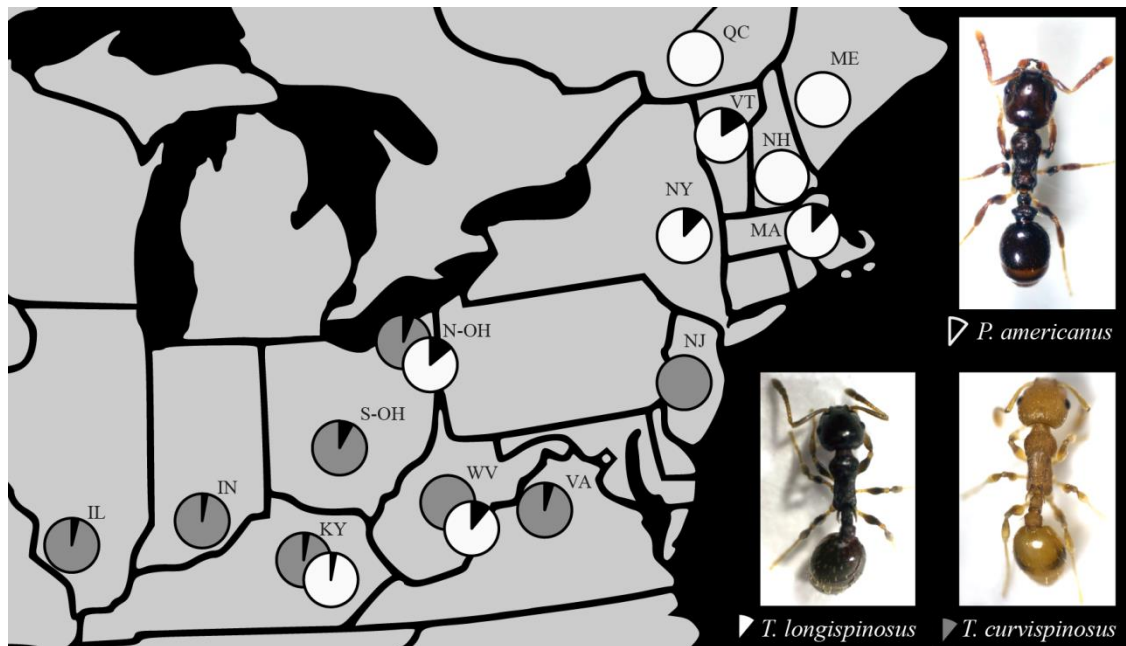

Figure 2.1 Geographic distribution of *Temnothorax longispinosus* (lower left picture) and *T. curvispinosus* (lower right picture) host populations and parasite pressure by the slavemaker ant, *Protomognathus americanus* (upper picture). Host worker ants are only 2-3mm in lengths, the slavemaker is about 20% larger. Pie diagrams represent the relative abundance of parasites and hosts and some are displaced to show both host species. Illinois (IL), Indiana (IN), Kentucky (KY), Maine (ME), Massachusetts (MA), New Hampshire (NH), New Jersey (NJ), New York (NY), Ohio North (N-OH), Ohio South (S-OH), Quebec (QC), Vermont (VT), Virginia (VA), West Virginia (WV). Map adapted from Jongepier et al. 2014, Chapter 3.

Conspecific opponents were chosen from the same population as the tested host colony. As some populations were unparasitized or showed low parasite pressure, most of the host colonies could not be tested against sympatric slavemakers. Thus, we standardized slavemaker origin: slavemaking workers from Illinois- or Ohio-colonies with *T. curvispinosus* slaves were used as opponents for all *T. curvispinosus* colonies and slavemaker workers from New York colonies with *T. longispinosus* slaves for all *T. longispinosus* colonies. *Temnothorax curvispinosus* colonies did not react differently towards slavemakers from Ohio or Illinois (binomial GLMM with colony identity, nested in population identity as random factor: $z = -0.824$, $P = 0.41$). *Temnothorax longispinosus* colonies from New York and *T. curvispinosus* colonies from Ohio were confronted with a sympatric parasite. To test for effects of allopatry vs. sympatry in host responses towards the slavemaker, each of the 64 *T. longispinosus* colonies from New York and West Virginia were tested against both a sympatric and an allopatric slavemaker in random order. The

aggressive responses of *T. longispinosus* colonies from New York and West Virginia did not differ between allopatric and sympatric slavemakers (z = 0.840, P = 0.401), supporting earlier studies which showed an absence of local adaptation (Brandt and Foitzik 2004; Foitzik et al. 2001). Moreover host colonies of all populations did not react differently aggressive towards slavemakers from either New York or West Virginia (i.e. NY or WV; binomial GLMM with colony identity, nested in population identity as random factor: z = -0.903, P = 0.367). The origin of the competing *Temnothorax* ant was also standardized, as most ant communities did not harbour both host species. As in our choice of slavemakers, we chose opponents either from New York (*T. longispinosus*) or from Ohio South (*T. curvispinosus*).

Statistics

We assessed whether the hosts′ aggressive responses were related to parasite pressure, opponent species, or host species using a generalized linear mixed model (GLMM; lmer function implemented in the lme4 R-package; Bates et al. 2014) with binomial distribution and logit link function. We fitted the relative aggression (i.e. the total number of aggressive workers versus the total number of non-aggressive workers that interacted with the opponent) as dependent variable. Parasite pressure, opponent species (conspecific, parasite or competing *Temnothorax* species) and host species (*T. longispinosus* or *T. curvispinosus*) as well as their interactions were included as fixed predictors. We fitted host colony identity, nested in population identity as random factors to account for pseudo-replication, as well as an observation level random factor to control for overdispersion (overdispersion parameter = 5.37).

To rule out potentially confounding effects of (1) host colony density on aggression towards a conspecific and (2) that of co-occurrence of the two host species on aggression towards competing *Temnothorax* species, we repeated the analyses of the effect of parasite pressure, species and their interaction on (1) aggression towards conspecifics, further including host density and its interaction with host species, and (2) aggression towards competing *Temnothorax* species, further including host sympatry and its interaction with host species (Tab. S2.3). For model selection, we used a backwards, stepwise selection procedure (α = 0.05). All analyses were performed in R version 3.0.2 (R Core Team 2013).

RESULTS

Parasite pressure mediates host aggression

Parasite pressure differentially affected the host's aggressive responses towards the different types of opponents (parasite pressure – opponent species interaction: χ^2_1= 19.352, P < 0.001). Host aggression against a conspecific opponent increased

with parasite pressure (z = 4.451, P < 0.001; Fig. 2.2a) and this positive relationship was independent of population density (Table S2.2). Hence, the relationship between parasite pressure and intraspecific antagonistic interactions was not driven by variation in density, but parasite pressure. In contrast, host aggression towards the parasite (z = 0.591, P = 0.555; Fig. 2.2b) or a competing *Temnothorax* species (z = -1.336, P = 0.182; Fig. 2.2c) was unrelated to parasite pressure. Taking host sympatry into account did not alter the relationship between parasite pressure and aggression towards a competing *Temnothorax* species (Table S2.3).
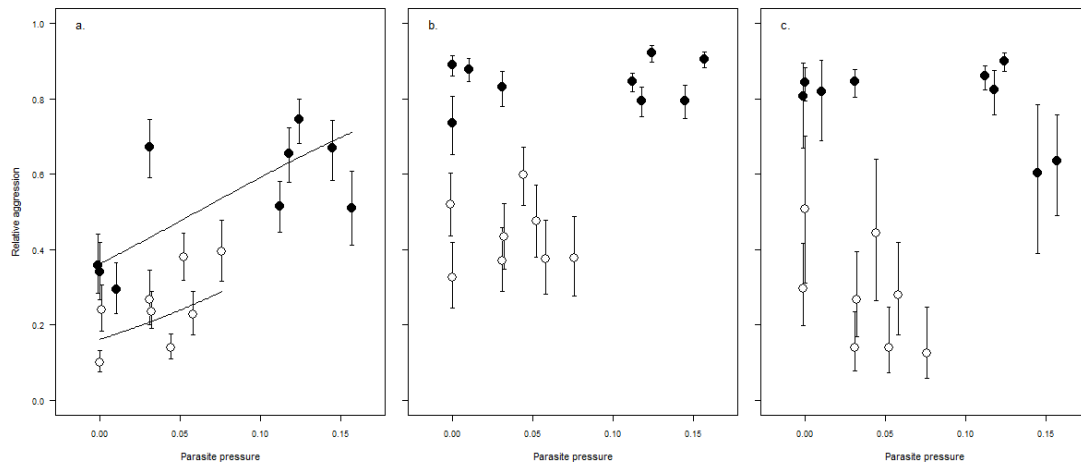

Figure 2.2 *Temnothorax* host colony aggression (proportion of aggressive interactions) in relation to parasite pressure (proportion of slavemaker colonies per host colony) exerted by the social parasite *Protomognathus americanus* across 17 host populations. a) Colony aggression towards a non-nestmate conspecific, b) colony aggression towards a *P. americanus* slavemaker, and c) colony aggression towards a competing *Temnothorax* species (either *T. longispinosus* or *T. curvispinosus*). Symbols represent the back-transformed logit-mean ± s.e. per population; solid symbols depict *T. longispinosus* populations, open symbols *T. curvispinosus* populations. Some symbols are slightly offset for clarity. Regression lines are derived from the following GLMM estimates and back-transformed to the original data scale. a) *Temnothorax curvispinosus*: est ± s.e. = 9.73 ± 2.11, z = 4.61, P < 0.001; *T. longispinosus*: 9.34 ± 2.10, z = 4.45, P < 0.001.

Differences in aggression between host species

The mediating role of parasite pressure on host aggressive responses was consistent across the two *Temnothorax* host species (host species – parasite pressure – opponent species interaction: χ^2_2 = 4.393, P = 0.112; host species – parasite pressure interaction: χ^2_2 < 0.001, P > 0.999). Nonetheless, the two host species differed in their aggressive responses in the absence of parasites (i.e. the intercepts in Fig. 2.2). *Temnothorax longispinosus* was more aggressive than *T. curvispinosus*, regardless of the opponent species (host species difference in aggression towards a conspecific: z = 4.611, P < 0.001; a parasite: z = 10.340, P <

0.001; and a competing *Temnothorax* species: $z = 7.815$, $P < 0.001$). Moreover, host species differed in their aggressive responses towards the different opponent species (host species - opponent species interaction: $\chi^2_2 = 19.671$, $P < 0.001$). *Temnothorax longispinosus* was as aggressive towards a worker of the competing *Temnothorax* species as towards the social parasite ($z = 1.830$, $P = 0.854$), whereas *T. curvispinosus* was more aggressive towards the latter ($z = -2.637$, $P = 0.008$). Both host species showed less aggression towards a conspecific host than towards a parasite (*T. longispinosus*: $z = 10.709$, $P < 0.001$; *T. curvispinosus*: $z = 7.425$, $P < 0.001$) or a competing *Temnothorax* species (*T. longispinosus*: $z = 6.598$, $P < 0.001$; *T. curvispinosus*: $z = 2.192$, $P = 0.028$).

DISCUSSION

Our study demonstrates that variation in social parasite pressure does not influence anti-parasite responses of hosts, but rather affects antagonistic interactions between host colonies of the same species. Across the geographic range of two host species, aggressive responses towards the social parasite were unrelated to its abundance. Rather, aggression towards conspecifics increased with parasite pressure, whereas aggressive responses towards a competing *Temnothorax* species, occupying a similar ecological niche, were unrelated to parasite pressure. The two hosts exhibited similar patterns of association of antagonistic behaviour with parasite pressure, which is indicative of convergent evolution in host behavioural responses and suggests a mediating role of parasites for intraspecific interactions.

Similar to hosts of avian brood parasites (Guigueno and Sealy 2011), *Temnothorax* ants use open aggression to fend off attacks of their social parasite, and colonies responding more aggressively to conspecifics fare better during slave raids (Pamminger et al. 2012; Kleeberg et al. 2014, Chapter 1). Why is aggression against conspecifics an important anti-parasite defence? Enslaved host workers participate in slave raids and many host colonies´ casualties stem from fights with slaves (Foitzik et al. 2001). Therefore, we would expect host colonies to show high aggression levels against conspecifics in parasitized communities. Indeed, in both species, aggression levels increase with parasite pressure. Moreover, host colonies in parasitized populations might be under selection to develop effective discrimination abilities to recognize enemies reliably (Delattre et al. 2012; Fürst et al. 2012). The increase in aggression towards conspecifics might therefore be in part explained by improved recognition capabilities of colonies from parasitized communities.

Beside parasite pressure, other ecological factors could influence the evolution of aggression. Within a *T. longispinosus* population, more aggressive colonies were found in high density locales (Modlmeier and Foitzik 2011). This

evidence and the fact that slavemakers can only persist in dense host populations (Brandt et al. 2005a), would suggest covariance between host density and parasite pressure across populations. However, because we could not find such an association, our data demonstrate that the mediating role of parasite pressure is not driven by differences in population densities. Moreover, other environmental factors are unlikely to explain the inter-population variation in aggression as the two host species show a divergent distribution of parasite pressure over their geographic ranges (Fig. 2.1). The primary host, *T. longispinosus*, is found in the north-eastern part and at higher elevations, whereas *T. curvispinosus* predominates at warmer sites. The slavemaker *P. americanus* occurring at the centre of the hosts' combined geographical distributions is rare or absent in northern *T. longispinosus* and southern *T. curvispinosus* populations (Pennings et al. 2011; Jongepier et al. 2014, Chapter 3). In communities in which the two hosts co-occur, exploitation by the slavemaker is biased towards the primary host, *T. longispinosus* (Brandt and Foitzik 2004). The inverted distribution of the two hosts and differences in parasite pressure in sympatric host populations renders it unlikely that environmental conditions dictate the distribution of host defences. Rather, convergence in behavioural defence traits across species can be explained by host-parasite interactions.

The less aggressive responses to conspecifics in our *Temnothorax* populations under low parasite pressure suggest that high aggression levels are associated with costs. Fitness costs of highly aggressive colonies could include the availability of a smaller workforce for social tasks, higher energy demands due to constant alert, or frequent fights and higher injury or death rates (Wilson 1970; Gobin et al. 2003; Georgiev et al. 2013). *Temnothorax* ants do not defend food sources or territories (Heinze et al. 1996), suggesting that aggression is utilized primarily for nest-site (Foitzik and Heinze 1998) and anti-parasite defence (Pamminger et al. 2011; Kleeberg et al. 2014, Chapter 1). Assuming that aggression simultaneously maintains a more fine-tuned recognition system, another possible cost could include recognition errors. High aggression could lead to elevated rejection errors, if an ant's acceptance threshold is too restrictive resulting in the rejection of nestmate workers (Reeve 1989).

In accordance with earlier studies showing enemy recognition (Alloway 1990; Scharf et al. 2011b), host colonies of both *Temnothorax* species reacted highly aggressively towards the slavemaking ant, independent of parasite pressure. Nevertheless, the host species differed in their aggression level towards the parasite, with the more heavily parasitized host *T. longispinosus* (Brandt and Foitzik 2004) showing the most aggressive responses towards a dead slavemaker. Interestingly, when confronted with invading slavemakers, *T. curvispinosus* colonies are better able to prevent the slavemaker scout from escaping to recruit nestmates and slaves and suffer lower fitness costs during raids (Brandt and Foitzik 2004; Jongepier et al. 2014, Chapter 3). This explains why the slavemaker prefers

T. longispinosus with its less coordinated behavioural defences (Brandt and Foitzik 2004). The response of host colonies towards a dead slavemaker was independent of parasite pressure. This result contrasts with our earlier study (Jongepier et al. 2014, Chapter 3), demonstrating that the number of workers involved in collective aggression against a live slavemaker decreased with parasite pressure. This difference could be explained by the parasite 's use of the Dufour's gland secretion, which elicits chaos and fights among defenders (Brandt et al. 2006; Bauer et al. 2009), a subject which we address in a parallel study (Jongepier E, Kleeberg I, Foitzik S, pers. comm., Chapter 4). In our current study, we deliberately used dead opponents to focus on the hosts' response to the parasite's chemical cues. But why are hosts more aggressive towards their social parasite than towards conspecifics (Alloway 1990; Scharf et al. 2011b), yet this response is unrelated to parasite pressure? One possible explanation could be the absence of costs of this defence in parasite-free populations. Alternatively, the high aggression towards the parasite could be a remnant from a parasitized past (Foitzik et al. 2003; D´Ettorre et al. 2004a).

The two host species reacted differently towards the competing *Temnothorax* species. *Temnothorax curvispinosus* responded with more aggression towards the social parasite *P. americanus* than towards the competing *T. longispinosus*. This indicates, besides improved coordinated defence behaviour (Jongepier et al. 2014, Chapter 3), better recognition abilities of the less preferred host. *Temnothorax longispinosus* was equally aggressive towards the parasite and the competitor, even though earlier studies on single populations revealed higher aggression against the slavemaker (Pamminger et al. 2011; Scharf et al. 2011b). Moreover, the aggressive response was unrelated to parasite pressure indicating that at least some populations show a less fine-tuned enemy recognition.

Some defensive strategies against social parasitism shift along a parasite pressure gradient and we found that these behavioural changes are consistent across the geographic range of the two host species that share a parasite. Interestingly, only aggression against conspecifics, which could represent enslaved hosts aiding in a slavemaker raid, co-varied with parasite pressure; aggression against workers of different species, either the social parasite itself or a competitor, did not. Possibly the absence of costs or remnants of a parasitized past can explain the high aggression towards the social parasite. In any case our study demonstrates that co-evolution between hosts and parasites, does not affect behavioural responses towards heterospecific competitors, but can shape intraspecific interactions.

ACKNOWLEDGEMENTS

SUPPORTING INFORMATION

Table S2.1 Collection sites and details for the 17 *Temnothorax* host populations. Parasite pressure was calculated by examining the number of slavemaking colonies with slaves of this host species per host colony (Jongepier et al. 2014).

Community	Coordinates	Collected colonies	con-specific	parasite	hetero-specific	Parasite pressure
Temnothorax curvispinosus						
Illinois	38°13′37.2′′N 89°44′58.0′′W	114	30	29	10	0.04
Indiana	38°11′52.8′′N 86°38′09.8′′W	94	30	30	11	0.03
Kentucky	37°48′07.8′′N 83°41′50.0′′W	161	30	30	10	0.03
New Jersey	40°00′25.2′′N 74°50′06.0′′W	119	31	30	11	0.00
Ohio North	41°50′20.4′′N 80°57′33.4′′W	156	31	30	11	0.06
Ohio South	40°14′13.8′′N 82°59′06.5′′W	594	24	24	06	0.08
Virginia	38°50′03.0′′N 78°11′09.6′′W	96	30	31	09	0.05
West Virginia	38°06′28.8′′N 80°07′52.9′′W	124	31	30	09	0.00
Temnothorax longispinosus						
Kentucky	37°48′07.8′′N 83°41′50.0′′W	65	21	21	07	0.03
Maine	44°08′33.0′′N 70°51′21.1′′W	104	30	30	09	0.00
Massachusetts	42°23′58.8′′N 71°11′40.7′′W	97	29	30	10	0.12
New Hampshire	43°26′24.6′′N 72°09′20.8′′W	105	30	30	05	0.00
New York	42°31′57.0′′N 74°08′45.2′′W	963	30	31/28*	08	0.12
Ohio North	41°50′20.4′′N 80°57′33.4′′W	99	33	33	09	0.14
Quebec	45°30′22.2′′N 73°36′56.8′′W	111	31	31	10	0.00
Vermont	43°58′15.0′′N 73°04′18.9′′W	102	30	30	09	0.16
West Virginia	38°06′28.8′′N 80°07′52.9′′W	113	36	36/33*	09	0.11

* First number refers to sympatric and second to allopatric parasite

Table S2.2 Model selection results from the generalized linear mixed model analysing relative colony aggression of *T. longispinosus* and *T. curvispinosus* against a non-nestmate conspecific ant in relation to parasite pressure and population density. Statistics indicated in bold were retained in the final model. All Δd.f. = 1.

Predictors	*Relative aggression towards conspecifics* χ2	P
Parasite pressure	11.433	**<0.001**
Species	9.555	**0.001**
Population density	0.201	0.654
Species x Population density	4.007	**0.045***
Species x Parasite pressure	0.005	0.943

**Temnothorax curvispinosus*: est ± s.e. = -0.13 ± 0.06, z = -2.105, P = 0.03; *T. longispinosus*: est ± s.e. = 0.02 ± 0.03, z = 0.622, P = 0.534

Table S2.3 Model selection results from the generalized linear mixed model analysing relative colony aggression of *T. longispinosus* and *T. curvispinosus* against a competing host ant in relation to parasite pressure and host sympatry. Statistics indicated in bold were retained in the final model All Δd.f. = 1.

Predictors	*Relative aggression towards competing Temnothorax ants* χ2	P
Parasite pressure	2.640	0.104
Species	28.462	**< 0.001**
Host sympatry	0.938	0.333
Species x Host sympatry	0.248	0.619
Species x Parasite pressure	0.782	0.376

Chapter **3**

Collective defence portfolios of ant hosts shift with social parasite pressure

Evelien Jongepier, **Isabelle Kleeberg**, Sylwester Job and Susanne Foitzik

Author contributions: EJ, IK and SJ collected the data and EJ analysed it. EJ wrote the first draft and EJ, IK and SF revised it until completion and responded to reviewer comments.

ABSTRACT

Host defences become increasingly costly as parasites breach successive lines of defence. Because selection favours hosts that successfully resist parasitism at the lowest possible cost, escalating coevolutionary arms races likely drive host defence portfolios towards ever more expensive strategies. We investigated the interplay between host defence portfolios and social parasite pressure by comparing 17 populations of two *Temnothorax* ant species. When successful, collective aggression not only prevents parasitation but also spares host colonies the cost of searching for and moving to a new nest site. However, once parasites breach the host's nest defence, host colonies should resort to flight as the more beneficial resistance strategy. We show that under low parasite pressure, host colonies more likely responded to an intruding *Protomognathus americanus* slavemaker with collective aggression, which prevented the slavemaker from escaping and potentially recruiting nestmates. However, as parasite pressure increased, ant colonies of both host species became more likely to flee rather than to fight. We conclude that host defence portfolios shift consistently with social parasite pressure, which is in accordance with the degeneration of frontline defences and the evolution of subsequent anti-parasite strategies often invoked in hosts of brood parasites.

Keywords: Host-parasite interaction; Defence portfolios; Brood parasites; Social insects; Frontline defences

INTRODUCTION

Why do some organisms express a multitude of defence strategies against exploiters, whereas others fail to employ seemingly adaptive defences? This long-standing question in evolutionary biology led to the development of the strategy blocking hypothesis, which poses that proficiency in one defensive strategy relaxes selection on other traits and thereby inhibits the evolution of further lines of defence (Planqué et al. 2002; Britton et al. 2007). Theory suggests that once the first line of defence is breached by exploiters, selection favours victims that mount further defences. This could result in complex defence portfolios, deployed in hierarchical sequence (Svennungsen and Holen 2010; Kilner and Langmore 2011). Consequently, in host-parasite systems, the evolution of host defences may not only depend on attributes of parasites, such as parasite pressure and their degree of specialisation, but also on the efficacy of other traits in the adaptive portfolios of hosts and parasites (Langmore and Kilner 2010; Kilner and Langmore 2011). Recent modelling approaches conclude that victims have unique evolutionary advantages when coevolution involves multiple traits in hosts and parasites (Gilman et al. 2012). These findings highlight the need for integrative studies on host defence portfolios to understand the trajectories and outcomes of co-evolutionary arms races.

In systems where parasites exploit the brood care behaviour of their host, defences that are expressed prior to parasitation spare the host costly investment in parasitic young (Feeney et al. 2012). Such frontline defences thus have the greatest potential to minimize the costs inflicted by parasites, while parasite attack becomes increasingly costly as successive lines of defence are breached (Feeney et al. 2012; Spottiswoode et al. 2012). Because selection favours hosts that successfully defend themselves at the lowest possible cost, the temporal sequence in which defences are employed is thought to reflect the order in which they evolved (Langmore and Kilner 2010). For instance, most examples of chick rejection - the most costly defence mode against avian brood parasites – occur in hosts with a long history of exploitation, such as those of the evolutionarily old Bronze Cuckoo, which breached preceding defences (Spottiswoode et al. 2012). Thus, with increasing evolutionary age of brood parasite-host associations, anti-parasite strategies appear to shift from frontline defences to those expressed later.

According to this rationale, frontline defences, that circumvent exploitation altogether (Feeney et al. 2012), mark the first phase in the evolutionary arms race between parasites and hosts. Parasites are then under selection to counter these defences. During the next phase, parasites breach the first defence line, potentially causing its decay. Simultaneously, hosts are under selection to mount further defences. If parasites are rare and reciprocal selection pressures are weak, the arms-race may not proceed beyond the first phase. However, at co-evolutionary hotspots, further escalation of the arms-race could result in counter adaptations by

the parasite, the degeneration of the first line of defence and the evolution of subsequent defensive strategies. Hence, over the geographic range of host species, parasite pressure is predicted to be associated with a hierarchy of defence strategies.

Although the evolutionary principles governing adaptive portfolios are generally applicable to exploiter-victim systems (Britton et al. 2007), the concept has almost exclusively received attention in avian brood parasites and their hosts (Langmore and Kilner 2010; Kilner and Langmore 2011; Feeney et al. 2012). Nonetheless, hosts of insect social parasites and avian brood parasites show striking similarities in the trajectories and outcomes of their co-evolutionary arms races (Kilner and Langmore 2011). Like avian hosts, social insects exhibit a range of morphological, behavioural and physiological adaptations to social parasites, which often co-occur in a single host species (Alloway 1990; Foitzik et al. 2001; Brandt et al. 2005a; Achenbach and Foitzik 2009; Pamminger et al. 2011). The apparent depth of these defence portfolios renders social parasitic hosts particularly suitable targets in the study of host defence portfolios (Kilner and Langmore 2011).

Contrary to avian brood parasites, social parasites target and exploit the brood care behaviour of entire societies (Hölldobler and Wilson 1990). The social life-style of the ant, wasp or bee host allows for the evolution of collective defences, which likely attenuate asymmetries in individual competitive ability of parasites and hosts. Indeed, organized group defences are one of the most characteristic features of insect societies and greatly contribute to their ecological success (Hermann 1984). Collective host defences can be represented by fight or flight behaviours (Wilson and Regnier 1971; Hölldobler and Wilson 1990). Hosts that can evade parasitism through aggressive nest defence avoid the costs associated with giving up their nest site, which is often a limited resource. Hence, aggression, as a first line of defence, is likely to convey the largest fitness benefits (Feeney et al. 2012). However, once parasites breach the host's nest defence, flight may remain the only beneficial mode of resistance.

The obligate social parasite *Protomognathus americanus* is an evolutionarily old parasite that exploits the brood care behaviour of its *Temnothorax* hosts (Beibl et al. 2005). Through regular, destructive raids, these slavemakers replenish their slave workforce by capturing the host's brood (Alloway 1979). Slave raids are preceded by a scouting event during which a single slavemaker worker discovers and inspects a host nest and returns to its colony to recruit nest mates. The slave raid that follows is often initiated by only one or few slavemakers, which recruit nestmates before or during the raiding attack. As host workers and the queen are often killed during a raid, few colonies survive a slavemaker attack (Foitzik and Herbers 2001; Blatrix and Herbers 2003). Hence, frontline defences, directed to fend off slavemaker scouts or raiding parties, are likely to be selected for. Indeed,

Temnothorax hosts exhibit both fight and flight behaviour during antagonistic interactions with slavemakers, both of which reduce the costs slavemakers inflict on their host (Wesson 1939; Alloway 1990; Foitzik et al. 2001; Brandt et al. 2005a; Pamminger et al. 2011).

Here, we investigate how host defence portfolios changed with social parasite pressure across 17 populations of two *Temnothorax* host species. Collection sites span most of the geographical range of the slavemaker and the two host species and include unparasitized host populations. Specifically, we ask whether fight and flight responses towards the introduction of a slavemaker ant into a host colony change according to parasite pressure in the population from which the host originated. We hypothesize that in populations where the slavemaker is rare or absent, hosts resort to collective aggressive nest defence, as the first line of defence. However, in highly parasitized populations, further escalation of the co-evolutionary arms race may have led to the expression of nest evacuation as a further defence strategy down the hierarchy. Hence, we predict that host defence portfolios shift from collective fight to flight behaviours with increasing parasite pressure.

METHODS

Colony collection and maintenance

From May to July 2012, we collected 3463 *Temnothorax longispinosus*, *T. curvispinosus* and *Protomognathus americanus* ant colonies from a total of 17 host populations from 14 sites in the United States and Canada (Fig. 3.1; Table S3.1). From each population we sampled ~100 colonies or more with the exception of the *T. longispinosus* population from Kentucky (65 colonies) due to the low local abundance of this species. Ants were collected shortly before the annual raiding season, which takes place between July and September. Hence, even in parasitized populations, colonies did not have slavemaker contact for at least one year. All colonies were counted, transferred to artificial glass nest sites (cavity size: 50 x 10 x 3 mm) and kept at a constant 25º C and a 12h : 12h light / dark cycle. They were housed in plastered nest boxes (10 x 10 x 3 cm) to prevent desiccation and fed weekly with honey and cricket. Ant collection permits were either obtained from parks/preserves or we asked private land owners for permission to collect ant colonies. Import and export licences are not required for the transport of our study species. We followed the guidelines of the Study of Animal Behaviour and the legal and institutional rules.
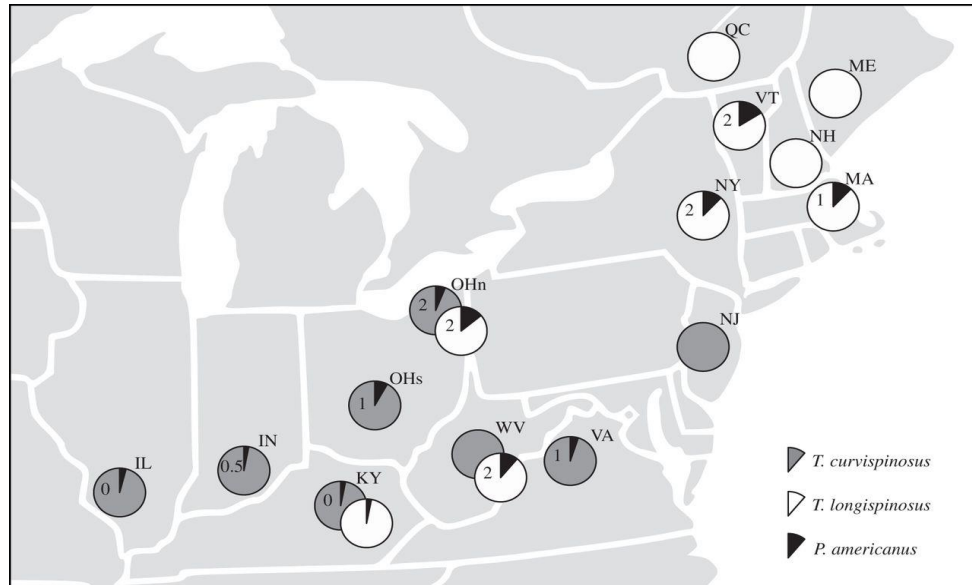
Figure 3.1 Distribution of experimental *Temnothorax* host populations and parasite pressure by the slavemaker ant *P. americanus*. Pie diagrams and numbers represent parasite prevalence and median slavemaker colony sizes (i.e. the median number of slavemaker workers), respectively, in Illinois (IL), Indiana (IN), Kentucky (KY), Maine (ME), Massachusetts (MA), New Hampshire (NH), New Jersey (NJ), New York (NY), Ohio North (OHn), Ohio South (OHs), Quebec (QC), Vermont (VT), Virginia (VA) and West Virginia (WV). Details on collection sites are provided in Table S1.

Parasite pressure estimates

Parasite pressure was estimated by i) parasite prevalence (i.e. the number of slavemaker colonies relative to the number of host colonies) and ii) median slavemaker colony size (i.e. the median number of slavemaker workers per slavemaker colony, the queen was not included in this count). Parasite prevalence reflects the likelihood of being attacked by a slavemaker whereas the median slavemaker colony size is indicative of the potential raiding party size. The latter is important because the decision to fight or flee is taken before host colonies have reliable information on the size of the raiding party, for instance because they face a scout or the first member of the raiding party. For median slavemaker colony size we assigned a value of zero to populations where the slavemaker was absent (excluding these populations from the analyses yielded qualitatively similar results).

Coevolutionary dynamics play out over long time-scales in species with long generation times such as *Temnothorax* ants, in which queens can live for several decades (Keller 1998). Whenever possible, we therefore included long-term collection data on parasite pressure from previously studied communities (i.e. Ohio

North, West Virginia, Vermont and New York; (Herbers and Foitzik 2002; Brandt and Foitzik 2004; Foitzik et al. 2004; Foitzik et al. 2009)).

Moreover, we have evidence for consistent parasite occurrence from some other communities that have been sampled sporadically in the past (i.e. *T. longispinosus* population from Massachusetts and *T. curvispinosus* populations from West-Virginia, Virginia and Ohio South; S. Foitzik, personal observation). Where available, long-term parasite pressure estimates were used in our analyses. Current parasite pressure and its relationship with host defences are reported in the supplementary material and Table S3.1.

Behavioural experiments

From each population, 32.0 ± 3.3 (mean ± s.d; Table S3.1), average-sized host colonies were selected for standardized fight-flight experiments. Colony sizes did not differ between *T. longispinosus* and *T. curvispinosus* colonies (Poisson GLMM with colony ID, nested in population ID as random factor: $\chi^2 = 2.88$, $P = 0.090$). Host colony sizes did not differ between 16 out of the 17 host populations (quasi-Poisson GLM: $F = 1.37$, Δd.f. = 15, $P = 0.157$). The exception was *T. longispinosus* colonies from New Hampshire, which were smaller than those from the other 16 populations (all $P < 0.005$). Excluding New Hampshire from all following analyses yielded qualitatively the same results.

For the experiments, a living *P. americanus* slavemaker worker was introduced into a host colony and the nest entrance was sealed for one hour. Upon opening the nest entrance, we recording the number of host workers individually attacking the slavemaker (i.e. biting or stinging) as well as the number of workers involved in collective slavemaker immobilization (i.e. holding). During the latter, multiple host workers immobilize the slavemaker by holding its legs and antennae in their mandibles for prolonged periods of time. Because a single ant is physically not strong enough to hold the larger slavemaker, collective immobilization requires cooperation between host workers. The holding behaviour exhibited by workers immobilizing the slavemaker distinguishes itself from biting (i.e. a form of individual attack) where workers show brief but forceful snapping with the mandibles. Although individual attack may harm the slavemaker, it does not necessarily prevent it from escaping and potentially recruiting nest mates. Contrastingly, collective immobilisation frequently causes dismemberment and subsequent death of the slavemaker and therefore can prevent the recruitment of a raiding party.

Host colonies were monitored for nest evacuation and slavemaker escape during the six hours following slavemaker introduction. Slavemaker escape status was assigned based on whether or not the slavemaker was able to leave the colony physically unharmed, as an unharmed scout is likely to return to its colony to recruit nest mates and initiate a slave raid. Preliminary tests showed that colony

evacuation status or slavemaker escape status did not change from six to 24 hours. Experiments were conducted at 25° C from August to September 2012, which coincides with the raiding season of *P. americanus* colonies. To eliminate potential test date and time-of-day effects, we randomly selected an equal number of colonies from each population per test day and randomized test order within test days.

Slavemaker origin

All slavemakers originated from colonies containing slaves of the species they were tested against. Since several host populations were unparasitized and could thus not be tested against a sympatric slavemaker, we standardized slavemaker population of origin. Hereto, we only used slavemakers from New York against *T. longispinosus* colonies and slavemakers from either Ohio South (N = 199) or Illinois (N = 58) against *T. curvispinosus* colonies. Colony evacuation probability (binomial GLM: Δdeviance = -1.87, Δd.f. = 1, P = 0.172) and the number of immobilizing workers (quasi-Poisson GLM: $F_{1,252}$ = 1.57, P = 0.211) did not differ between colonies facing a slavemaker from Ohio or Illinois.

Temnothorax longispinosus colonies from New York and *T. curvispinosus* colonies from Ohio were confronted with a sympatric slavemaker whereas colonies from the remaining populations faced allopatric slavemakers. To assess whether slavemaker sympatry affected colony responses, we tested each of the 64 experimental *T. longispinosus* colonies from New York and West Virginia against both a sympatric and an allopatric slavemaker in random order with a seven day interval. As in previous behavioural studies (Foitzik et al. 2001; Brandt and Foitzik 2004), we found no effect of slavemaker sym- or allopatry (GLMMs with colony identity, nested in population identity as random factor: number of aggressive workers: χ^2 = 0.01, P = 0.931; evacuation probability: χ^2 = 0.048, P = 0.826).

Statistics

We assessed whether collective defences to an intruding slavemaker and the efficiency of such responses were associated with parasite pressure, host species identity and host colony size. Hereto, we analysed the likelihood of collective immobilization (i.e. the holding of a slavemaker by more than one host worker), colony evacuation and slavemaker escape using generalized linear mixed models (GLMM; lmer function implemented in the lme4 package (Bates et al. 2014)) with binomial error distribution and logit link function. In addition, we tested for differences in individual and collective aggressive defences by analysing the number of workers either attacking or immobilizing the slavemaker. Because there is only a quantitative difference between holding by a single worker and immobilization by multiple workers, we assessed all instances of holding, including those where only a single worker was involved. Holding by a single worker was however rare, as 93% of the 329 holding events involved multiple workers. For these analyses we used a set of GLMMs (Bates et al. 2014) with Poisson error

distribution and log link function. The effect of parasite pressure was evaluated using separate analyses of parasite prevalence and median slavemaker colony size, as these estimates of parasite pressure were highly correlated (Spearman-ρ = 0.76, S = 69.11, P = 0.004). In all analyses, the parasite pressure measure, species identity and their interaction were included as fixed predictors, as was host colony size. Colony identity, nested in population identity was included as random factor to account for pseudo-replication. Only non-evacuating colonies suffer from allowing a slavemaker to escape, recruit and return to raid the colony. Hence, we excluded evacuating host colonies from the analysis of slavemaker escape status.

Analyses including parasite prevalence and slavemaker colony size were based on all 17 and a subset of 16 host populations, respectively, because colony sizes of slavemakers with *T. longispinosus* slaves from Kentucky were not recorded. For all analyses we used a backwards-stepwise procedure for model selection (α = 0.05). Model selection tables are provided in the supplementary tables. All analyses were performed in R version 3.0.0 (R Core Team 2014).

RESULTS

Defence portfolios and parasite pressure

Host defence portfolios shifted from collective fight to flight with parasite pressure (Fig. 2). Both the likelihood of collective slavemaker immobilization (estimate ± s.e. = -4.36 ± 2.05, z = -2.13, P = 0.033) and the number of immobilizing host workers (Fig. 3.2a, P = 0.004) decreased with parasite prevalence. Contrastingly, the evacuation probability of host populations increased with parasite prevalence (Fig. 3.2c; P = 0.004). Thus, host populations that are under severe parasite pressure are more likely to flee rather than fight when they encounter a slavemaker in their nest, which is supported by a strong negative relationship between colony evacuation probability and the number of immobilizing workers (estimate ± s.e. = -0.499 ± 0.074, z = -6.78, P < 0.0001).

Contrary to collective fight and flight responses, individual aggressive defences (i.e. the number of attacking workers) were unrelated to parasite prevalence (χ^2 = 0.97, Δd.f. = 1, P = 0.324). Parasite prevalence was not associated with the likelihood that the slavemaker escaped (χ^2 = 0.08, Δd.f. = 1, P = 0.780).
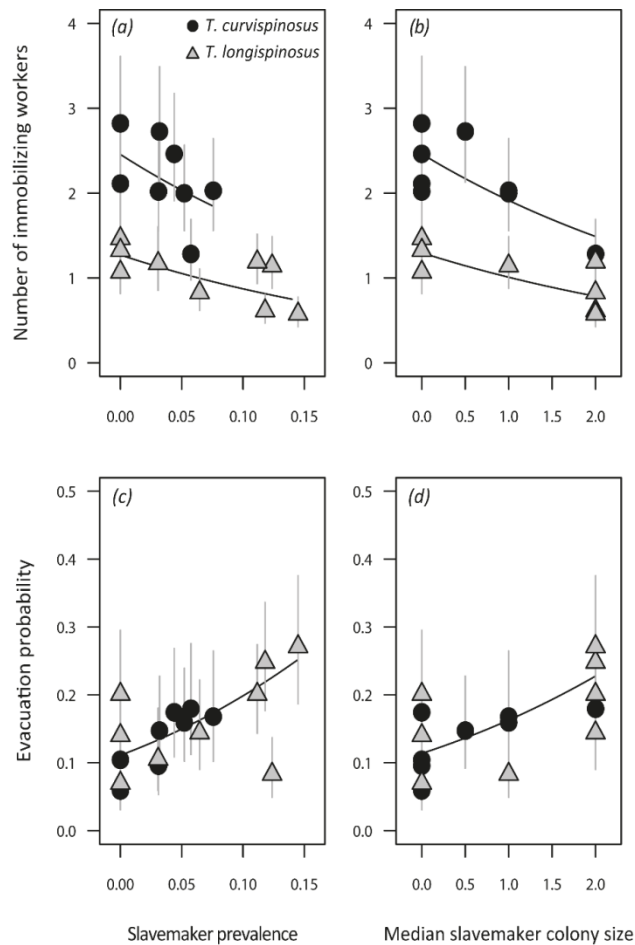
Figure 3.2 Collective host defences in relation to social parasite pressure. Parasite pressure is represented by the parasite prevalence *(a,c)* and the median slavemaker colony size *(b,d).* Symbols represent the estimate ± s.e. per population, standardized for average host colony size (i.e. population estimates + colony size estimate × average host colony size). Regression lines are derived from the following GLMM estimates and back-transformed to the original data scale. *(a)* Estimate ± s.e. = −3.77 ± 1.31, z = −2.89, *P* = 0.004; *(b)* −0.25 ± 0.07, z = −3.49, *P* < 0.001; *(c)* 6.84 ± 2.40, z = 2.85, *P* = 0.004 and *(d)* 0.42 ± 0.14, z = 3.02, *P* = 0.003.

Species differences in defence portfolios

Changes in host defence strategies with parasite prevalence were consistent across the two hosts, indicated by the absence of interaction effects with species identity (all *P* > 0.05; see supplementary tables for model selection results). Nonetheless, the two host species resorted to different aggressive defence strategies (Fig. 3.3a-b), independent of parasite pressure. The collective defence of immobilizing the slavemaker with multiple host workers more often occurred in *T. curvispinosus* than in *T. longispinosus* colonies (estimate ± s.e. = 0.810 ± 0.202, z = 4.01, *P* < 0.0001) and more workers were involved in slavemaker immobilization in *T. curvispinosus* (Fig. 3.3a; *P* < 0.0001). Contrastingly, *T. longispinosus* colonies primarily showed

individual attack (Fig. 3.3b; $P < 0.0001$). Slavemaker escape probability decreased with the number of immobilizing host workers (estimate ± s.e. = -0.46 ± 0.05, z = -9.64, $P < 0.0001$), but was unrelated to the number of attacking host workers (z = 0.01, P = 0.990). Hence, slavemakers were more likely to escape from *T. longispinosus* colonies (Fig. 3.3c; $P < 0.0001$). Host species did not differ in evacuation probability (χ^2 = 0.05, Δd.f. = 1, P = 0.816).
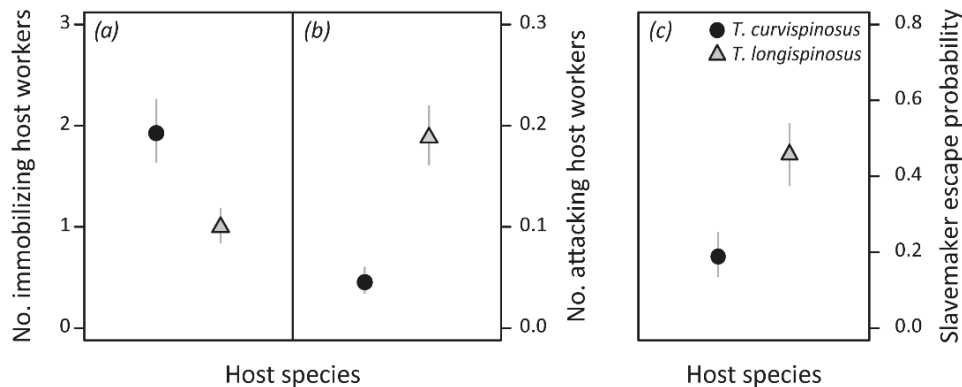
Figure 3.3 Differences in aggressive defence strategies and efficiency between host species. Symbols represent the GLMM estimates ± s.e. *(a) z = −5.45, P < 0.0001; (b) z = 4.54, P < 0.0001; (c) z = 5.30, p < 0.0001.*

Defence strategies and colony size

Large host colonies evacuated less often (estimate ± s.e. = -0.074 ± 0.013, z = -5.60, $P < 0.0001$) and were more likely to show collective immobilization (0.052 ± 0.008, z = 6.21, $P < 0.0001$), in which more workers were involved (0.038 ± 0.004, z = 9.06, $P < 0.0001$). Moreover, large host colonies were less likely to let the slavemaker escape (-0.052 ± 0.009, z = -5.35, $P < 0.0001$).

Contrastingly, host colonies that co-occurred with large slavemaker colonies evacuated more often (Fig. 3.2d; P = 0.003), were less likely to show collective immobilization (-0.324 ± 0.116, z = -2.80, P = 0.005) and fewer workers immobilized the slavemaker (Fig. 3.2b, $P < 0.001$). The number of workers showing individual attack was unrelated to colony sizes of the host (χ^2 = 1.02, Δd.f. = 1, P = 0.312) or the slavemaker (z = 0.62, P = 0.538).

DISCUSSION

We have demonstrated that host defence portfolios shift from collective fight to flight with social parasite pressure over large geographic ranges. Host populations in which the slavemaker is rare or absent more frequently show collective

aggression whereas highly parasitized populations are more likely to respond to an intruding slavemaker by evacuating their nest site. These changes in collective defence strategies were consistent across the two host species, despite clear interspecific differences in the geographic distribution of populations that occurred in sympatry with the slavemaker. This finding renders it unlikely that environmental conditions govern the evolution of defence strategies in the hosts. Rather, convergence in the association between parasite pressure and defence strategies across species suggests universal patterns in host-parasite coevolution. We further found distinct differences in defence portfolios between the two hosts, pointing to a lower efficiency of averting parasitic exploitation by the preferred host *T. longispinosus*.

The strategy blocking hypothesis poses that as long as early lines of defence are effective there is limited selection for subsequent costly defences due to diminishing returns. In accordance we found that host populations that responded to an intruding slavemaker by collective immobilization were less likely to abandon their nest site. Indeed, flight may involve substantial costs as nest sites are known to be limiting for *T. longispinosus* (Herbers 1986). Colonies that can successfully evade parasitation through collective slavemaker immobilization should thus not abandon their nest site, which is indeed what we found. Likewise, avian host species that are highly aggressive towards adult brood parasites are less likely to reject parasitic eggs, for instance through nest desertion (Robertson and Norman 1976; Neudorf and Sealy 1992) (but see (Moksnes, Røskaft, Braa, et al. 1991b; Røskaft et al. 2002)).

Interestingly, our study shows that collective aggression towards the parasite decreased with parasite prevalence. This contrasts with studies on other host-parasite systems which show that hosts facing high parasite pressure exhibit more aggressive defences (Briskie et al. 1992; Lindholm and Thomas 2000; Hale and Briskie 2007; Welbergen and Davies 2009; Thorogood and Davies 2013). Previous studies on *Temnothorax* ants further indicate that parasitized populations are not less aggressive per se. On the contrary, aggression during raiding attacks was more pronounced in host populations that were exposed to high parasite pressure (Foitzik et al. 2001). In addition, colony aggression towards conspecific workers strongly increased with parasite prevalence (Kleeberg et al. 2015, Chapter 2). Our finding that *Temnothorax* hosts do not always employ the aggressive potential revealed in different contexts, would support that collective aggression is disadvantageous as a frontline defence against the social parasite under severe parasite pressure. Moreover, the fact that *Temnothorax* ants from parasitized populations can be highly aggressive in different situations (Kleeberg et al. 2014, Chapter 1; Foitzik et al. 2001) renders it unlikely that low aggression has led to high parasite pressure rather than the other way around. Nonetheless, we cannot rule out that variation in parasite pressure between host populations is the result of differences in host defence strategies and not its cause.

Despite its ubiquity, aggressive host defences are not universal and, in some circumstances, non-adaptive (Zamora-Munoz et al. 2003; Feeney et al. 2012). In avian hosts, the lack of aggression has often been attributed to small host body size which prohibits successful nest defence. Indeed, enemy attack can involve considerable costs and fights only escalate when the strength asymmetry between opponents is small (Savolainen and Vepsäläinen 1988). Aggressive defences may thus be selected against if the chance of winning antagonistic encounters is limited. In our study, large host colonies were more likely to respond with collective immobilization than with nest evacuation when confronted with a slavemaker. We also found the highest collective aggression in colonies originating from populations where slavemaker colonies were typically small. The collective defence of immobilizing a slavemaker is probably disadvantageous when facing a large raiding party, as valuable time and workforce is lost on the retention of one out of multiple opponents, which cannot be utilized for brood or queen rescue. Thus, the inability to win a fight may render evacuation the only feasible option. Analogously, physical constraints to remove a potential threat to avian hosts of brood parasites may leave desertion and re-nesting as the only beneficial mode of defence (Davies et al. 1989; Moksnes, Røskaft, and Braa 1991b; Hosoi and Rothstein 2000; Peer and Sealy 2004).

Although the shift from fight to flight was consistent across host species, the two host species also showed distinct defence strategies towards an intruding slavemaker. *Temnothorax longispinosus* mainly responded by individual attack on the slavemaker, whereas *T. curvispinosus* more often showed collective defence by pinning the slavemaker down. Only the latter strategy reduced the likelihood that the slavemaker escaped and subsequent raiding risk. Brandt and Foitzik demonstrated higher aggression in *T. curvispinosus* towards slavemakers, a higher fraction of slavemakers killed and more brood saved during raiding attacks (Brandt and Foitzik 2004). Such interspecific differences in host defence strategies have also been reported in hosts of brood parasites (Robertson and Norman 1976) and social parasitic hosts (Mori et al. 1995) and may reflect host preference by the parasite (Mori et al. 1995). Although we cannot rule out that differences in defence strategies and efficiencies between our two host species resulted from the use of different slavemaker populations, it could provide a mechanistic explanation for *P. americanus'* preference for its primary host, *T. longispinosus* (Blatrix and Herbers 2003; Brandt and Foitzik 2004).

In theory, evolutionary divergence in defence portfolios could arise in the absence of intrinsic differences between host species, provided they are at different stages in the co-evolutionary arms-race with their parasite (Britton et al. 2007). In practice, however, host species invariably differ in ecology, life-history and morphology, which may impose differential constraints on the evolution of specific host defences (Servedio and Hauber 2006). Such differences may greatly restrict formal tests of the predictions of the strategy blocking hypothesis using

interspecific comparisons. As an alternative we assessed the interplay between parasite pressure and host defence portfolios between multiple populations of the same host species. Such comparisons have proven highly valuable in the study of single-trait pair co-evolutionary arms races (Brodie et al. 2002; Thompson and Cunningham 2002), especially when they cover the entire geographic range of host species, including populations where the parasite is absent (Gomulkiewicz et al. 2007). Nonetheless, intraspecific variation in host defence portfolios has rarely been studied (Lindholm and Thomas 2000), let alone across geographically distant populations.

In conclusion, we demonstrate that host defence portfolios shift consistently along a social parasite pressure gradient. Collective aggression, as a first line of defence against the slavemaker, is less frequently employed by host populations that are under severe parasite pressure. Instead, these populations resort to an alternative collective defence strategy in the form of nest evacuation. Degeneration in the first line of defence and the evolution of subsequent anti-parasite strategies has been invoked in a number of hosts of both brood and social parasites (Kilner and Langmore 2011). However, the present study is the first to demonstrate consistent shifts in host defence portfolios along a social parasite pressure gradient.

SUPPORTING INFORMATION

Defence portfolios and current parasite pressure

Host evacuation probability increased with both the current parasite prevalence (estimate ± s.e. = 2.850 ± 1.091, z = 2.61, P = 0.009) and the current median slavemaker colony size (estimate ± s.e. = 0.194 ± 0.075, z = 2.58, P = 0.010). However, the decrease in collective slavemaker immobilization we observed when taking available long-term parasite pressure estimates into account, could not be shown for current parasite pressure. That is, neither the probability of collective immobilization nor the number of host workers involved in collective immobilization was related to current parasite pressure (probability: χ^2 = 1.03, Δd.f. = 1, P = 0.311; number of workers: χ^2 = 1.80, Δd.f. = 1, P = 0.180) or current median slavemaker colony size (probability: z = -1.01, P = 0.310; number of workers: z = -1.40, P = 0.161).

To assess whether any of the populations that were repeatedly sampled exhibited an atypical response to current parasite pressure, we sequentially removed each of those populations and repeated the analyses of collective immobilization probability and the number of immobilizing workers. This showed that the likelihood of collective immobilization decreased with current parasite pressure when we excluded the *T. longispinosus* from West Virginia (parasite prevalence: estimate ± s.e. = -5.209 ± 2.131, z = -2.44, P = 0.015; median slavemaker colony size: -0.316 ± 0.107, z = -2.94, P = 0.003), but not when we excluded any of the other host populations (all P > 0.05). Likewise, the number of workers involved in collective slavemaker immobilization decreased with current parasite pressure when we excluded the *T. longispinosus* from West Virginia (parasite prevalence: -4.443 ± 1.374, z = -3.23, P = 0.001; median slavemaker colony size: -0.257 ± 0.072, z = -3.67, P < 0.001), but was unrelated to the current parasite pressure when including West Virginia but excluding any of the other populations (all P > 0.05).

These results suggests that, given the current parasite pressure in West Virginia, *T. longispinosus* colonies responded differently towards an intruding slavemaker than the remaining host populations. Comparing long-term and current parasite pressure shows that, in West Virginia, *T. longispinosus* hosts have witnessed a substantial increase in both parasite prevalence (long-term: 0.11, current: 0.35) and median slavemaker colony size (long-term: 2, current: 5). By comparison, parasite pressure remained relatively constant in the other four populations for which long-term data was available (supplementary table S1). This may suggest that *T. longispinosus* defence strategies in West Virginia have been selected for under different conditions that those experienced at present.

Table S3.1 Collection sites and details for 17 *Temnothorax* host populations of the slavemaker *Protomognathus americanus*. Median slavemaker colony sizes refer to the median number of slavemaker workers per colony for each of the populations. Median slavemaker colony sizes of 0 indicate that the majority of colonies in the population contained only a slavemaker queen but no slavemaker workers. For some populations long-term parasite pressure estimates were available which are indicated by the first parasite prevalence and median slavemaker colony size entry. The second entry refers to the parasite pressure recorded during colony collection. Slavemaker colony sizes were not recorded for *T. longispinosus* parasites from Kentucky.

Population	County	Coordinates		Collect. colonies	Experim. colonies	Parasite prevalence	Median slavemaker colony size
Temnothorax curvispinosus							
Illinois	St. Clair	38°13'62''	089°44'97.6''	114	33	- / 0.04	- / 0.0
Indiana	Perry	38°11'88''	086°38'16.3''	94	34	- / 0.03	- / 0.5
Kentucky	Estill	37°48'13''	083°41'83.3''	161	31	- / 0.03	- / 0.0
New Jersey	Burlington	40°00'42''	074°50'10.0''	119	32	- / 0.00	-
Ohio North	Ashtabula	41°50'34''	080°57'55.7''	156	31	0.06 / 0.04	- / 2.0
Ohio South	Delaware	40°14'23''	082°59'10.9''	594	29	- / 0.08	- / 1.0
Virginia	Warren	38°50'05''	078°11'16.0''	96	32	- / 0.05	- / 1.0
West Virginia	Pocahontas	38°06'48''	080°07'88.2''	124	32	- / 0.00	-
Temnothorax longispinosus							
Kentucky	Estill	37°48'13''	083°41'83.3''	65	22	- / 0.03	-
Maine	Oxford	42°23'98''	070°51'35.1''	104	33	- / 0.00	-
Massachusetts	Middlesex	42°23'98''	071°11'67.9''	97	33	- / 0.12	- / 1.0
New Hampshire	Sullivan	43°26'41''	072°09'34.6''	105	35	- / 0.00	-
New York	Albany	42°31'95''	074°08'75.3''	963	32	0.12 / 0.19	2.0 / 3.0
Ohio North	Ashtabula	41°50'34''	080°57'55.7''	99	39	0.14 / 0.14	2.0 / 1.0
Quebec	Montreal	45°30'37''	073°36'94.7''	111	32	- / 0.00	-
Vermont	Addison	43°58'25''	073°04'31.6''	102	32	0.07 / 0.16	2.0 / 1.0
West Virginia	Pocahontas	38°06'48''	080°07'88.2''	113	32	0.11 / 0.35	2.0 / 5.0

Table S3.2 Model selection results from the generalized linear mixed models analysing host collective defences and slavemaker escape probability in relation to social parasite pressure. Parasite pressure is represented by the number of slavemaker colonies per host colony (i.e. parasite prevalence) and the median number of slavemaker workers per colony (i.e. slavemaker colony size). The effect of the two measures of parasite pressure was evaluated using separate models. Statistics indicated in bold were retained in the final models. All Δd.f. = 1.

	Collective immobilization probability[a]		Host evacuation probability[b]		Slavemaker escape probability[c]	
Predictors	χ^2	*P*	χ^2	*P*	χ^2	*P*
Host colony size	**43.952**	**<0.0001**	**43.276**	**<0.0001**	**28.262**	**<0.0001**
Species	**11.805**	**<0.001**	0.053	0.816	**21.941**	**<0.0001**
Parasite prevalence	**3.957**	**0.047**	**7.768**	**0.005**	0.078	0.780
Parasite prevalence x Species	0.071	0.790	0.874	0.350	0.262	0.609
Host colony size	**44.192**	**<0.0001**	**42.345**	**<0.0001**	**28.914**	**<0.0001**
Species	**11.661**	**<0.001**	0.095	0.758	**18.152**	**<0.0001**
Slavemaker colony size	**36.904**	**<0.0001**	**28.975**	**<0.0001**	**24.263**	**<0.0001**
Slavemaker colony size x Species	0.073	0.787	0.014	0.906	0.865	0.353

Sample size for the models including parasite prevalence ($N_{experiments}/N_{colonies}/N_{populations}$): a) 599/527/17; b) 606/534/17; c) 442/407/17. Sample size for the models including slavemaker colony size: a) 577/506/16; b) 584/513/16; c) 425/391/16.

Table S3.3 Model selection results from the generalized linear mixed models analysing host aggressive defences in relation to social parasite pressure. Parasite pressure is represented by the number of slavemaker colonies per host colony (i.e. parasite prevalence) and the median number of slavemaker workers per colony (i.e. slavemaker colony size). The effect of the two measures of parasite pressure was evaluated using separate models. Statistics indicated in bold were retained in the final models. All Δd.f. = 1.

	Number of immobilizing workers		Number of attacking workers	
Predictors	χ^2	*P*	χ^2	*P*
Host colony size	76.173	<0.0001	1.022	0.312
Species	23.227	<0.0001	30.322	<0.0001
Parasite prevalence	8.340	0.004	0.972	0.324
Parasite prevalence x Species	0.075	0.784	3.563	0.059
Host colony size	75.090	<0.0001	1.067	0.302
Species	23.140	<0.0001	23.329	<0.0001
Slavemaker colony size	56.246	<0.0001	24.328	<0.0001
Slavemaker colony size x Species	0.022	0.883	3.160	0.076

Sample size for the models including parasite prevalence ($N_{experiments}/N_{colonies}/N_{populations}$): 599/527/17. Sample size for the models including slavemaker colony size: 577/506/16.

Chapter **4**

The ecological success of a social parasite increases with manipulation of collective host behaviour

Evelien Jongepier, **Isabelle Kleeberg** and Susanne Foitzik

Evelien Jongepier, **Isabelle Kleeberg** and Susanne Foitzik

ABSTRACT

Many parasites alter the behaviour of their host to their own advantage; yet, hosts often vary in their susceptibility to manipulation. The ecological and evolutionary implications of such variation can be profound, as resistant host populations may suffer lower parasite pressures than those susceptible to manipulation. To test this prediction, we assessed parasite-induced aggressive behaviours across 16 populations of two *Temnothorax* ant species, many of which harbour the slavemaker ant *Protomognathus americanus*. This social parasite uses its Dufour's gland secretions to manipulate its hosts into attacking nestmates, which may deter defenders away from itself during invasion. We indeed find that colonies that were manipulated into attacking their Dufour-treated nestmates were less aggressive towards the slavemaker than those that did not show slavemaker-induced nestmate-attack. Slavemakers benefitted from altering their hosts' aggression, as both the likelihood that slavemakers survived host encounters and slavemaker prevalence in ant communities increased with slavemaker-induced nestmate attack. Finally, we show that *T. longispinosus* colonies were more susceptible to manipulation than *T. curvispinosus* colonies. This explains why *T. curvispinosus* responded with more aggression towards invading slavemakers, why they were less likely to let slavemakers escape and why they were less frequently parasitized by the slavemaker than *T. longispinosus*. Our findings highlight that large-scale geographic variation in resistance to manipulation can have important implications for the prevalence and host preference of parasites.

Keywords: Manipulative parasites, Social parasites, Parasite prevalence, Slavemaking ants, Dufour's gland

INTRODUCTION

Parasites can manipulate the behaviour, morphology and physiology of their hosts (Poulin and Thomas 1999), which in turn impacts the wider community of species (Lefèvre et al. 2009; Lafferty and Kuris 2012; Sato et al. 2012). Although the magnitude of such effects chiefly depends on the success of manipulative parasites themselves, the evolutionary dynamics that govern their distribution and abundance are still poorly understood (Poulin 2010). For instance, host manipulation is, by definition, beneficial to the parasite (Moore 2013). Yet, empirical evidence that host manipulation - rather than infection - promotes the success of parasites is lacking (Mouritsen and Poulin 2003). Moreover, several reviews have alluded to host counter-adaptations driving variation in parasite-induced changes (Poulin et al. 1994; Thomas et al. 2005; Wellnitz 2005; Poulin 2010; Daoust et al. 2015), but how host resistance to manipulation relates to the success of parasites remains unknown.

Many manipulative parasites target their host's behaviour, resulting in parasite-induced behavioural alterations, ranging from slight changes in the host's initial traits to the display of completely novel behaviours (Moore 2002; Lefèvre and Thomas 2008; Libersat et al. 2009). Classical examples include terrestrial insects that jump into water where the parasite can complete its life cycle (Thomas et al. 2002); or ants that perch among patches of berries, their bright red abdomen raised to attract the parasite's avian host (Yanoviak et al. 2008). There is increasing evidence for qualitative and quantitative variation in the expression of parasite-induced behaviours, even within manipulative parasite-host systems (Thomas et al. 2005; Bauer et al. 2009; Poulin 2010; Thomas et al. 2011). Elucidating the causes of such variation is challenging because parasite-induced behaviours are expressed by the host alone, although they may be under the control of the parasite, the host or both (Moore 2013). Moreover, parasite-induced behavioural alterations are inherently dependent on the initial characters of the host, not only because they are measured as a change in those characters, but also because initial characters often represent the very substrate of manipulation (Moore 2002; Blanchet, Méjean, et al. 2009a; Blanchet, Thomas, et al. 2009b). Inter-individual variation in the expression of parasite-induced behaviours thus cannot be fully understood without also considering the host's initial characters.

Theory predicts that manipulative strategies should evolve under many different scenarios (Poulin 1994; Parker et al. 2009; Vickery and Poulin 2010), and manipulative parasites are indeed found in numerous host and parasite taxa (Moore 2002). These include social parasites, such as slavemaking ants, which manipulate the behaviour of entire societies to gain access to the workforce of their ant, bee or wasp hosts (Hölldobler and Wilson 1990). Entering a host colony, social

parasites can use offensive chemicals to protect themselves from aggression by the more numerous host defenders (Lenoir et al. 2001). These propaganda or appeasement substances are often produced in the parasite's Dufour's gland. They can repel or pacify defending host workers (Topoff et al. 1988; D'Ettorre et al. 2000), cause panic and confusion within host colonies (Regnier and Wilson 1971) or elicit attacks between host nestmates (Allies et al. 1986; Brandt et al. 2006). Contrary to most other manipulative parasites, social parasites manipulate their hosts without becoming an integral part of the host's body and manipulation occurs prior to parasitic exploitation. Because this negates common difficulties in measuring the aggressive potential of naturally parasitized populations in the absence of manipulation (i.e. the host's initial character; Blanchet et al. 2009a; b), hosts of social parasites pose excellent opportunities to investigate parasite-induced behavioural alterations.

Here, we test whether variation in resistance to manipulation in *Temnothorax* ant hosts predicts the success of the slavemaker ant *Protomognathus americanus*. *Temnothorax* hosts aggressively defend themselves against slavemakers, reducing the slavemakers' success during nest foundation (Pamminger et al. 2012) and slave raids (Kleeberg et al. 2014, Chapter 1). Despite the apparent advantages of aggression, *Temnothorax* populations suffering high slavemaker prevalence in the field were found to be less, not more aggressive towards the slavemaker than populations where the slavemaker was rare or absent (Jongepier et al. 2014, Chapter 3). This decrease in host aggression with slavemaker prevalence was not due to geographic variation in the aggressive potential of *Temnothorax* colonies (i.e. aggression in the absence of manipulation; Kleeberg et al. 2015, Chapter 2). Hence, we hypothesise that populations that are better defended against slavemakers are more resistant to manipulation of their aggressive responses, resulting in lower slavemaker prevalence in the field. To test this, we compared the aggressive responses of 16 populations of two *Temnothorax* species, covering much of the geographic range of the slavemaker, as well as communities where the slavemaker was absent. We assessed host susceptibility to slavemaker manipulation by testing whether colonies attacked a nestmate treated with the slavemaker's Dufour's gland secretions. We used the same host colonies for which we previously assessed aggression towards live, potentially manipulative (Jongepier et al. 2014, Chapter 3) and dead, non-manipulative slavemakers (Kleeberg et al. 2015, Chapter 2). Hence, we could integrate the results of the new experiment with our previous findings to (i) explore the relationship between slavemaker-induced nestmate attack and aggression towards the slavemaker itself, using a mixed-model approach, (ii) demonstrate slavemaker survival benefits of altered host aggression, and hence confirm that slavemakers manipulate their hosts' aggressive defences, and (iii) compare the role of slavemaker-induced aggression with that of the hosts' aggressive potential in governing the global distribution and abundance of the slavemaker. We predict that slavemaker-induced

aggressive responses, rather than the hosts' aggressive potential, hamper *Temnothorax* defences. Given that more aggressive *Temnothorax* populations suffer lower slavemaker pressure (Jongepier et al. 2014, Chapter 3), we further predict that slavemaker-induced constraints on host defences result in an increase in slavemaker survival and prevalence. Our results not only support these predictions, but also suggest that the biased exploitation of the preferred host *T. longispinosus* (Brandt and Foitzik 2004) is driven by interspecific differences in host resistance to manipulation.

METHODS

Collection and maintenance

From May to July 2012, we collected *T. curvispinosus* and *T. longispinosus* colonies from 16 populations in the USA and Canada (Fig. 4.1; Jongepier et al. 2014, Chapter 3). All colonies were counted, transferred to artificial nest sites and housed in plastered nest boxes to prevent desiccation. They were kept at a constant 25°C with a 12h : 12h light /dark cycle and fed semi-weekly with honey and cricket. For our experiments, we selected 11.44 ± 0.96 (mean ± s.d.) colonies per population, yielding in total 105 *T. longispinosus* colonies originating from nine populations and 78 *T. curvispinosus* colonies from seven populations. All experimental colonies were collected as free-living *Temnothorax* colonies and hence without slavemakers. We cannot rule out that some experimental colonies were part of a larger polydomous colony, although based on prior estimates of the incidence of polydomy in *T. longispinosus* (Foitzik et al. 2004), we estimate that less than 1% of the 183 experimental colonies did not represent an independent sample in our experiments. Since our populations show substantial genetic differentiation (Brandt et al. 2007; Pennings et al. 2011), it is unlikely that gene flow between populations eliminates geographic differences in the hosts' anti-slavemaker defences.

Study design

From August to October 2012, each experimental colony was subjected to three standardized aggression tests to assess their aggressive potential and slavemaker-induced aggression. These tests measured 1) colony attack on a nestmate treated with the slavemaker's Dufour's gland secretions (new experiment); 2) colony aggression towards a live, potentially manipulative slavemaker (based on a subset of the data presented in Jongepier et al. 2014, Chapter 3); and 3) colony aggression towards a dead, and hence non-manipulative slavemaker (based on a subset of data presented in Kleeberg et al. 2015, Chapter 2). All slavemakers used as opponents in the aggression tests originated from colonies containing slaves of the species they were tested against. To enable us to test aggression by hosts from communities

where slavemakers are rare or absent, slavemaker population of origin was standardized, testing *T. longispinosus* colonies against slavemakers from New York and *T. curvispinosus* colonies against slavemakers from Ohio (further details are provided in section 3).

1) One day prior to the tests against a Dufour-treated nestmate, a *Temnothorax* worker and a slavemaker worker were removed from their respective colonies and frozen at -20° C. Slavemakers were dissected in distilled water under a stereomicroscope. Glands were obtained by pulling the stinger with a Dumont forceps, attached to which are the poison gland and the Dufour's gland. The Dufour's gland was then separated from the stinger and the poison gland by pinching the Dufour's duct with a forceps and pulling away the rest with a second forceps. The entire Dufour's gland was applied directly, without the use of a solvent, to the gaster of the host by exerting slight pressure on the forceps. This ruptures the delicate membrane of the Dufour's gland without damaging the gaster of the host ant. The Dufour-treated worker was placed into its original colony for a standardized colony aggression test, during which we recorded the number of attacking workers (i.e. stinging, biting, holding or dragging) every 15 s for 5 min. A previous study confirmed that *Temnothorax* colonies showed virtually no aggression towards a nestmate treated with either water or the Dufour's gland secretion of an infertile, non-nestmate conspecific worker, contrary to the aggression they showed towards a nestmate treated with the slavemaker's Dufour's gland content (Brandt et al. 2006). The lack of aggression towards a nestmate treated with the Dufour's gland of a non-nestmate indicates that a colony's response does not result from Dufour-treatment masking the colony identity of the Dufour-treated worker, otherwise the non-nestmate's Dufour's gland extracts would likewise elicit aggression by *Temnothorax* colonies. Since 46% of the colonies did not respond with aggression to their Dufour-treated nestmate, we grouped colonies based on whether they did or did not attack their nestmate for analyses. Slavemakers and host colonies were paired at random (i.e. slavemakers were selected blindly with respect to individual and colony-level traits of the slavemaker), ruling out consistent biases due to inter-individual variation in the quantity or quality of Dufour's gland secretions. We used a new slavemaker Dufour's gland for each aggression test.

2) To assess the response to the slavemaker itself, colonies were subjected to an aggression test against a live slavemaker, previously published in Jongepier et al. (2014, Chapter 3). In summary, we introduced a live slavemaker worker into the experimental colony and recorded the number of workers showing aggression towards the slavemaker (i.e. stinging, biting, holding or dragging). In addition, we recorded whether the colony evacuated its nest site within six hours of slavemaker introduction and whether the slavemaker managed to escape, physically unharmed and therefore able to recruit nestmates and initiate a slave raid.

3) To control for differences in aggressive potential between colonies we further included data on colony aggression towards a dead slavemaker, previously published in Kleeberg et al. (2015, Chapter 2). Hereto, we introduced a freshly frozen slavemaker worker and recorded the number of aggressive workers (i.e. stinging, biting, holding or dragging) every 30 s for 5 min. Because ants mainly rely on cuticular hydrocarbon profiles for enemy recognition (Sturgis and Gordon 2012), aggression towards a dead, freshly frozen opponent that still bears the recognition cues on its cuticle, and a live opponent are correlated in the absence of manipulation (i.e. when facing a non-manipulative, conspecific worker; Modlmeier & Foitzik, 2011). Hence, by comparing colony aggression towards a dead, non-manipulative slavemaker and a live slavemaker that could employ its manipulative arsenal, we were able to distinguish between the colony's aggressive potential towards slavemakers, and responses elicited by slavemaker behaviour, including its use of Dufour's gland secretions.

To rule out potential test day or time-of-day effects, we randomly selected an equal number of colonies from each population per test day and randomized test order within test days. Given that the median number of slavemaker workers per slavemaker colony is only two (Jongepier et al. 2014, Chapter 3), the test order and the time interval between tests were chosen as to minimize both the number of required slavemakers for the 538 tests (detailed below) and the risk of carry-over effects between tests. That is, all colonies were first tested against a live slavemaker such that surviving slavemakers could be used in the tests involving dead slavemakers. Because the contradictory results of these first two tests formed an important motivation to further explore the role of host manipulation by the slavemaker, tests against a Dufour-treated nestmate were performed last. Three days prior to the tests against a dead slavemaker, colonies were subjected to an aggression test against a non-nestmate conspecific worker as part of a parallel experiment (Kleeberg et al. 2015, Chapter 2). It is however unlikely that this test has affected the outcome of our experiments since Pamminger et al. (2011) found no effect of conspecific encounter on *Temnothorax* colony aggression. However, they did show that slavemaker contact induces elevated aggression by *Temnothorax* colonies for up to two weeks, which is why we only subjected colonies to the aggression test against the Dufour-treated nestmate after a three week time interval.

Parasite prevalence and origin

Parasite prevalence was defined as the proportion of slavemaker colonies out of the ~100 slavemaker and free-living host colonies (i.e. without slavemakers) collected per sampling location (Fig. 4.1; Jongepier et al. 2014, Chapter 3; Kleeberg et al. 2015, Chapter 2). As mentioned in the previous section, slavemaker population of origin was standardized, which allowed us to include ant communities where the slavemaker was rare or absent as well as control for geographic variation in the

parasite's ability to manipulate their host. Nonetheless, standardization could potentially cause confounding effects due to local adaptation in the two populations that were tested against a sympatric slavemaker, or the lack thereof in the remaining 14 populations. Several studies, however, found no evidence that *Temnothorax* hosts are better adapted to their local slavemaker (Foitzik et al. 2001; Brandt and Foitzik 2004), which included tests of local host adaptation in aggression towards live and dead slavemakers (Jongepier et al. 2014; Kleeberg et al. 2015, Chapter 2-3). While we did not test for local host adaptation in the hosts' Dufour-treated nestmate attack, we could show that the exclusion of populations tested against a sympatric slavemaker did not qualitatively change the outcome of our analyses, nor were the hosts' aggressive responses related to the geographic distance between the *Temnothorax* population and the slavemaker population of origin (Dufour-treated nestmate attack: $t_{14} = 0.514$, $P = 0.615$; aggression towards a live parasite: $t_{14} = -0.447$, $P = 0.662$).
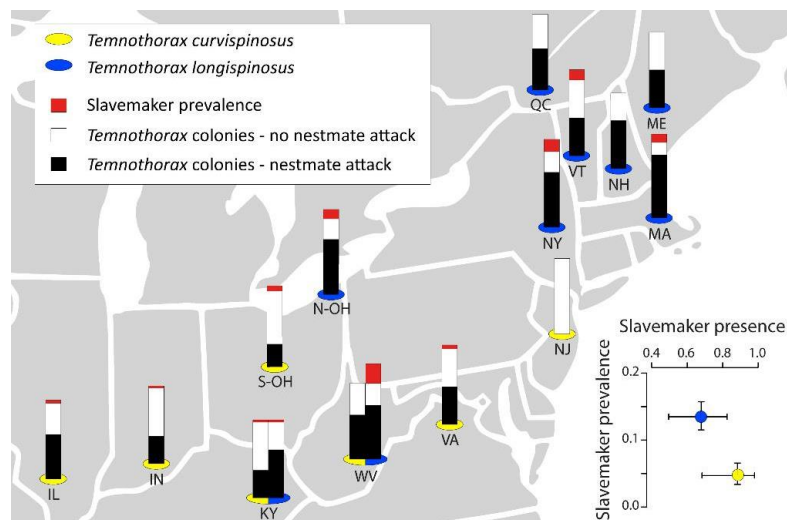
Figure 4.1 Slavemaker prevalence and the proportion of colonies attacking their Dufour-treated nestmate for each of the 16 *Temnothorax* populations. Ant communities represent Illinois (IL), Indiana (IN), Kentucky (KY), Maine (ME), Massachusetts (MA), New Hampshire (NH), New Jersey (NJ), New York (NY), Ohio South (S-OH), Ohio North (N-OH), Quebec (QC), Vermont (VT), Virginia (VA), West Virginia (WV). Inset depicts slavemaker prevalence and presence associated with the two *Temnothorax* species (estimates ± SE). *Temnothorax longispinosus* suffered higher parasite prevalence (quasi-binomial GLM: t14 = 2.90, p =0.012) although it did not co-occur more often with the slavemaker (binomial GLM: z14 = -0.85, p = 0.395).

Sample sizes

In total, we performed 538 aggression tests; 183 against a Dufour-treated nestmate, 205 against a dead slavemaker and 150 against a live slavemaker. All 183 colonies included in the analyses were tested against a Dufour-treated nestmate. Of these experimental colonies, 147 were additionally tested against a dead as well as a live

slavemaker, 33 colonies were tested against a dead but not a live slavemaker and 3 colonies were tested against a live but not a dead slavemaker. Sample sizes differ between tests for two main reasons. Firstly, 33 colonies were replaced after the aggression tests against a live slavemaker due to high worker mortality (not as a consequence of the aggression test but in the three week interval between tests). Secondly, *T. longispinosus* colonies from New York and West-Virginia ($N = 25$) were tested against a sympatric and an allopatric dead slavemaker to assess the potential role of slavemaker sympatry. Since colony aggression was not related to slavemaker sympatry (Kleeberg et al. 2015, Chapter 2), we included both aggression tests in our analyses.

Data analyses

Slavemaker-induced nestmate attack and anti-slavemaker aggression - To assess whether a colony's ability to defend itself aggressively against a slavemaker was related to its susceptibility to slavemaker manipulation, we compared the aggressive responses of colonies that attacked their Dufour-treated nestmate to those that did not attack their Dufour-treated nestmate. If slavemaker-induced changes in host aggression constrain a colony's anti-slavemaker defences, we predict that colonies that attack their Dufour-treated nestmate are less aggressive towards a live, potentially manipulative slavemaker, but not towards a dead, non-manipulative slavemaker, compared to colonies that did not attack their nestmate. Hence, an interaction between Dufour-treated nestmate attack and opponent type (dead slavemaker / live slavemaker) would support our hypothesis that slavemaker-induced changes in aggression, rather than a colony's aggressive potential towards slavemakers, constrains colony defence. We used Generalized Linear Mixed Models (GLMM; glmer function implemented in the lme4 package; Bates et al. 2014) following a Poisson distribution with a log-link function. The number of aggressive workers (i.e. stinging, biting, holding, dragging) served as dependent variable whereas Dufour-treated nestmate attack (attack / no attack), opponent type (dead slavemaker / live slavemaker) and their interaction were fitted as fixed predictors. Colony ID, nested in population ID, was included as random factor to account for our repeated measure design and to avoid pseudo-replication. In addition, we assessed whether the two *Temnothorax* species differed in their aggression towards nestmates, live and dead slavemakers. For the analysis of Dufour-treated nestmate attack (attack / no attack) we used a binary GLMM with logit-link function, including species (*T. curvispinosus / T. longispinosus*) as fixed predictor and population ID as random factor. For the analysis of aggression towards slavemakers we used a Poisson GLMM with log-link function, fitting species (*T. curvispinosus / T. longispinosus*), opponent type (dead slavemaker / live slavemaker) and their interaction as fixed predictors. Colony ID, nested in population ID served as random factor.

Slavemaker-induced change in aggression and slavemaker survival - We tested whether and how geographic variation in host aggression was related to individual slavemaker survival. For each of the 16 host populations, we calculated the probability that slavemakers escaped following their introduction into a host colony. This probability was analysed using Generalized Linear Models (GLM) following a binomial distribution with a logit-link function. Using three separate analyses, we fitted either the proportion of *Temnothorax* colonies attacking their Dufour-treated nestmate or their average aggression towards live or dead slavemakers, in addition to species and their interaction. We included the evacuation probability of *Temnothorax* colonies as covariate, since we have previously shown that slavemakers are more likely to escape if a colony evacuates its nest site upon slavemaker encounter (Jongepier et al. 2014, Chapter 3). Reported p-values are Holm-Bonferroni corrected for multiple testing (Holm 1979).

Slavemaker-induced change in aggression and slavemaker prevalence - We tested whether and how geographic variation in host aggression was related to slavemaker prevalence using a quasi-binomial GLM with logit-link function. Using three separate analyses, we fitted either the proportion of *Temnothorax* colonies attacking their Dufour-treated nestmate or their average aggression towards live or dead slavemakers, in addition to species and their interaction. Reported p-values are Holm-Bonferroni corrected for multiple testing (Holm 1979).

For all analyses, we used a backwards-stepwise model selection procedure (α = 0.05). Neither of our GLMMs were overdispersed (residual deviance/residual d.f. < 1.4). All analyses were performed in R v. 3.1.1 (R Core Team 2014).

RESULTS

Slavemaker-induced nestmate attack and anti-slavemaker aggression

Aggression towards live and dead slavemakers differed between colonies that attacked their Dufour-treated nestmate or not (opponent type x Dufour-treated nestmate attack: χ^2_1 = 29.70, P < 0.0001). Colonies that attacked their Dufour-treated nestmate showed less aggression towards a live slavemaker than colonies that did not attack their Dufour-treated nestmate (Fig. 4.2a; z = -2.27, P = 0.005). Contrastingly, dead slavemakers elicited more aggression from colonies that attacked their Dufour-treated nestmates compared to those that did not attack their Dufour-treated nestmate (z = 3.68, P < 0.001). Although a positive association between nestmate attack and aggression towards a dead, non-manipulative slavemaker may be indicative of intrinsic differences in aggressive potential between colonies, the opposite response when facing a live slavemaker is indicative of slavemaker-induced changes in the aggressive defences of colonies that are susceptible to manipulation.

The two *Temnothorax* species differed in whether or not they attacked their Dufour-treated nestmate (Fig. 4.2b; $\chi^2_1 = 8.29$, $P = 0.004$), as well as their aggressive responses towards live or dead slavemakers (species x opponent type: $\chi^2_1 = 43.22$, $P < 0.0001$). Among *T. longispinosus* colonies, 64.8% attacked their Dufour-treated nestmate, compared to only 39.7% of the *T. curvispinosus* colonies. Moreover, *T. curvispinosus* colonies were more aggressive towards a live slavemaker than *T. longispinosus* ($z = -3.18$, $P = 0.001$; also shown in Jongepier et al. 2014, Chapter 3). This difference was not driven by species-specific variation in aggressive potential towards slavemakers, since *T. longispinosus* colonies were more, not less aggressive towards a dead slavemaker ($z = 4.41$, $P < 0.0001$; also shown in Kleeberg et al. 2015, Chapter 2).
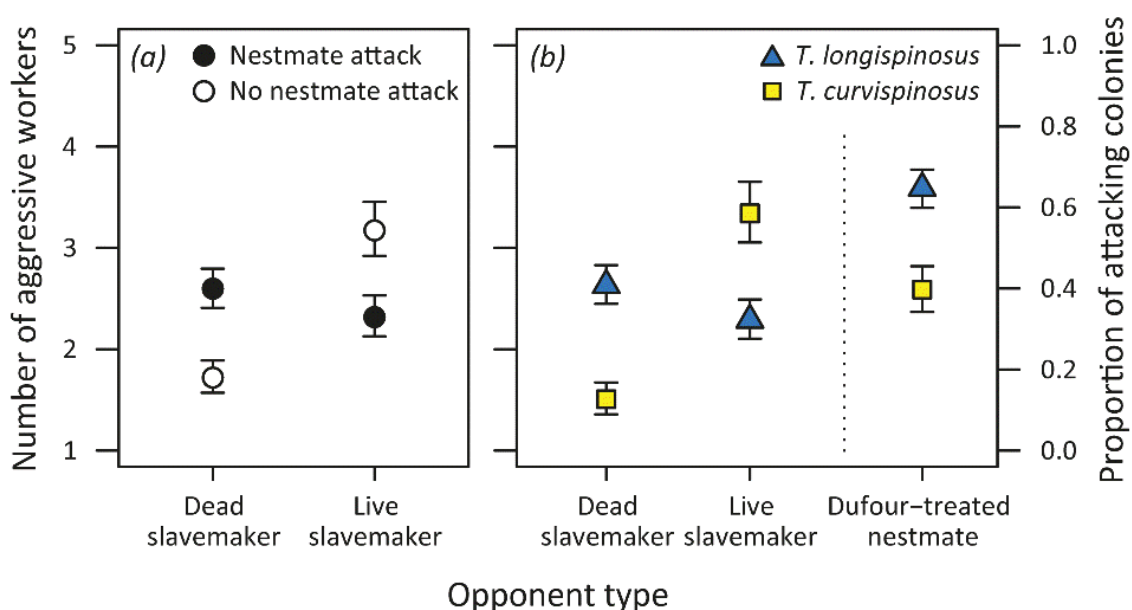
Figure 4.2 The relationship between aggression towards slavemakers, slavemaker-induced nestmate attack and *Temnothorax* ant species. (a) The number of *Temnothorax* workers showing aggression towards a dead and a live slavemaker, in relation to whether or not those colonies attacked their Dufour-treated nestmate. (b) Differences between the two *Temnothorax* species in the number of workers showing aggression towards a dead and a live slavemaker, as well as the proportion of colonies attacking their Dufour-treated nestmate. Symbols represent estimates ± s.e. of the GLMMs presented in the text.

Slavemaker-induced change in aggression and slavemaker survival

Slavemaker escape probability increased with the proportion of colonies that attacked their Dufour-treated nestmate (Fig. 4.3a; $t_{14} = 2.67$, $P_{corr} = 0.024$) and decreased with the average aggression towards live slavemakers (Fig. 4.3b; $t_{14} = -2.49$, $P_{corr} = 0.026$, also shown in Jongepier et al. 2014, Chapter 3). Contrastingly, slavemaker escape was unrelated to the hosts' aggression towards a dead, non-manipulative parasite (Δ deviance = 0.82, Δ d.f. = 1, $P_{corr} = 0.365$).

Although slavemakers were more likely to escape unharmed from *T. longispinosus* than *T. curvispinosus* colonies (Jongepier et al. 2014, Chapter 3), this difference appears to be mainly driven by the higher resistance to manipulation in *T. curvispinosus*: Slavemakers were equally likely to escape from *T. longispinosus* and *T. curvispinosus* colonies after controlling for interspecific differences in Dufour-treated nestmate attack (species difference with the proportion of colonies that attacked their Dufour-treated nestmate as covariate: Δ deviance = 0.09, Δ d.f. = 1, P = 0.769) or aggression towards live slavemakers (species differences with the average aggression towards live slavemakers as covariate: Δ deviance = 0.03, Δ d.f. = 1, P = 0.857). Moreover, we show consistency across the two *Temnothorax* species in the relationships between slavemaker escape and Dufour-treated nestmate attack (species x proportion of colonies attacking their Dufour-treated nestmate: Δ deviance = 0.65, Δ d.f. = 1, P = 0.419) or aggression towards live slavemakers (species x average aggression towards live slavemakers: Δ deviance = 2.07, Δ d.f. = 1, P = 0.150).

Slavemaker-induced change in aggression and slavemaker prevalence

Slavemaker prevalence increased with the proportion of colonies attacking their Dufour-treated nestmate (Fig. 4.3c; t_{14} = 3.63, P_{corr} = 0.009) and decreased with the average aggression towards live slavemakers (Fig. 4.3d; t_{14} = -3.18, P_{corr} = 0.014; also shown in Jongepier et al. 2014, Chapter 3). Slavemaker prevalence was unrelated to the average aggression towards dead, non-manipulative slavemakers (F = 0.73, Δ d.f. = 1, P_{corr} = 0.406; also shown in Kleeberg et al. 2015, Chapter 2).

The higher slavemaker prevalence in *T. longispinosus* compared to *T. curvispinosus* populations (Fig. 4.1) appears to be mainly driven by a higher resistance to manipulation in the latter. That is, slavemaker prevalence did not differ between *T. longispinosus* or *T. curvispinosus* populations when taking the proportion of colonies attacking their Dufour-treated nestmate (F = 0.15, Δ d.f. = 1, P = 0.709) or the average aggression towards live slavemakers into account (F = 0.78, Δ d.f. = 1, P = 0.394). Moreover, we show consistency across the two *Temnothorax* species in the relationship between slavemaker prevalence and Dufour-treated nestmate attack (species x proportion of colonies attacking their Dufour-treated nestmate: F = 2.67, Δ d.f. = 1, P = 0.128), as well as consistency in the relationship between slavemaker prevalence and aggression towards live slavemakers (species x average aggression towards live slavemakers: F = 0.45, Δ d.f. = 1, P = 0.514).
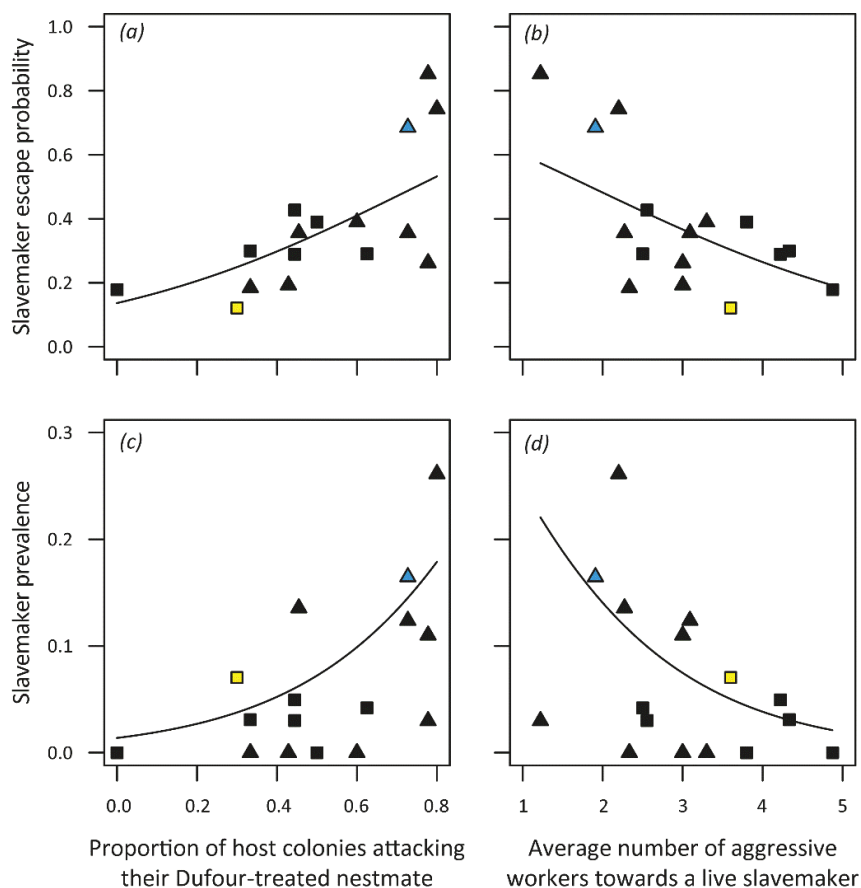
Figure 4.3 Slavemaker-induced nestmate attack and anti-slavemaker aggression of Temnothorax ant colonies in relation to (a-b) slavemaker escape and (c-d) slavemaker prevalence per population. Slavemaker-induced nestmate attack is represented by the proportion of colonies attacking their Dufour-treated nestmate and anti-slavemaker aggression by the average aggression towards a live slavemaker (b, d). Each symbol represents a *Temnothorax* population; squares: *T. curvispinosus*, triangles: *T. longispinosus*. Populations depicted in yellow (S-OH) and blue (NY) depict the population of origin of the slavemaker used in the aggression tests. Regression lines represent the back-transformed estimates of the binomial GLMs.

DISCUSSION

We demonstrated that *Temnothorax* colonies that resist manipulation by the slavemaker *P. americanus* are better able to defend themselves during slavemaker intrusion. Although the social parasite can manipulate its hosts into attacking nestmates and seems able to divert aggression away from itself, not all host colonies appear equally defenceless against slavemaker manipulation. In particular, colonies of the preferred host *T. longispinosus* were more often manipulated into attacking their nestmates than the less preferred host *T. curvispinosus*. This explains why *T. curvispinosus* was more aggressive towards live, potentially manipulative parasites

(this study; Jongepier et al. 2014, Chapter 3), but not towards a non-manipulative parasite (this study; Kleeberg et al. 2015, Chapter 2). Parasite-induced host behaviours do not necessarily benefit parasites, they could also be adaptive to hosts or represent mere pathological side effects of infection (Poulin et al. 1994; Poulin 1995; Thomas et al. 2005). In our study, the attack of nestmates is clearly non-adaptive to the host and pathological side effects are unlikely given that slavemaker manipulates the host prior to parasitic exploitation (i.e. "infection"). Instead, our findings indicate that slavemakers manipulate their hosts by altering host behaviour to their own advantage. That is, both the survival of individual slavemaker workers and the prevalence of slavemaker colonies decreased with resistance to manipulation by their local host. Our comparison of 16 *Temnothorax* populations thus suggests that large-scale geographic variation in resistance to manipulation has important implications for the prevalence and host preference of the parasite.

Although most work to date has focused on the role of more conventional defence mechanisms on the outcome of host-parasite interactions (Greischar and Koskella 2007), we show that both slavemaker survival and the success of slavemaker colonies were unrelated to their hosts' defences against non-manipulative slavemakers (this study; Kleeberg et al. 2015, Chapter 2). Instead, slavemaker prevalence seems to be constrained by the hosts' ability to withstand manipulation. To our knowledge, only a single other study investigating how host manipulation, rather than infection, was related to the population dynamics of parasites in a natural community. It showed that the benefits of host manipulation by trematode parasites are small (Mouritsen and Poulin 2003). Our findings instead suggest that host manipulation plays an important role in the ecological and evolutionary dynamics between antagonists, which begs for large scale comparative approaches in other host-parasite systems to ascertain their generality.

The decrease in slavemaker prevalence with resistance to manipulation by *Temnothorax* populations contrasts with previous studies showing a positive association between parasite prevalence and host resistance (Foitzik et al. 2003; Franceschi et al. 2010). For instance, ant hosts originating from highly parasitized populations were less likely to be manipulated by the parasitic ant *Harpagoxenus sublaevis* to fight against nestmates than those from less severely parasitized populations (Foitzik et al. 2003). Likewise, naturally parasitized amphipod populations were less susceptible to manipulation than parasite-naïve ones, suggesting that resistance has evolved in response to parasite pressure, rather than the other way around (Franceschi et al. 2010). Franceschi et al. (2010) do show variation between populations in the manipulative ability of parasites, although this was unrelated to parasite transmission success. To what extent local adaptation and geographic variation in the manipulative ability of *P. americanus* contributes to their success is currently unknown. However, many slavemaker populations appear

to attain a lower prevalence than expected from the hosts' slavemaker-induced attack of nestmates or their aggression towards the parasite itself. Future studies on geographic heterogeneity in the slavemaker's manipulative ability may shed light on this unexplained variation in slavemaker prevalence.

Our findings suggest that slavemaker-induced changes in aggression in the two *Temnothorax* species have similar epidemiological consequences. The positive relationship between slavemaker-induced nestmate attack and slavemaker survival or prevalence was consistent across *T. longispinosus* and *T. curvispinosus* hosts. Likewise, the negative relationship between aggression towards live, potentially manipulative slavemakers and slavemaker survival or prevalence did not differ between host species (this study; Jongepier et al. 2014, Chapter 3). Nonetheless, species did differ in their slavemaker-induced aggressive responses, with the preferred host, *T. longispinosus*, being more susceptible to slavemaker manipulation. This corroborates with a previous study showing that a *T. longispinosus* population responded with stronger agitation to the release of the slavemaker's Dufour's gland secretions than a *T. curvispinosus* population (Brandt et al. 2006). Similar interspecific differences were shown in other host-parasite interactions, including amphipod-acanthocephalan (Tain et al. 2007), spider-parasitoid wasps (Eberhard 2010; Korenko and Pekár 2011) and other ant-slavemaker systems (Bauer et al. 2009). In some cases, parasites readily switch between host species, preferentially targeting the most abundant host (Korenko et al. 2011), whereas in others, host preference reflects differences in the ability of parasites to manipulate host behaviour (Tain et al. 2007). In our study system, parasite prevalence is higher in *T. longispinosus* populations, even in communities where *T. curvispinosus* is more abundant (Brandt and Foitzik 2004). Hence, slavemaker exploitation is not biased towards the most abundant species, but rather towards the host that is least defended against manipulation by slavemakers.

Although Thomas et al. (2011) argued that spatial heterogeneity in host-parasite associations most often arises from variation in the traits of hosts and parasites themselves, slavemaker prevalence may also be governed by other factors such as local habitat preference. While we cannot fully rule out the role of confounding effects in our correlative study, this alternative explanation is unlikely given the distribution and abundance of the slavemaker. That is, *T. longispinosus* suffers higher parasite prevalence, both at a global scale and in communities harbouring both host species , yet, slavemakers more frequently co-occur with *T. curvispinosus* (be it non-significantly; Fig. 4.1). This suggests that local environmental conditions do not preclude slavemaker presence, although better resistance to manipulation by *T. curvispinosus* colonies may limit their prevalence.

The fitness costs of manipulation and resistance have been subject to recent speculation, although empirical evidence is still wanting (Thomas et al. 2005; Poulin 2010). At present, little is known about the costs and benefits of resistance in our

study system, except that populations that resist manipulation likely benefit from the lower parasite prevalence. The diverse functions of Dufour's gland secretions in social insects may provide important cues for such costs. For instance, ants employ their Dufour's gland secretions as trail, recruitment and alarm pheromones, (Bradshaw et al. 1979; Coll et al. 1987; Blatrix et al. 2002), as markers of territory boundaries (Salzemann et al. 1992), to protect eggs from worker policing (Vander Meer and Morel 1995) and in fights over reproductive dominance (Heinze et al. 1998). Although resistance to Dufour-manipulation provides advantages during parasite encounter, it might likewise interfere with any of these other processes important to the social structure and functioning of a colony. Indeed, the Dufour's gland extracts of fertile *T. longispinosus* workers induces similar levels of intra-colonial aggression among host workers as the parasite's gland content, suggesting that the parasite exploits the fertility signal used to maintain the reproductive hierarchy within *Temnothorax* host colonies (Brandt et al. 2006). The geographic variation in slavemaker-induced behavioural changes reported in this study may provide the ideal background against which to measure the potential costs of resistance to manipulation by slavemakers.

In conclusion, our study suggests that host manipulation can have profound effects on the success of parasites. Whether parasites manipulate their host to promote access to the host's resources or to increase their chances of transmission, host counter-adaptations thus have the potential to alter the population dynamics of manipulative parasites and their hosts. Such ecological feedbacks directly impact the selective pressures acting on the host and hence the eco-evolutionary dynamics between parasites and hosts (Boots et al. 2009). Determining which factors control the spatial dynamics of manipulative parasites therefore contributes to our understanding of the co-evolutionary process itself.

Chapter **5**

Temnothorax pilagens sp. n. – a new slavemaking species of the tribe Formicoxenini from North America (Hymenoptera, Formicidae).

Bernhard Seifert, **Isabelle Kleeberg**, Barbara Feldmeyer, Pamminger Tobias, Evelien Jongepier and Susanne Foitzik

ABSTRACT

A new species of the ant genus *Temnothorax* Forel, 1890 – *Temnothorax pilagens* **sp. n.** is described from eastern North America. *T. pilagens* **sp. n.** is an obligate slave-making ant with two known hosts: *T. longispinosus* (Roger, 1863) and *T. ambiguus* (Emery, 1895). A differential diagnosis against *Temnothorax duloticus* (Wesson, 1937), the other dulotic congener from the Nearctic, is presented and a biological characteristics of the new species is given.

Keywords: *Temnothorax*, Nearctic region, dulosis, slave-raiding behavior, morphometrics

INTRODUCTION

Three slave-making species of the *Temnothorax* genus group (Bolton 2003) of the ant tribe Formicoxenini are known from North America. Each of the three represents an unmistakable combination of phenotypic characters. They all use species of the genus *Temnothorax* Forel, 1890 as hosts and cluster genetically with species of this genus (Beibl et al. 2005). The first species and outgroup to all the others (Beibl et al. 2005), *Protomognathus americanus* (Emery, 1895), is characterized by an elongated, semi-rectangular head capsule with extremely long antennal scobes that fully accommodate the short and flattened scape when it is folded back. These characters are a convergence to the Holarctic genus *Harpagoxenus* that belongs to the distantly related *Leptothorax* genus group. The second one, *Temnothorax duloticus* (Wesson, 1937), shows an acute, frontoventrally directed dent on the postpetiolar sternite, a high petiole with a bulky, truncate node that slopes down to the caudal cylinder with a distinct step. This particular petiolar shape and the reduction of antennal segments to 11 resemble the situation in the subgenus *Mychothorax* Ruzsky, 1904 of the genus *Leptothorax* Mayr, 1855. However, *T. duloticus* differs from the latter by the absence of a curved transverse carina on the stipes of the maxillae. On the third species, as yet taxonomically undescribed, only little information exists to date (Herbers and Foitzik 2002; Beibl et al. 2005). Its phenotype is an unmistakable combination of an acute, frontoventrally directed dent on postpetiolar sternite, a stout, hump-backed mesosoma, small scape length, a high petiole that is in lateral aspect roughly triangular, a wide petiole and reduced mandibular dentition. Robin Stuart was the first who recognized the new species (Herbers and Foitzik 2002). We follow his proposal to name this slave-making species *Temnothorax pilagens* **sp. n.** and provide here the formal taxonomic description and differential diagnosis plus a short comparative life history.

MATERIAL

Type Material

Holotype worker labelled "USA:44.7560°N, 86.0711°W, Michigan: Sleeping Bear National Lakeshore, 180 m, 2013.05.27 – M509" and "Holotype *Temnothorax pilagens* Seifert et al."; 3 paratype workers from the holotype nest and the same collecting data; 1 paratype gyne labelled "USA:44.7560°N, 86.0711°W, Michigan: Sleeping Bear National Lakeshore, 180 m, 2013.05.27 – M502"; 4 paratype workers, each on a separate pin, labelled "USA: 44.8435°N, 86.0612°W, Michigan: North Bar Lake Dunes, 185 m, 2013.05.31 – Q534.3", "USA: 44.8435°N, 86.0612°W, Michigan: North Bar Lake Dunes, 185 m, 2013.05.31 – Q534.1", "USA: 44.8435°N, 86.0612°W, Michigan: North Bar Lake Dunes, 185 m, 2013.05.31 – Q534.2", "USA: 44.8435°N,

86.0612°W, Michigan: North Bar Lake Dunes, 185 m, 2013.05.30 – Q520"; 1 paratype gyne labelled "USA:44.7560°N, 86.0711°W, Michigan: Sleeping Bear National Lakeshore, 180 m, 2013.05.27 – M502". Different codes after the date sequence refer to different nests. All material is stored in the Senckenberg Museum of Natural History in Goerlitz.

Comparative material of *Temnothorax duloticus* (Wesson, 1937) consisted of five workers and one gyne labelled "USA: 39.9927°N, 83.2575°W, Ohio: Prairie Oaks Metro Park, 270 m, 2013.06.04 – Z698" and three workers labelled "USA: 40.1469°N, 83.0381°W, Ohio: Highbanks Metro Park, Olentangy River, 239 m, 2013.06.07 - G827".

METHODS

Recording of morphological data

Twenty morphometric characters currently being used in taxonomy of Palaearctic *Temnothorax* (Seifert 2006; Csösz et al. 2013) were investigated. In bilaterally recorded characters, arithmetic means of both body sides were calculated. All measurements were made on mounted and dried specimens using a pin-holding stage, permitting full rotations around X, Y, and Z axes. A Leica M165C high-performance stereomicroscope equipped with a 2.0 planapochromatic objective (resolution 1050 lines/mm) was used at magnifications of x120-384. The mean relative measuring error over all magnifications was 0.3%. A Schott KL 1500 cold–light source equipped with two flexible, focally mounted light–cables, providing 30°–inclined light from variable directions, allowed sufficient illumination over the full magnification range and a clear visualization of silhouette lines. A Schott KL 2500 LCD cold–light source in combination with a Leica coaxial polarized–light illuminator provided optimal resolution of tiny structures and microsculpture at highest magnifications. Simultaneous or alternative use of the cold-light sources depending upon the required illumination regime was quickly provided by regulating voltage up and down. A Leica cross-scaled ocular micrometer with 120 graduation marks ranging over 52 % of the visual field was used. To avoid the parallax error, its measuring line was constantly kept vertical within the visual field. Measurements of body parts always refer to real cuticular surface and not to the diffuse pubescence surface.

Z-stack photographs were made with a Leica Z6 APO photomicroscope equipped with 2.0x planapochromatic objective and the automontage software Leica application suite version 3.

CL maximum cephalic length in median line; the head must be carefully tilted to the position with the true maximum. Excavations of occiput and/or clypeus reduce CL.

CS cephalic size; the arithmetic mean of CL and CW, used as a less variable indicator of body size.

CW maximum cephalic width; the maximum is found in *Temnothorax* and *Leptothorax* usually across and including the eyes.

EYE eye-size index: the arithmetic mean of the large (EL) and small diameter (EW) of the elliptic compound eye is divided by CS, i.e. EYE=(EL+EW)/(CL+CW). All structurally visible ommatidia are considered.

FCDV tangens of divergence angle of frontal carinae measured along a 50 μm section from FRS level caudad. A cross-scaled ocular micrometer and full magnification is used.

FRS distance of the frontal carinae immediately caudal of the posterior intersection points between frontal carinae and the lamellae dorsal of the torulus. If these dorsal lamellae do not laterally surpass the frontal carinae, the deepest point of scape corner pits may be taken as reference line. These pits take up the inner corner of scape base when the scape is fully switched caudad and produce a dark triangular shadow in the lateral frontal lobes immediately posterior of the dorsal lamellae of scape joint capsule (Fig. 1 in Seifert 2006).

MGr depth of metanotal groove or depression, measured from the tangent connecting the dorsalmost points of promesonotum and propodeum; here given as per cent ratio of CS.

MH in workers: with mesosoma in lateral view and measured orthogonal to "longitudinal mesosomal axis", MH is the longest measurable section line of mesosoma at mesopleural level (not height above all). "Longitudinal mesosomal axis" in lateral view is defined as straight line from the centre of propodeal lobe to the border point between anterior pronotal shield and propleuron. In gynes it is the longest section line directed perpendicular to the straight dorsal profile line of mesosoma (formed by mesonotum and scutellum). The lower reference point is usually lowest part of mesopleuron.

MW maximum mesosoma width (worker); maximum mesosoma width anteriorly of the tegulae (gynes).

ML in workers: mesosoma length from caudalmost point of propodeal lobe to transition point between anterior pronotal slope and anterior propodeal shield (preferentially measured in lateral view; if the transition point is not

well defined, use dorsal view and take the centre of the dark-shaded borderline between pronotal slope and pronotal shield as anterior reference point). In gynes: length from caudalmost point of propodeal lobe to the most distant point of steep anterior pronotal face.

PEH   maximum petiole height. The straight section of ventral petiolar profile at node level is the reference line perpendicular to which the maximum height of petiole node is measured.

PEL   diagonal petiolar length in lateral view; measured from anterior corner of subpetiolar process to dorsocaudal corner of caudal cylinder.

PEW   maximum width of petiole.

PoOc   postocular distance. Use a cross-scaled ocular micrometer and adjust the head to the measuring position of CL. Caudal measuring point: median occipital margin; frontal measuring point: median head at the level of the posterior eye margin. Note that many heads are asymmetric and average the left and right postocular distance (Fig.2 in Seifert 2006).

PPW   maximum width of postpetiole.

SL   maximum straight line scape length excluding the articular condyle as arithmetic mean of both scapes.

SP   maximum length of propodeal spines; measured in dorsofrontal view along the long axis of the spine, from spine tip to a line, orthogonal to the long axis, that touches the bottom of the interspinal meniscus (Fig.3 in Seifert 2006). Left and right SP are averaged. This mode of measuring less ambiguous than other methods but results in some spine length in species with reduced spines.

SPBA   the smallest distance of the lateral margins of the spines at their base. This should be measured in dorsofrontal view, since the wider parts of the ventral propodeum do not interfere with the measurement in this position. If the lateral margins of spines diverge continuously from the tip to the base, a smallest distance at base is not defined. In this case, SPBA is measured at the level of the bottom of the interspinal meniscus.

SPST   distance between the centre of propodeal stigma and spine tip. The stigma centre refers to the midpoint defined by the outer cuticular ring but not to the centre of real stigma opening that may be positioned eccentrically.

SPTI   the distance of spine tips in dorsal view; if spine tips are rounded or thick take the centres of spine tips as reference points.

TrScuC   density of transverse microsculpture elements in centromedian vertex. Count the transverse elements crossing a median line of ± 120 μm at a central place with most such elements. Unit: number of elements / mm.

RESULTS

***Temnothorax pilagens* n. sp.**

http://zoobank.org/816821D0-8B10-4BEE-889D-CA45A80ABFDD

http://species-id.net/wiki/Temnothorax_pilagens

Etymology. The species epithet refers to the slave raiding behaviour of the new ant species (from Latin: pilare, English: to pluck, plunder, pillage).

Description and differential diagnosis. The differential diagnosis is done in relation to the congeneric slave-making species *Temnothorax duloticus*. Measurements and indices in the text of description are the arithmetic means of the whole samples (for full data see Table 5.1).

Worker (Figs. 5.1, 5.3 and 5.5, Table 5.1): Body size close to the genus average of *Temnothorax*, mean CS 645 μm. Head relatively broader [CL/CW 1.048 but 1.078 in *duloticus*], in dorsal aspect with strongly convex postocular sides and nearly linear, converging genae. Postocular distance smaller [PoOc/CL 0.364 but 0.394 in *duloticus*]. Antennae with 11 segments only, scape strikingly shorter [SL/CS 0.721 but 0.801 in *duloticus*]. Vertex finely longitudinally rugulose, distance between rugulae on central vertex 12 μm. The rugulae are connected by very delicate transverse anastomosae, which have on central vertex a mean distance of 12-14 μm. Clypeus finely longitudinally carinulate and in full-face view with straight or feebly emarginated anteromedian margin. Only the apical and subapical dent of the masticatory margin of the mandibles are fully developed and acute, the following dents are reduced to an undulating line of 3-6 shallow waves [in *duloticus* at least the first three dents are fully developed and the whole dentition is more similar to the normal *Temnothorax* situation]. Genae each with 2-6 semi-erect to erect setae [these are absent in *duloticus*]. Mesosoma massive, in lateral view with strongly convex dorsal profile, appearing hump-backed – i.e., much more compact and shorter than in *duloticus* [ML/CS 1.174 but 1.272 in duloticus]. Spines significantly shorter and thicker [SPST/CS 0.364 and SP/CS 0.300 but 0.425 and 0.361 respectively in duloticus]; spines in lateral view semi-erect, deviating from longitudinal axis of the mesosoma by 27-35°; in dorsal view diverging by 36-39° and with a larger basal distance [SPBA/CS 0.382, but 0.317 in *duloticus*]. The entire

mesosoma exhibits a rugulose-microreticulate sculpture. Petiolar node in lateral view with a straight or weakly concave frontal profile forming with the short dorsal plane an angle of 81-91°; caudal petiolar profile steeply but linearly sloping down to junction with postpetiole [in *duloticus* there is a distinct step in the caudal slope caused by a prolongation of the caudal cylindric part of petiole]. Petiole clearly shorter [PEL/CS 0.452 but 0.517 in *duloticus*].

Figure 5.1 *Temnothorax pilagens* **sp. n.**, worker, head of holotype in dorsal view.

Figure 5.2 *Temnothorax duloticus,* worker, head in dorsal view.

Postpetiolar sternite in lateral view with a strongly developed, triangular dent, directed anteroventrad, comparable to situation in *duloticus*. Dorsum of petiole node in dorsal view 1.7-2.0 fold wider than long, postpetiole in dorsal view roughly trapezoidal and much wider than in any independent *Temnothorax* species, PPW/CS 0.491. Whole surface of petiolar and postpetiolar nodes coarsely microreticulate. Surface of 1st gaster tergite smooth and shining, but with a very delicate (sculpture lines only 0.5 µm thick), patchily missing microreticulum [in *duloticus* there is nowhere a connected microreticulum – it is reduced to isolated, scattered structures in the form of an "X" or of a matchstick man]. All dorsal body surfaces with setae of medium length. Dorsal head dark brown. Mesosoma, waist and appendages yellowish, propodeum, meso- and metapleuron sometimes darker brownish. Gaster tergites yellowish, often with small brown bands at posterior margin; the first tergite usually shows big brown patches on each side that may fuse medially in some specimens, then covering 70% of total surface.

Gyne (only one gyne was evaluated in both *pilagens* **sp. n.** and *duloticus*): Head size similar to the genus average of *Temnothorax*, mean CS 673 µm. Head very short [CL/CW 0.982 but 1.044 in *duloticus*], in full-face view with strongly convex

postocular sides, a feebly concave occipital margin and linear, converging genae. Postocular distance very short [PoOc/CL 0.341 but 0.401 in *duloticus*]. Antennae with 11 segments only, scape very short [SL/CS 0.683 but 0.748 in duloticus]. Vertex longitudinally rugulose, distance between rugulae on central vertex 15 μm, the interspaces between rugulae with reticulate microsculpture. Clypeus finely longitudinally carinulate and in full-face view with feebly notched anteromedian margin. The three apical dents of the mandibular masticatory margin are fully developed and acute, the following four dents are reduced to denticles. Mesomoma very small for *Temnothorax* in general, but not smaller than in *duloticus* [ML/CS 1.484, MW/CS 0.904, MH/CS 0.868].

Table 5.1 Nineteen shape characters and one size character in *Temnothorax pilagens* **n. sp.** and *T. duloticus* including data extracted from the photo of a paratype specimen of *T. duloticus*. F- and p-values of an univariate ANOVA are given and the shape variables are arranged by decreasing F.

	T. pilagens (*N* = 8)	ANOVA *F P*	*T. duloticus* (*N* = 6)	*T. duloticus* photo of paratype
CS [μm]	645 ± 29 [603,684]	0.22 n.s.	639 ± 22 [616,678]	562
PoOc/CL	0.364 ± 0.006 [0.354,0.371]	91.76 <0.001	0.394 ± 0.005 [0.388,0.400]	0.377
SL/CS	0.721 ± 0.019 [0.684,0.742]	87.55 <0.001	0.801 ± 0.008 [0.785,0.807]	0.831
ML/CS	1.174 ± 0.027 [1.142,1.229]	55.96 <0.001	1.272 ± 0.020 [1.242,1.300]	1.296
PEL/CS	0.452 ± 0.011 [0.438,0.466]	50.18 <0.001	0.517 ± 0.023 [0.487,0.538]	0.538
SPST/CS	0.368 ± 0.015 [0.343,0.383]	43.71 <0.001	0.425 ± 0.017 [0.397,0.440]	no data
SP/CS	0.300 ± 0.014 [0.268,0.311]	41.90 <0.001	0.361 ± 0.022 [0.327,0.392]	no data
SPBA/CS	0.382 ± 0.027 [0.351,0.411]	21.23 0.001	0.317 ± 0.021 [0.296,0.354]	0.281
MPGR/CS [%]	0.13 ± 0.27 [0.0,0.8]	14.60 0.002	0.89 ± 0.47 [0.1,1.4]	1.09
PnHL/CS	0.158 ± 0.008 [0.146,0.172]	11.86 0.005	0.179 ± 0.014 [0.164,0.197]	no data
PEH/CS	0.425 ± 0.014 [0.400,0.444]	9.00 0.011	0.451 ± 0.019 [0.425,0.476]	0.440
CL/CW	1.048 ± 0.023 [1.019,1.084]	7.00 0.021	1.077 ± 0.014 [1.058,1.099]	1.076
MW/CS	0.656 ± 0.014 [0.635,0.672]	2.77 n.s.	0.644 ± 0.014 [0.630,0.671]	0.630
PPW/CS	0.491 ± 0.018 [0.452,0.506]	2.06 n.s.	0.503 ± 0.012 [0.489,0.520]	0.468
TrScuC [n / mm]	77.2 ± 5.4 [69,85]	2.02 n.s.	73.5 ± 4.0 [71,80]	70
EYE/CS	0.236 ± 0.008 [0.229,0.249]	1.73 n.s.	0.231 ± 0.005 [0.225,0.237]	no data
PEW/CS	0.297 ± 0.010 [0.284,0.313]	1.34 n.s.	0.304 ± 0.012 [0.284,0.318]	0.306

SPTI/CS	0.469 ± 0.026 [0.434,0.505]	0.51 n.s.	0.479 ± 0.030 [0.445,0.527]	no data
FRS/CS	0.389 ± 0.007 [0.378,0.399]	0.10 n.s.	0.390 ± 0.010 [0.376,0.404]	0.381
MH/CS	0.569 ± 0.018 [0.550,0.600]	0.10 n.s.	0.572 ± 0.026 [0.534,0.604]	0.577

Figure 5.3 *Temnothorax pilagens* **sp. n.**, worker, holotype in lateral

Figure 5.4 *Temnothorax duloticus,* worker, lateral view.

Spines well-developed and acute but significantly shorter and relatively thicker than in *duloticus* [SPST/CS 0.364 and SP/CS 0.265 but 0.457 and 0.360 respectively in *duloticus*]; spines in lateral view very weakly erected, deviating from longitudinal

axis of mesosoma by 20°; in dorsal view with a very large basal distance and weakly diverging [SPBA/CS 0.482, SPTI/CS 0.479; in *duloticus* more clearly diverging, SPBA/CS 0.434 and SPTI/CS 0.530]. Whole mesosoma with rugose-microreticulate sculpture that is on mesonotum and mesopleuron less developed. Petiolar node in lateral view very high and with a weakly concave frontal profile forming with the short dorsal plane an angle of 80°; caudal petiolar profile steeply and almost linearly sloping down to junction with postpetiole [in *duloticus* there is a distinct step in the caudal slope caused by a significant prolongation of the caudal cylindric part of petiole]. Petiole clearly shorter than in *duloticus* [PEL/CS 0.496 vs. 0.551]. Postpetiolar sternite in lateral view with a strongly developed, triangular dent, directed anteroventrad, comparable to situation in *duloticus*. Dorsum of petiole node in dorsal view 1.9 fold wider than long, postpetiole in dorsal view broadly cordate and much wider than in any independent *Temnothorax* species, PPW/CS 0.491. Whole surface of petiolar and postpetiolar nodes strongly microreticulate. Surface of 1st gaster tergite smooth and shining but with a very delicate (sculpture lines only 0.5 µm thick), patchily missing microreticulum [in duloticus the microreticulum more incomplete - frequently reduced to isolated, scattered structures in the form of an "X" or of a matchstick man]. All dorsal body surfaces with setae of medium length, the longest on occiput are 74 µm long. Head, mesosoma and waist brown, appendages yellowish to yellowish brown. Gaster tergites yellowish brown, a lighter yellowish patch is at the base of 1st tergite.

Figure 5.5 *Temnothorax pilagens* **sp. n.,** worker, mesosoma of holotype in dorsal view.

Figure 5.6 *Temnothorax duloticus*, worker, mesosoma in dorsal view.in dorsal view.

DISCUSSION

The original description of *T. duloticus* and the photos in antweb.org (CASENT0103163) of a paratype specimen of the type colony from Ohio: Jackson Country: White's Gulch clearly show the heterospecifity of *T. pilagens*. The conspecifity of our two *T. duloticus* samples with the paratype is indicated by agreement in mesosomal and petiolar shape characters and by the similarity of NUMOBAT data. Both spines of the paratype are broken off - excluding to assess the characters SP, SPTI and SPST. Furthermore, the spatial adjustment of the photo excluded estimating the characters EYE and PnHL. The remaining 15 characters (see Table 5.1) could be extracted from the image – albeit with some distortion. Using these characters and running the paratype in an LDA as wild-card, it is allocated with a posterior probability of p = 1.000 to the same cluster with our *T. duloticus* samples. The same clear allocation is provided by the 1st factor of a principal component analysis being –0.87 ± 0.20 [-1.07,-0.42] in eight specimens of *T. pilagens* sp. n. and 0.94 ± 0.37 [0.34,1.28] in seven specimens of *T. duloticus*, with the paratype scoring 1.28.

Several discriminatory characters allow easy separation of *T. pilagens* **n. sp.** and *T. duloticus* workers. Despite low sample size, there are highly significant differences in 55% of the tested characters (Table 5.1). The characters SL/CS or PoOc/CL alone should provide a safe and parsimonious numeric species delimitation. There is also no doubt that experienced observers can distinguish the two dulotic species by simple eye-inspection integrating subjective impressions on mesosomal, petiolar, cephalic and spine shape and mandibular dentition (compare Figs. 5.1-5.6).

Short biological characteristics of *Temnothorax pilagens* sp. n.

Biology and host species. Obligate slave-making ant with two known hosts: *T. longispinosus* (Roger, 1863) and *T. ambiguus* (Emery, 1895). Mitochondrial DNA phylogeny indicates sister species relationship with *T. longispinosus* (Beibl et al. 2005). *Geographical range.* Nearctic. North-eastern parts of the United States and possibly south-eastern Canada. Habitat. Forest, woodland, parks. Preferentially wooded sites with little understory, and a high density of suitable nest sites, such as acorns, hickory nuts and sticks. *Abundance.* Patchy - occurrence depends on high density of suitable host populations; so far only known from three sites in the Northern US: Niquette Bay State Park, Vermont (44.3513°N 73.1156°W; 8 colonies collected in 1986; Herbers & Foitzik 2002), E.N. Huyck Preserve, Rensselaerville, New York (42.3133°N 74.1012°W; 7 colonies collected in 2002 and 2003; Beibl et al. 2005) and Sleeping Bear National Lakeshore, Empire, Michigan (44.7560°N, 86.0711°W, 6 and 44 colonies collected in 2011 and 2013, respectively). In all three populations, *T. pilagens* was enslaving *T. longispinosus* and *T. ambiguus*; many nests with slaves of both host species. In Vermont and New York, this species has not been re-collected recently, despite regular search by our group. Plotting data from 2011 in Michigan: between 0.08 and 0.02 slave-making colonies per m^2 (at an average of 4.66 host colonies per m^2). *Temnothorax pilagens* occurs more often in sites with both host species, than in areas with *T. longispinosus* colonies only (Fisher Test; *P* = 0.019). We did not sample *Temnothorax* communities with *T. ambiguus* only. *T. pilagens* colonies more often contained a mixed slave workforce than slaves of a single host species (Chi_1^2 = 49.59, *P* < 0.001). *Nest construction.* As its hosts, *T. pilagens* nests occur in preformed cavities in acorns, hickory nuts or sticks. *Colony demography.* Strictly monogynous. Most likely polydomous at least during the summer season: 72% of the nests were queenless with queenright nests close-by and neighboring nests merged in the laboratory without aggression. Nests contain on average four slave-making workers (ranging from 0 to 16 *T. pilagens* workers) and 13 *Temnothorax* slaves (ranging from 2 to 50 workers) - but see Herbers & Foitzik (2002) for a nest with 27 *T. pilagens* and 55 slave workers. *Colony foundation.* Four colonies with no *T. pilagens* workers, but a founding queen were collected. All four contained *T. longispinosus* slaves only. *Slave-raids.* Obligatory slave hunter of *Temnothorax longispinosus* and *T. ambiguus*. Raids are performed either by a scout alone or via group recruitment of up to four slavemaking workers forming a raiding column headed by a scout. Slave raids resemble more those of its congener *T. duloticus* than that of *P. americanus* (Alloway 1979). This is especially true for the frequent and effective use of the stinger in fights: well-aimed stings from a caudal direction between head and thorax cause paralysis in hosts followed by quick death. Effective sting use is likely facilitated by morphological adaptations, such as strongly developed flexor-muscles in the petiole and postpetiole allowing for easy gaster flexion. Similar behavioural strategies and morphological traits are also found in *T. duloticus*, but not in *P. americanus*. Hosts attacked by *T. pilagens*

show little or only delayed flight responses. Occasionally, host workers try to drag slavemakers out of the nest, and only respond aggressively when attacked by them. The low host responsiveness towards invading *T. pilagens* indicate reduced or supressed enemy recognition. Due to their most effective stinging behaviour, *T. pilagens* can cause high rates of host casualties (ranging from 5% to 100%). As the high variation in the rate of casualties indicates, raids can be highly aggressive or relatively peaceful; the latter was often found in raids against queenless host nests. Slave-makers do not only take brood from the attacked host nests, but in 6 of the observed 11 raids, they also carry adult host workers back to their nest and integrated them into the slave workforce.

Chapter **6**

The placid slavemaker: avoiding detection and conflict as an alternative, peaceful raiding strategy

Isabelle Kleeberg and Susanne Foitzik

ABSTRACT

Host entry is a crucial step in a parasite's life cycle. When parasites manage to circumvent host detection, exploitation of host resources is facilitated, as host defenses have not to be counteracted. Social parasites exploit animal societies and likewise, detection avoidance can be beneficial. Yet, due to strong selection pressures, hosts of socially parasitic slavemaking ants often recognize them as enemies, so that slavemakers use open force to raid host colonies. These fights however, prohibit the enslavement of adult host workers ("eudulosis"), which cannot be manipulated to work for them. Instead, they steal brood during raids, and enslave those upon emergence. In contrast to the violent raids of most slavemakers, no aggression occurs during most of the raids of the newly described slavemaker *Temnothorax pilagens*. Thereby, *T. pilagens* regularly induces adult host workers to be part of their slave workforce. We demonstrate that non-enslaved colonies of its host species respond to this slavemaker with little aggression. We further investigate how the slavemaker circumvents recognition and show that chemical resemblance of host profiles might explain the low aggressive responses. But which parameters determine whether slave raids escalate, resulting in carnage among defenders? Our experiments reveal that host aggression is counterproductive as aggressive host colonies suffer from more fatalities during raids, but cannot save more brood. The slavemaker, however, benefits from not eliciting fights, as it doubles its enslaved workforce by capturing brood plus adult host workers. Hence undercutting recognition allows the slavemaker to avoid raid escalation with its associated fitness benefits.

INTRODUCTION

Conflict management is crucial for social animals to prevent escalation of fights among group members (Aureli et al. 2002). Conflict escalation within social groups can be avoided through the establishment of dominance hierarchies, the use of ritualized appeasement gestures or displays or through chemical signaling (Preuschoft and van Schaik 2000; van Wilgenburg et al. 2005; Ratnieks et al. 2006; Hick et al. 2014). Encounters between social groups are more difficult to prevent from escalating, but if both sides benefit from it, mediating behavioral mechanisms can evolve (e.g. Neat et al. 1998). For example, the ritualized tournaments of desert honeypot ants allow to avoid costly fights between colonies of similar size (Hölldobler 1976). Social insects often rely on chemical information to decide whether an interaction will remain friendly or escalate into a fight. In particular, ants use sophisticated chemical communication systems to differentiate friends from foes (van Zweden and D´Ettorre 2010). Still, ant societies can fall victim to social parasites, who exploit their altruistic behaviors (Thomas et al. 2005). Social parasites have to gain entry into their well-protected societies and those interactions often result in escalated conflicts. Aggressive conflicts between groups of antagonistic species are even more difficult to keep from escalating, but if enemies manage to stay undetected, open fights can be avoided, benefitting the parasite, but not the host.

Many social parasites have evolved refined chemical strategies to avoid host recognition. Chemical insignificance is a rare strategy where the parasite lacks recognition cues on its cuticle (Lenoir et al. 2001). Host ants facing these chemically "invisible" parasites will not recognize them and unintentionally integrate them into their colony (e.g. D´Ettorre and Errard 1998). As hydrocarbons also function as desiccation barriers (Gibbs and Rajpurohit 2010), there are clear constraints to this strategy, which might explain why it is so rare. More commonly used is chemical mimicry, where social parasites actively biosynthesize substances to mimic the hosts' profile (Dettner and Liepert 1994; Lenoir et al. 1997). A restriction for this strategy is that the parasite has to focus on a single host, whose profile it will imitate. Chemical camouflage is more flexible and more common, because social parasites using this strategy acquire the host´s odor through rubbing, allogrooming, and trophallaxis or from nest material (Dettner and Liepert 1994; Lenoir et al. 2001; D´Ettorre et al. 2002; Tsuneoka and Akino 2012). The problem here is the first host contact, when the parasites had as yet no time to acquire host chemicals. Chemical manipulation of host behavior is a fourth and commonly used strategy by which parasites use offensive chemicals to disrupt the host defense systems. While entering host colonies social parasites use substances released from the Dufour´s, the Pygidial or the Poison gland, which serve as manipulative alarm signals or chemical weapons (Lenoir et al. 2001) and as appeasement substances to

circumvent aggressive escalations, such as attacks directed towards the social parasite (Mori et al. 2000; Brandt et al. 2006)

Slavemaking ants are social parasites that have to successfully invade host colonies recurrently during their life cycle, in contrast to many other social parasites, such as the workerless inquilines, that do so only once in their life. Most slavemakers are obligate parasites that are contingent on their slave work force, which oversees colony maintenance including caring for the parasites' offspring. During destructive slave raids, slavemaking ants invade free-living host colonies to steal brood (Foitzik and Herbers 2001). These raids often escalate into violent fights, as hosts fiercely defend their young. Slavemaking ants can show high prevalence, so that the resulting parasite pressure can lead to the evolution of fine-tuned host defense strategies, including enemy recognition, flight and fighting strategies (Foitzik et al. 2001; Bauer et al. 2009; Jongepier et al. 2014, Chapter 3; Kleeberg et al. 2014, 2015, Chapters 1-2). These host defenses might be one of the reasons why slavemakers generally do not employ a sneaking strategy or avoid aggressive escalations during raids. Instead they enter host colonies forcefully and openly attack adult hosts. The downside of this aggressive strategy is that adult host workers cannot be manipulated to join slavemaking nests; instead slavemakers rely entirely on the enslavement of the hosts´ brood (Schumann 1992; Mori et al. 2001; Herbers and Foitzik 2002).

Temnothorax longispinosus, *T. curvispinosus* and *T. ambiguus*, hosts of the slavemakers *Protomognathus americanus*, *T. duloticus* and the focal species here, *T. pilagens*, generally recognize slavemakers as enemies (Alloway 1990) and respond to invasions with counterattacks (Alloway 1979; Pamminger et al. 2011). Next to aggression as a beneficial anti-parasite defense (Pamminger et al. 2012; Kleeberg et al. 2014, Chapter 1), host colonies also react with fast nest evacuation (Alloway 1979; Jongepier et al. 2014, Chapter 3). Slavemakers try to undermine these adaptive host responses through behavioral (e.g. nest entrance guarding; Alloway 1979) or chemical strategies, such as manipulation through the use of the Dufour´s gland (Jongepier et al. 2015, Chapter 4; Brandt et al. 2006). Slave raids by *T. duloticus* and *P. americanus* often escalate in open fights with the consequence that many hosts and slavemakers die during these encounters (Foitzik and Herbers 2001). Here, we focus on the newly described North American slavemaker *T. pilagens* (Seifert et al. 2014, Chapter 5), a close relative of the two other slavemakers (Beibl et al. 2005, note *T. pilagens* was then undescribed and referred to as *T.* spp.), which employs two different raiding strategies as preliminary observations indicate. a) peaceful raid: during most raids, neither the slavemaker *T. pilagens* nor colonies of its two *Temnothorax* hosts show aggression. Besides stealing the host brood unmolested, the slavemaker carries the adult host workers into its own nest and integrates them into its slave workforce ("eudulosis", Kutter 1957). So far eudulosis has only been described in the slavemakers *Strongylognathus afer*, *Formica naefi* and infrequently in *Polyergus rufescens*, and it is as yet unclear how

adult enslavement is accomplished (Kutter 1957; D´Ettorre and Heinze 2001; Sanetra and Guesten 2001). Ants learn their colony odor as young adults and hence attack chemically distinct slavemakers rather than being induced to work for them. This makes eudulosis rather difficult to accomplish and might be a reason why it is so rare. b) escalated raid: Occasionally however, even in *T. pilagens*, raids can escalate. Preliminary observations indicate that *T. pilagens* switches its behavior and stings most adult hosts to death. Especially the peaceful strategy of *T. pilagens* is clearly distinct from the strategies used by the closely related slavemakers *P. americanus* and *T. duloticus*. Eudulosis, that is the reprogramming of adult workers to serve as slaves, is fascinating. However, for the fitness of host workers, which are either victim to a peaceful or an escalated raid, it might make no difference whether they are enslaved or killed, as enslaved host workers do not reproduce (Gladstone 1981).

First observations of the peaceful raiding strategy of *T. pilagens* let us predict, that this slavemaker is able to prevent raid escalation by outwitting the hosts´ recognition system. Here we analyze how *T. pilagens* manages the apparent conflict, chemically or behaviorally, to maximize its raiding success. We predict that *Temnothorax* hosts should show less aggression towards their sympatric *T. pilagens* slavemaker than towards the other two slavemaking species. To circumvent recognition and aggression, *T. pilagens* is expected to exhibit chemical adaptations, be it mimicry or insignificance, and we therefore analyzed its cuticular hydrocarbon (CHC) profile and compared it to that of its hosts and to that of the two related slavemakers *T. duloticus* and *P. americanus*. Furthermore, we staged raids in the laboratory to unveil the causes and consequences of the two strategies. We hypothesize that raids only escalate if host colonies recognize and attack the slavemaker, which will be counteracted by a killing frenzy of *T. pilagens*. Avoiding raid escalation and keeping the interaction peaceful should be more beneficial for the slavemaker, as they not only can steal brood unmolested, but can moreover exploit adult hosts.

METHODS

Ant collection and maintenance

From May to July 2013, we obtained colonies of three slavemaking ant species and their three host species *T. longispinosus*, *T. ambiguus* and *T. curvispinosus* in the North-eastern US by opening suitable nest sites (acorns and hickory nuts) on the forest floor. We collected colonies of *T. pilagens* and of its two hosts *T. ambiguus* and *T. longispinosus* at the Sleeping Bear Dunes National Lakeshore, Michigan. Colonies of the slavemaker *P. americanus* and of its host *T. longispinosus* were gathered at the Edmund Niles Huyck Preserve in Rensselearville, New York. At our study site in

Columbus, Ohio, we not only collected colonies of *P. americanus* and its host *T. curvispinosus*, but also those of a second slavemaking species, *T. duloticus* (Collection sites Table S6.1). During a field trip in May 2014, we collected additional *T. pilagens* and *T. ambiguus* colonies for the raiding experiments at the same location in Michigan (Table S6.1). We only used *T. ambiguus* host colonies for raiding experiments, as this species was more heavily parasitized. Out of 231 collected slavemaker colonies, only a single colony contained solely *T. longispinosus* slaves, 221 colonies contained only *T. ambiguus* hosts and nine colonies harbored slaves of both host species, suggesting that *T. ambiguus* is the preferred host species. Ant colonies were transferred in their natural nest sites into Ziploc bags and provided with leaf litter, bits of tuna and cookie crumbs. They were stored at 7°C until being transported back to our laboratory, transferred to artificial nest sites, counted and housed in three-chambered plastic boxes. Colonies were fed weekly with honey and crickets and stored in a climate chamber at 15°C constant. Colonies used for the raiding experiments were kept at 25°C constant to stimulate raiding activities of slavemaker colonies (Buschinger et al. 1980).

Host colony aggression tests

A series of colony aggression tests was conducted to investigate whether host colonies of *T. pilagens* respond with lower aggression towards one slavemaker than towards the other slavemaker species. Moreover, we contrasted host colony responses with those of other *Temnothorax* populations towards their respective sympatric slavemakers to rule out that sympatry could explain potential differences (Table 6.1). Twenty similar-sized host colonies (X ± SD = 26 ± 4.26 workers), each of the two hosts from Michigan, *T. ambiguus* and *T. longispinosus*, were subjected to five aggression trials. Each colony was confronted with a freshly frozen worker of their sympatric slavemaker *T. pilagens*, of two allopatric slavemakers *T. duloticus* (from Ohio with *T. curvispinosus* slaves) and *P. americanus* (one worker each from Ohio and New York from colonies with *T. curvispinosus* and *T. longispinosus* slaves, respectively) and towards a non-nestmate conspecific ant (Table 6.1). Aggression towards a non-nestmate conspecific ant has been described as constitutive aggression, reflecting the ant colonies' baseline aggressive potential (Kleeberg et al. 2014, Chapter 1). Hence, we are able to test whether host colonies show reduced or elevated aggression depending on the type of opponent. Dead opponents (frozen at -20°C) were used to eliminate behavioral variation due to the opponents' responses and to prohibit the slavemakers' usage of the Dufour´s gland secretion to manipulate host behavior (Brandt et al. 2006). Moreover, we were interested in the focal colony´s response to the chemical stimulus. The order of the five aggression trials was randomized, and a three day interval in-between each aggression test was strictly adhered to. We could only test 12 colonies per host species against a *T. pilagens* worker due to a lack of slavemaking colonies sampled in 2013. Additionally, we had to use each *T. pilagens* opponent twice. To adhere to the same experimental procedure, we also used opponents of the other species twice.

Aggressive responses did not differ between the first and the second use of an opponent (GLMM with binomial distribution and logit link function; aggressive responses were fitted as dependent variable and whether an opponent was used the first or the second time as fixed predictor. Colony ID was included as random factor: $\chi^2_1 = 0.57$, $P = 0.45$).

To compare the aggressive responses to hosts of other slavemakers, we additionally tested 20 similar-sized free-living host colonies (X ± SD = 24 ± 6.16) of *T. curvispinosus* from Ohio and *T. longispinosus* from New York against a freshly frozen conspecific non-nestmate worker and against their sympatric slavemaker(s) (Table 6.1). *T. curvispinosus* was tested against a sympatric *T. duloticus* and a *P. americanus* worker and *T. longispinosus* against a sympatric *P. americanus* worker (Table 6.1). The same number of colonies per species was tested in random order per day.

Table 6.1 Experimental set-up and sample sizes of host colony aggression tests. Slavemaker species are given in bold.

Focal host community	Focal host species	Opponent community	Opponent species	Slave species	N of colonies
Michigan	*T. longispinosus*	Michigan	*T. longispinosus*		20
		Michigan	***T. pilagens***	*T. longispinosus + T. ambiguus*	12
		New York	***P. americanus***	*T. longispinosus*	20
		Ohio	***P. americanus***	*T. curvispinosus*	20
		Ohio	***T. duloticus***	*T. curvispinosus*	20
Michigan	*T. ambiguus*	Michigan	*T. ambiguus*		20
		Michigan	***T. pilagens***	*T. longispinosus + T. ambiguus*	12
		New York	***P. americanus***	*T. longispinosus*	20
		Ohio	***P. americanus***	*T. curvispinosus*	20
		Ohio	***T. duloticus***	*T. curvispinosus*	20
New York	*T. longispinosus*	New York	*T. longispinosus*		20
		New York	***P. americanus***	*T. longispinosus*	20
Ohio	*T. curvispinosus*	Ohio	*T. curvispinosus*		20
		Ohio	***P. americanus***	*T. curvispinosus*	20
		Ohio	***T. duloticus***	*T. curvispinosus*	20

The respective opponent was placed into the host colony´s nest site, 1cm from the entrance. All interactions with the opponent were recorded for 5 minutes and the number of interacting ants and their behavior were noted. Antennation was considered a non-aggressive interaction, whereas mandible spreading, biting, stinging, holding and dragging were recorded as aggressive interactions. For statistical analyses we calculated relative aggression as the proportion of aggressive interactions from all interactions with the opponent. Additionally, we calculated an aggression index based on Errard & Hefetz (1997). As the results of the two approaches are qualitatively the same, we report the aggression index findings in the supplementary material (Supplement S6.3). The same colony aggression essays were used to assess the aggressive potential of host and slave colonies one day prior to the raiding experiments (see Raiding trials).

We first assessed whether the aggressive responses of host species from Michigan were related to the opponent species and/or host species, using a generalized linear mixed model (GLMM; lmer function implemented in the lme4 for R package; Bates et al. 2014) with binomial distribution and logit link function. We fitted the relative aggression (i.e. the total number of aggressive workers versus the total number of non-aggressive workers that interacted with the opponent) as dependent variable. Opponent species (conspecific, *T. pilagens*, *T. duloticus* and *P. americanus* from OH / NY) and host species (*T. ambiguus* or *T. longispinosus*) as well as their interaction were included as fixed predictors. We fitted host colony identity as a random factor to account for the repeated measure design and to avoid pseudo-replication, as well as an observation level random factor to control for overdispersion. For model selection, we used a backwards, stepwise selection procedure (α = 0.05). Therefore we removed non-significant terms, starting with the least significant interaction. Terms that significantly reduce the explanatory power of the model after removal, were retained in the minimal model.

In addition, to compare aggression of the three host species towards their respective social parasites with the aggression towards a non-nestmate conspecific (constitutive aggression), we assessed whether the aggressive responses of all three host species (*T. ambiguus* from MI, *T. longispinosus* from MI, *T. longispinosus* from NY and *T. curvispinosus* from OH) were related to the opponent type (conspecific or respective slavemaker species) and host species (*T. ambiguus*, *T. longispinosus* and *T. curvispinosus*), using a generalized linear mixed model with binomial distribution and logit link function. *T. curvispinosus* was tested against two slavemaker species, *T. duloticus* and *P. americanus*. As we did not find any differences in the aggressive responses towards those two slavemaker species (GLMM with binomial error distribution and Host ID as random factor: χ^2_1 = 1.705, P = 0.191), we pooled the aggression data as being one factor ("slavemaker" for *T. curvipinosus* hosts now including aggression towards *T. duloticus* and *P. americanus*). We fitted relative aggression as dependent variable. Opponent type (slavemaker vs. conspecific) and host species as well as their interaction were included as fixed predictors. Host colony nested in population identity were included as random factors to account for repeated measures and to avoid pseudo-replication, as well as an observation level random factor to control for overdispersion. To correct for multiple testing (the use of the same data set in two models), the significance level for the analyses of aggression of host species from Michigan (*T. longispinosus* and *T. ambiguus*) was adjusted using the MFDR (mean false discovery rate) approach to αc = 0.0375 according to αc = (n+1) / (n*2) * 0.05 where n denotes the number of times a data set was used. All analyses were performed in R version 3.0.2 (R Core Team 2013).

Chemical analyses

We extracted and analyzed the CHCs of between 13 and 26 workers per species sampled in 2013 (exact sample sizes see Tab. S6.2) using a GC-MS (Agilent Technologies, GC: Agilent 7890A; MS: Agilent 5975). The chemical data were further processed by integrating peak areas with the software MSD ChemStation E.02.02 (Agilent) (also see supplement S6.3 for more details on the temperature program and data analyses). Chemical substances were further identified (Table 6.2).

Using a permutational multivariate analysis of variance based on the Bray-Curtis Similarity Index (PERMANOVA) with the software Primer 6.0 (Primer-E Ltd), we constructed models for the chemical profiles per slavemaker-host combination, and included species as fixed factor and colony ID as a random factor (number of permutations: 999). Under a chemical mimicry or camouflage scenario, we would expect chemical similarities between individuals of the same host species to be roughly similar to that between slavemaker and host. We designed a second model with the hosts from Michigan and all slavemaker species, included species as fixed factor and colony ID as a random factor (number of permutations: 999).

Adding an n-C18 standard allowed us to calculate the absolute amounts of CHCs, which we compared between species. This was of interest as low amounts of CHCs in social parasites might reflect chemical insignificance (Lenoir et al. 2001). To control for interspecific body size differences, we divided the total amounts of CHCs by the mean head width per species (Supplement S6.3). Analyses were done using a generalized linear model with quasi-Poisson distribution with log link function. We fitted the total amounts of hydrocarbons [µg] divided by the mean body size as dependent variable and included species as fixed predictor. In a separate, but similar model we only included Michigan hosts and all slavemaker species. Analyses were performed in R version 3.0.2 (R Core Team 2013).

Raiding trials

To analyze how the aggressive potential of the slavemakers, slaves and host colonies influence the course and outcome of slave raids, we conducted a series of standardized experiments. A day prior to each raiding trial we separated every *T. pilagens* slavemaker worker from its colony and tested them individually in aggression assays against a living *T. ambiguus* worker from an unparasitized colony (N = 293). The slavemaker was placed into a small arena (Ø1.1cm) for two minutes to calm down until we introduced the living host worker. Host workers were beforehand isolated from their colony in a small petri dish to calm down for at least 2 minutes before confronting it with the slavemaker. The aggression assays were then observed for 3 min and 20 sec. We documented the number of interactions with the opponent and recorded the behaviors displayed by the slavemaker (similar to the colony aggression tests). We calculated the relative aggression as the

proportion of aggressive interactions of all interactions and as slavemaker workers were tested individually, we averaged the relative aggression per slavemaker colony, based on the individual slavemaker aggression tests for further statistical analyses. Additionally each host and slave colony (but containing only *T. ambiguus* workers) was tested in a standardized colony aggression essay against a dead non-nestmate conspecific ant (also see *Host colony aggression tests*). Colonies were tested against a non-nestmate conspecific ant, as it was shown recently that the aggressive potential of host colonies determines the outcome of raids by the slavemaker *P. americanus* (Kleeberg et al. 2014, Chapter 1). Each slavemaker and slave was individually color-marked for identification and more importantly to distinguish between slaves and newly enslaved adult host workers.

Raids were staged in a climate chamber at 25°C. Before and after the raiding trials, every colony was carefully counted, including the number of brood and workers to estimate how much brood and / or host workers were captured by the slavemaker colony. Each morning, we chose a color-marked host and slavemaker colony of about the same size (number of slaves and hosts; Spearman´s rank correlation: rho = 0.381, *P* < 0.001) and placed them into a triangular plastered arena with an escape nest. The distance between the slavemaker colony and the host colony was 12 cm and between the host colony and the escape nest 15.5 cm. During the day the experimental set-up was observed constantly until a raid occurred and we recorded the number of fights between hosts and slaves or slavemakers. After a raid was preformed we recorded the number of brood items saved by the host or captured by the slavemaker, the number of enslaved adult host ants and the number of dead hosts and slavemakers. All raiding experiments were conducted between July and September 2014. Out of 172 raiding set ups, 43 raids were conducted by a total of 39 colonies. Each colony conducted a single raid, with the exception of three colonies, which raided twice and thrice respectively.

We assessed whether the hosts´, the slaves´ or the slavemakers´ aggressive potential (assessed one day prior to the raiding trials) was related to the outcome of a raid. Therefore we separately analyzed the proportion of brood saved, brood captured, dead hosts and dead slavemakers and the proportion of integrated adult hosts using a set of generalized linear models (GLMs) with quasi-binomial error distribution and logit link function. We included host colony and slave colony aggression as well as the mean slavemaker aggression per colony as predictors. Similarly we analyzed the number of fights between slaves and hosts and between slavemakers and hosts using generalized linear models with quasi-Poisson error distribution and log link function. Again, we included host, slave and slavemaker aggression as predictors. Additionally we tested associations between adult enslavement and other raiding outcomes and hence analyzed the proportion of enslaved adult host ants using a generalized linear model with quasi-binomial error distribution and log link function. We included the number of fights between slavemakers and hosts, the proportion of captured brood and of killed slavemaking

and host ants and whether or not the host queen was present/ still alive after a raid (queenless vs. queenright) as predictors.

Finally, we studied demographic effects on colony aggression of hosts and slaves. We analyzed the relative aggression of either the host colony or the slave colony (with slavemakers removed) in relation to colony size using generalized linear models with quasi-binomial error distribution and logit link function. Here we included the number of workers, queens (as binary factor) and brood as predictors. For all model selections we used a backwards stepwise selection procedure based on the F-statistics ($\alpha = 0.05$). All analyses were performed using R version 3.0.2 (R Core Team 2013).

Evacuation response of hosts of the slavemaker T. pilagens

A potential defense strategy against raids of slavemaking ants is fast nest evacuation (Jongepier et al. 2014, Chapter 3). Hence we tested whether free-living host colonies of *T. pilagens* (host colonies that are sympatric with *T. pilagens*, but are presently not parasitized) evacuate their colony after confrontation with their sympatric slavemaker using standardized evacuation experiments. We chose between 48 *T. ambiguus* and 50 *T. longispinosus* colonies from Michigan (collected in 2014) and tested them either against a living *T. pilagens* worker (MI), a living, allopatric *P. americanus* worker (NY) or a living, heterospecific and non-parasitic *T. curvispinosus* worker (OH). Twenty host colonies of *T. ambiguus* were tested against *T. pilagens*, 14 against *P. americanus* and 14 against *T. curvispinosus*. Twenty host colonies of *T. longispinosus* were tested against *T. pilagens*, 15 against *P. americanus* and 15 against *T. curvispinosus*.

Next to their sympatric slavemaker species we chose the allopatric slavemaker *P. americanus* to test whether the potential absence of evacuation behavior is a general pattern of Michigan hosts against any type of intruder or whether *T. pilagens* is able to circumvent host evacuation during raids. Additionally we tested host colonies against a heterospecific host worker *T. curvispinosus* to control for potential disturbance effects of the experimental treatment itself. Each host colony was offered a new nest site within its three chambered plastic box. The living opponent was placed into the colony and the nest site was closed with tissue for 1 hour to ensure interactions of the intruder with the colony (Jongepier et al. 2014, Chapter 3). After opening the nest-site we waited 24h and documented whether the colony evacuated into a new nest site.

We analyzed the hosts evacuation response (whether or not a host colony evacuated after 24h) using a generalized linear model with binomial error distribution and logit link function. We included host colony species (*T. longispinosus* or *T. ambiguus*) and opponent species (*T. pilagens*, *P. americanus* or *T. curvispinosus*) as predictors with interaction allowed. For all model selections we used a backwards stepwise selection procedure ($\alpha = 0.05$).

RESULTS

Colony aggression of Michigan hosts towards the three slavemaker species

Colonies of the two host species *T. ambiguus* and *T. longispinosus* from Michigan did not differ in their aggressive responses (χ^2_1 = 0.008, *P* = 0.925), but the aggressive responses of both host species differed depending on the opponent species (χ^2_1 = 32.646, *P* < 0.001). Both hosts were more aggressive towards the two allopatric slavemakers than towards a conspecific non-nestmate (towards *P. americanus* (NY): *z* = 3.324, *P* < 0.001; towards *P. americanus* (OH): *z* = 3.883, *P* < 0.001; towards *T. duloticus* (OH): *z* = 2.124, *P* < 0.001). In contrast, they were slightly less aggressive towards their sympatric slavemaker, *T. pilagens* than towards non-nestmate conspecifics, albeit non-significantly (*z* = -1.821, *P* = 0.068). Moreover, *Temnothorax* hosts were more aggressive towards all other slavemaker species than towards their sympatric slavemaker *T. pilagens* (towards *P. americanus* (NY): *z* = 4.613, *P* < 0.001; towards *P. americanus* (OH): *z* = 5.086, *P* < 0.001; towards *T. duloticus* (OH): *z* = 3.607, *P* < 0.001; Fig. 6.1a). The aggressive responses of MI hosts did not differ between *P. americanus* from Ohio, New York and *T. duloticus* from Ohio (*T. duloticus* compared to *P. americanus* (OH): *z* = 1.777, *P* = 0.075 and to *P. americanus* (NY): *z* =1.210, *P* = 0.226; *P. americanus* (OH) compared to *P. americanus* (NY): *z* = -0.571, *P* = 0.568).

Colony aggression towards conspecifics and sympatric slavemakers

A comparison of all hosts revealed an interaction between host species and opponent type (slavemaker vs. conspecific x host species - interaction: χ^2_3 = 12.442, *P* = 0.006; Fig. 6.1b). While both *T. pilagens* hosts were about as aggressive towards a conspecific than towards their sympatric slavemaker, with *T. ambiguus* being slightly less aggressive (*T. longispinosus*: *z* = -0.701, *P* = 0.483; *T. ambiguus*: *z* = -1.769, *P* = 0.077), the two host populations, *T. longispinosus* NY and *T. curvispinosus* OH, showed higher aggression towards their respective slavemaker species than towards conspecifics (*T. longispinosus* – *P. americanus* (NY): *z* = 2.670, *P* = 0.007; *T. curvispinosus* - *T. duloticus* (OH) / *P. americanus* (OH): *z* = 2.377, *P* = 0.017), supporting earlier findings of enemy recognition (Alloway 1990). All three host species did not differ in their constitutive aggression, tested towards a non-nestmate conspecific ant (χ^2_3 = 4.451, *P* = 0.108), with the exception of *T. curvispinosus* being less aggressive than *T. ambiguus* (*t* = -2.064, *P* = 0.042).
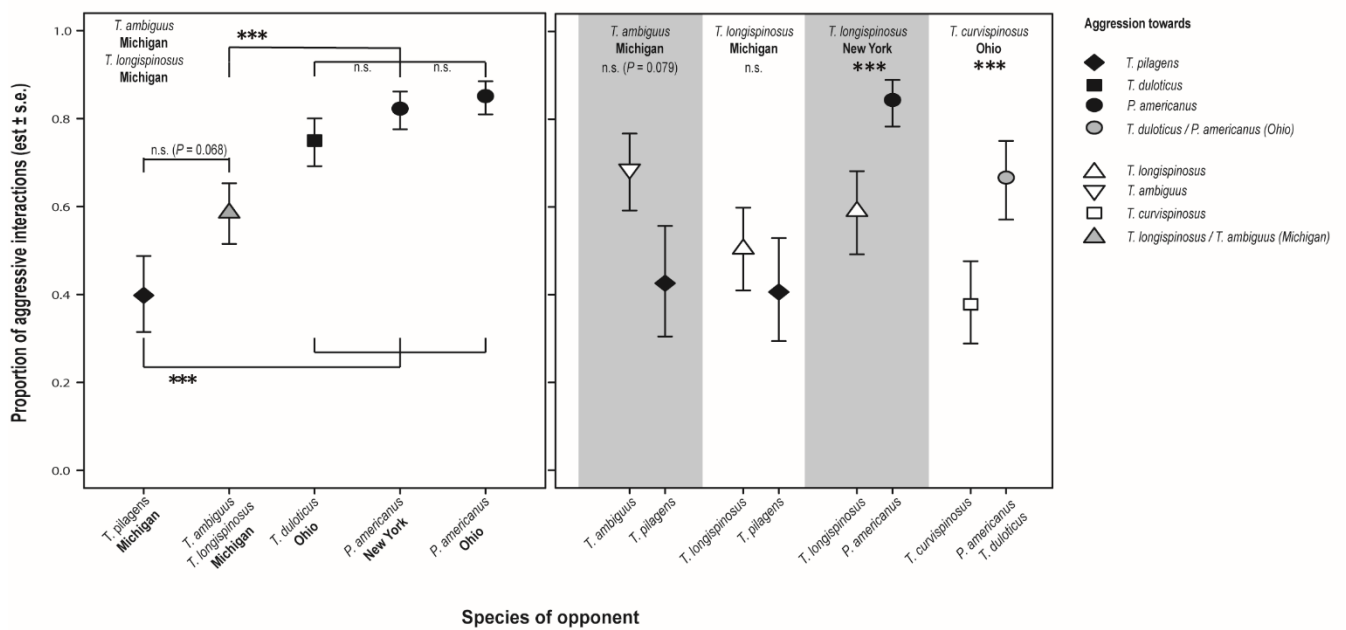
Figure 6.1 Host colony aggression, measured as the proportion of aggressive interactions, towards different opponent species. (a) Colony aggression of *T. ambiguus* and *T. longispinosus* hosts from Michigan towards the sympatric slavemaker *T. pilagens*, a non-nestmate conspecific ant and the allopatric slavemakers *T. duloticus* from Ohio and *P. americanus* from either New York or Ohio. As the two host species from Michigan did not differ in their aggressive responses ($\chi^2 = 0.008$, $P = 0.925$), the data for both species were pooled for illustration. (b) Colony aggression of *T. ambiguus* and *T. longispinosus* from Michigan, *T. longispinosus* from New York and *T. curvispinosus* from Ohio towards their respective sympatric slavemaking species and towards a non-nestmate conspecific ant. Solid symbols represent aggression towards slavemaking species and hollow symbols towards non-nestmate conspecifics. Different symbols represent different opponent species. Symbols moreover represent the back-transformed logit-mean ± s.e.. Significance levels: ✶ < 0.05; ✶✶< 0.01; ✶✶✶< 0.001.

Chemical similarities between host and slavemaker species

We identified in total 36 hydrocarbons on the cuticle of the ants, of which 35 were shared between all species analyzed here. Only one substance (11,15-DiMeC27; Table 6.2) was not present on the cuticle of *T. pilagens*, but on all others. Free-living *T. ambiguus* host colonies differed in their chemical profile as much from each other as they differed from their *T. pilagens* slavemaker ($F_1 = 1.595$, $P = 0.149$; Fig. 6.2a). In contrast, chemical distances towards the slavemaker were higher in the second host species *T. longispinosus* ($F_1 = 3.659$, $P = 0.01$, Fig. 6.2b). We moreover found clear differences between *P. americanus* and its hosts *T. longispinosus* ($F_1 = 5.046$, $P < 0.001$, Fig. 2c) and *T. curvispinosus* was chemically distinct from both of its slavemakers ($F_2 = 4.868$, $P < 0.001$; *P. americanus*: $t = 2.847$, $P < 0.001$; *T. duloticus*:

t = 1.853, P = 0.014, Fig. 6.2d). Both hosts of *T. pilagens* were chemically different to the two other allopatric slavemaker species (F_5 = 4.594, P < 0.001; Table S6.3).
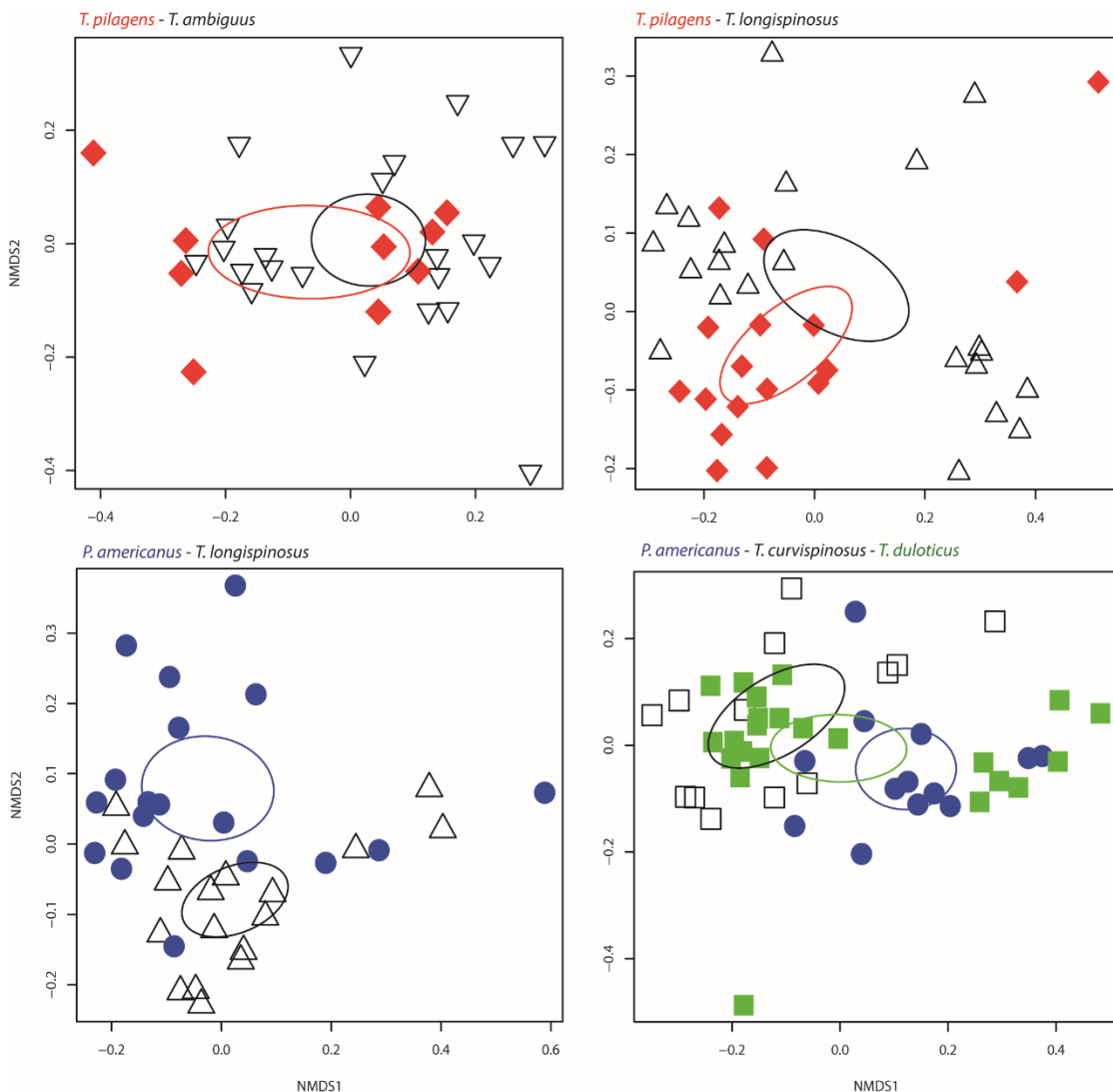
Figure 6.2 NMDS Plots of cuticular hydrocarbon resemblances amongst workers of each slavemaker-host system. (a) *T. pilagens* (solid diamonds in red) and workers of its main host species *T. ambiguus* (open triangles) (2D – stress = 0.15) (b) *T. pilagens* (solid diamonds in red) and workers of its second host species *T. longispinosus* (open triangles) (2D – stress = 0.11) (c) *P. americanus* (solid circles in blue) and workers of its main host *T. longispinosus* (open triangles) from NY (2D – stress = 0.12) (d) *T. duloticus* (solid squares in green) and *P. americanus* (solid circles in blue) and workers of its´ host *T. curvispinosus* (open squares) from OH (2D – stress = 0.15). Resemblances are based on Bray-Curtis similarity and stress is a quality measure of multidimensional scaling. Ellipses show 95% confidence intervals.

Total amounts of cuticular hydrocarbons

T. pilagens did not carry less hydrocarbons on its cuticle than its hosts, as expected under a chemical insignificance scenario, rather it exhibited higher amounts (*T.*

ambiguus: t = -3.651, P < 0.001; *T. longispinosus*: t = - 3.366, P < 0.001). *P. americanus* from New York and *T. duloticus* from Ohio had similar amounts than *T. ambiguus* (*P. americanus*: t = - 0.054, P = 0.957; *T. duloticus*: t = - 2.279, P = 0.781) and *T. longispinosus* (*P. americanus*: t = - 0.309, P = 0.758; *T. duloticus*: t = - 0.142, P = 0.887). *P. americanus* from Ohio had higher amounts than *T. longispinosus* (t = - 2.204, P = 0.029) and *T. ambiguus* (t = - 2.392, P = 0.018). There were also no differences in total amounts of hydrocarbons between the other hosts and their respective slavemaker species (*T. curvispinosus* - *P. americanus* (OH): t = - 1.330, P = 0.185; *T. curvispinosus* - *T. duloticus* (OH): t = 0.559, P = 0.577; *T. longispinosus* - *P. americanus* (NY): t = - 0.309, P = 0.758).

Table 6.2 The 36 identified cuticular hydrocarbons, including their mean relative abundance per host and slavemaker species (mean ± S.D.). Slavemaker species are given in bold.

Substance	*T. pilagens*	*P. americanus*	*T. duloticus*	*T. ambiguus*	*T. longispinosus*	*T. curvispinosus*
n-C23	**0.17 ± 0.36**	**1.42 ± 1.78**	**0.60 ± 0.69**	0.96 ± 1.42	0.91 ± 1.26	0.71 ± 1.30
n-C24	**0.25 ± 0.66**	**0.26 ± 0.73**	**0.20 ± 0.38**	0.37 ± 1.06	0.03 ± 0.10	0.27 ± 0.54
n-C25	**3.58 ± 3.21**	**12.22 ± 8.51**	**6.83 ± 6.12**	3.74 ± 3.55	5.21 ± 4.91	6.56 ± 7.16
9/11/13-MeC25	**0.06 ± 0.15**	**0.87 ± 1.12**	**1.97 ± 1.72**	0.51 ± 2.31	0.22 ± 0.56	0.90 ± 1.10
5/7-MeC25	**0.10 ± 0.24**	**0.33 ± 0.65**	**1.55 ± 1.21**	0.40 ± 0.73	0.67 ± 1.45	0.58 ± 0.86
3-MeC25	**0.79 ± 1.58**	**0.46 ± 0.83**	**1.22 ± 0.76**	0.26 ± 1.21	0.79 ± 1.58	2.53 ± 3.28
n-C26	**0.31 ± 0.48**	**0.97 ± 0.98**	**0.76 ± 0.75**	0.30 ± 0.47	0.53 ± 0.92	0.75 ± 0.78
alkene	**0.02 ± 0.07**	**0.48 ± 0.83**	**0.61 ± 0.71**	0.10 ± 0.35	0.41 ± 0.82	0.28 ± 0.57
n-C27	**13.86 ± 16.11**	**26.44 ± 13.89**	**25.31 ± 15.46**	13.08 ± 15.81	20.29 ± 20.64	18.01 ± 18.04
11/13/15-MeC27	**1.94 ± 1.10**	**4.25 ± 2.76**	**7.50 ± 4.40**	1.96 ± 1.20	4.19 ± 2.63	4.92 ± 3.34
7-MeC27	**0.97 ± 0.66**	**2.16 ± 1.47**	**1.04 ± 0.94**	0.56 ± 0.61	0.57 ± 0.67	1.36 ± 1.05
5-MeC27	**3.88 ± 1.34**	**5.30 ± 2.90**	**8.31 ± 5.75**	4.10 ± 2.14	3.25 ± 2.04	4.54 ± 2.59
11,15-DiMeC27	**0.00**	**0.12 ± 0.38**	**0.22 ± 0.57**	0.39 ± 1.25	0.17 ± 0.58	0.14 ± 0.63
3-MeC27	**3.09 ± 0.91**	**4.52 ± 1.86**	**3.38 ± 2.90**	3.91 ± 1.87	2.98 ± 2.55	5.03 ± 3.82
n-C28	**1.06 ± 0.72**	**0.59 ± 1.06**	**0.65 ± 0.84**	1.18 ± 1.40	0.83 ± 1.66	0.76 ± 1.68
4-MeC28	**1.09 ± 2.02**	**0.56 ± 1.06**	**0.55 ± 0.94**	2.82 ± 2.57	3.05 ± 3.73	2.03 ± 3.18
n-C29	**0.28 ± 0.62**	**2.91 ± 1.98**	**2.51 ± 2.41**	2.95 ± 1.61	2.61 ± 2.19	2.04 ± 1.52
11/13/15/17-MeC29	**6.14 ± 2.65**	**6.60 ± 4.05**	**12.88 ± 6.37**	11.60 ± 5.86	8.96 ± 5.53	6.23 ± 5.01
7-MeC29	**6.02 ± 2.04**	**2.76 ± 1.68**	**2.28 ± 2.10**	4.66 ± 2.48	3.05 ± 2.89	7.03 ± 12.19
5-MeC29	**2.90 ± 1.13**	**2.41 ± 1.95**	**4.38 ± 3.73**	3.85 ± 2.90	4.10 ± 3.73	1.50 ± 1.15
11,21/15,19/13,17/11,15-DiMeC29	**1.54 ± 1.65**	**0.69 ± 1.35**	**2.15 ± 2.45**	2.20 ± 2.60	1.78 ± 2.02	1.76 ± 3.68
3-MeC29	**1.81 ± 1.66**	**0.30 ± 0.77**	**0.61 ± 0.76**	1.63 ± 1.42	1.38 ± 1.69	1.29 ±1.45
n-C30	**0.62 ± 1.41**	**1.56 ± 4.46**	**0.30 ± 0.50**	2.42 ± 2.88	0.93 ± 1.37	1.74 ± 3.29
14-MeC30	**1.96 ± 1.34**	**0.92 ± 1.09**	**1.60 ± 2.38**	1.98 ± 1.47	1.13 ± 1.26	1.15 ± 1.15
3-MeC30	**2.50 ± 1.19**	**0.16 ± 0.39**	**0.60 ± 0.73**	2.17 ± 1.51	0.93 ± 1.22	0.35 ± 0.69
11/13/15-MeC31	**13.45 ± 5.11**	**3.74 ± 5.26**	**4.49 ± 4.23**	6.85 ± 7.71	8.12 ± 9.37	3.48 ± 7.20
11,15/11,13-DiMeC31	**12.26 ± 4.93**	**3.89 ± 4.39**	**0.23 ± 0.60**	10.82 ± 8.02	8.85 ± 6.09	3.56 ± 5.45
4,11,14-MeC31	**2.25 ± 1.65**	**0.54 ± 1.06**	**0.56 ± 1.02**	1.80 ± 2.33	1.01 ± 1.45	0.67 ± 1.36
n-C32	**2.32 ± 3.01**	**0.26 ± 0.64**	**0.34 ± 0.59**	0.24 ± 0.57	0.50 ± 1.01	0.61 ± 0.99
13/14-MeC32	**0.30 ± 0.54**	**0.36 ±0.70**	**0.26 ± 0.59**	0.62 ± 1.15	0.71 ± 1.18	0.75 ±1.09
11/13/15/17-MeC33	**4.41 ± 2.88**	**3.92 ± 3.40**	**1.41 ± 2.04**	3.96 ± 2.79	4.55 ± 3.88	4.45 ± 3.77
13,15/15,17-DiMeC33	**3.50 ± 4.29**	**2.03 ± 3.26**	**1.96 ± 2.74**	3.65 ± 4.73	2.82 ± 3.68	4.46 ± 7.89
11,13/11,15-DiMeC33	**5.44 ± 8.06**	**2.04 ± 3.78**	**1.00 ± 2.14**	3.17 ± 5.97	1.19 ± 3.15	3.41 ± 6.11
5,15/12,18-DiMeC33	**0.49 ± 1.13**	**2.58 ± 3.00**	**1.30 ± 2.34**	0.33 ± 1.10	1.12 ± 1.69	1.46 ± 2.46
13,15,17-MeC35	**0.35 ± 1.00**	**0.93 ± 1.71**	**0.38 ± 0.82**	0.08 ± 0.40	0.50 ± 1.02	0.86 ± 1.37
11,19/15,19-DiMeC35	**0.30 ± 0.79**	**0.44 ± 1.56**	**0.05 ± 0.30**	0.36 ± 1.25	1.64 ± 2.87	3.81 ±5.91

Raids of T. pilagens

In a quarter (25.6%) of the 43 staged raids, *T. ambiguus* host colonies were able to save all brood and adult host workers. More frequently however, the slavemaker was not only able to capture brood, but also adult host workers, which happened in about two-thirds (65.1%) of the raids. Slavemaker workers invariably carried the adult host workers into their nest-site and placed them near or on the brood pile of the slavemaker colony, only then they would continue with stealing the host brood. All newly acquired host workers were fully accepted in the slavemaker colonies, albeit in a few cases (9.5%) they were first removed by already-established slaves. These cases were resolved peacefully, as evicted hosts were again carried back into the colony by slavemakers. The number of host casualties greatly varied between raids, with 0-80% killed ants. The fewer host ants were killed, the more adult hosts were enslaved (t = -2.621, P = 0.014; Fig. 6.3a). Additionally if more adult hosts were enslaved also more brood could be captured by the slavemaker colony (t = 4.193, P < 0.001; Fig. 6.3c; Table S6.4). If the host queen was absent after a raid, either because it was killed during the raid (16.27% of all raids) or the host colony was originally queenless (46.51%), the slavemaker colony managed to enslave more adult host ants (t = 2.891, P = 0.007; Fig. 6.4). The number of fights between slavemakers and hosts (F_1 = 0.004, P = 0.949) and the proportion of killed slavemakers (F_1 = 2.681, P = 0.115) was unrelated to the enslavement of adult host ants (for model selection results see Table S6.4). The aggression tests of individual slavemakers revealed that *T. pilagens* shows only little aggression towards its host *T. ambiguus*. On average, only 7.48% ± 16.96% (X ± SD) of the interactions between *T. pilagens* and a non-enslaved host were aggressive.

More aggressive host colonies suffered from more casualties (t = 2.222, P = 0.036; Fig. 6.3b; for model selection results see Table S6.5), but slavemaker (F_1 = 0.807, P = 0.379) and slave aggression (F_1 = 2.543, P = 0.124) was not associated with the proportion of host workers killed. Moreover, more aggressive host colonies were unable to save more brood (F_1 = 0.527, P = 0.476), in contrast to hosts of *P. americanus*, where colony aggression can be beneficial (Kleeberg et al. 2014, Chapter 1; Pamminger et al. 2012). All other parameters related to the outcome of a raid were independent of the aggression behavior of hosts, slaves or slavemaker workers (Supplement S6.2).
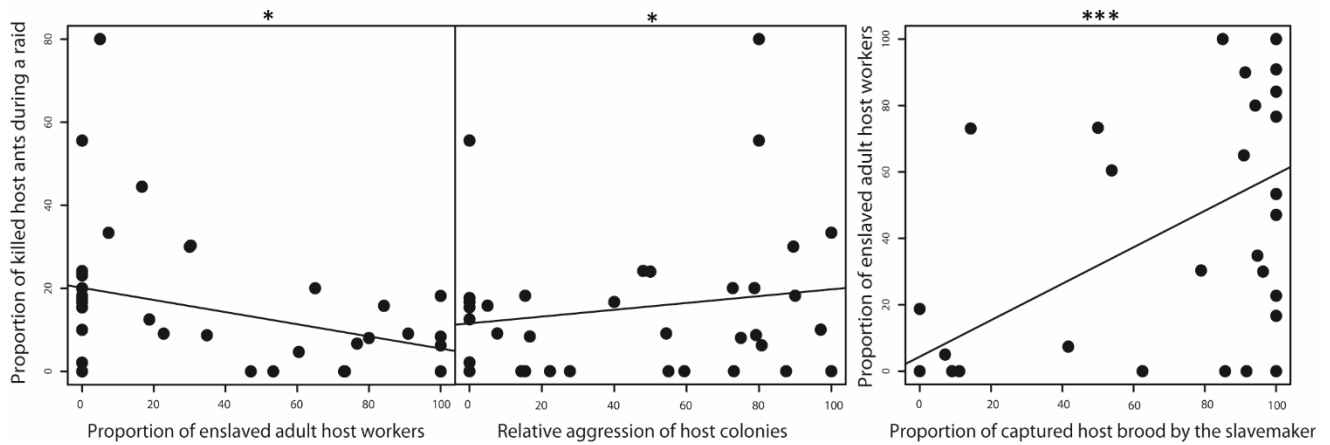
Figure 6.3 Raiding outcomes. (a) The relationship between the proportion of enslaved adult host ants and host casualties during a raid. Fewer ants were killed if the slavemaker colony enslaved more adult host ants. (b) The relationship between host colony aggression and host casualties during a raid. More ants died if the colony was more aggressive. (c) The relationship between the proportions of captured brood by the slavemaker and enslaved adult host ants. If the slavemaker was able to enslave more adult host ants it was also able to capture more brood. Significance levels: ✶ < 0.05; ✶✶< 0.01; ✶✶✶< 0.001.

Demographic effects on host and slave colony aggression

Host colonies were more aggressive if they contained more workers ($t = 2.541$, $P = 0.012$), more brood items ($t = 3.395$, $P < 0.001$) and if they were queenright ($t = 2.693$, $P = 0.007$). The aggression of slaves however was only elevated if they contained more brood ($t = 4.032$, $P < 0.001$) and more slavemakers ($t = 2.020$, $P = 0.045$), but in the presence of a slavemaker queen, slaves were less aggressive ($t = -2.080$, $P = 0.039$).

Evacuation response of hosts of the slavemaker T. pilagens

Both hosts of *T. pilagens* did not differ in their evacuation response (host colony species effect: $\chi^2_1 = 0.224$, $P = 0.635$), however they more often evacuated their colony after confrontation with the allopatric slavemaking species *P. americanus* (opponent species effect: $\chi^2_1 = 10.072$, $P = 0.006$) than after an encounter with a *T. pilagens* ($z = -2.286$, $P = 0.022$) or *T. curvispinosus* worker ($z = -1.977$, $P = 0.048$). There was no difference in the evacuation response between confrontations with *T. pilagens* and *T. curvispinosus* ($z = -0.231$, $P = 0.817$).
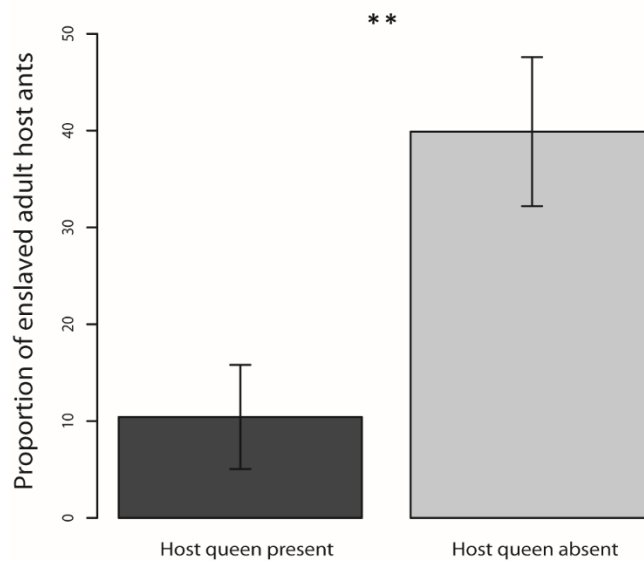
Figure 6.4 The relationship between adult host worker enslavement and the presence of a host queen after a raid. The light grey bar includes raids with originally queenless host colonies and raids where the host queen was killed by the slavemaker. The dark grey bar includes raids with queenright host colonies, whose queen survived the raid. Significance levels: ✶ < 0.05; ✶✶< 0.01; ✶✶✶< 0.001.

DISCUSSION

Our study clearly demonstrates that hosts of the slavemaker *T. pilagens* do not recognize and attack their enemy nor do they evacuate their colony after confrontations with the slavemaker, explaining observations of peaceful raids including the enslavement of adult hosts (Seifert et al. 2014, Chapter 5; this study). Most likely *T. pilagens* utilizes chemical mimicry or camouflage to outwit the host´s recognition system as the chemical profiles of *T. pilagens* closely resemble its main host *T. ambiguus*. The two related slavemakers *T. duloticus* and *P. americanus* differ in their chemical profiles from their *Temnothorax* hosts, which clearly recognize their enemies (Alloway 1990; Brandt et al. 2005b; this study). Slave raids by *T. pilagens* against more aggressive host colonies are likely to escalate, resulting in more host casualties and clearly not promoting the rescue of brood as it has been shown for hosts of *P. americanus* (Kleeberg et al. 2014, Chapter 1). However, *T. pilagens* showed very little proactive aggression and slavemaker aggression was independent of the course or outcome of raids, suggesting that the hosts' aggressive potential is the main driver for the outcome of a raid by *T. pilagens*. The peaceful strategy is beneficial for this slavemaker as it facilitates the capture of brood and eudulosis, leading to a substantial and importantly, an instantaneous increase in its slave workforce.

Open aggression by host colonies can be an effective defense during raids against the slavemaker *P. americanus* (Pamminger et al. 2012; Kleeberg et al. 2014, Chapter 1). Moreover, in two *Temnothorax* host species, colony aggression positively correlates with parasite pressure over their entire range, indicating that slavemaker presence selects for high aggression in hosts (Kleeberg et al. 2015, Chapter 2). Similar high aggression in the presence of social parasites was found in *Myrmica* ants (Fürst et al. 2011). In the host *T. ambiguus* aggression does not pay during raids against *T. pilagens*, but the reverse. More aggressive host colonies suffered higher fatalities and could not rescue more brood. Aggression can only benefit hosts that are able to recognize their enemy and our data indicate that this is not the case for hosts of *T. pilagens*, which show little aggression towards their parasite. Indeed, high aggression did only benefit hosts of *P. americanus* if they obtained information on the impending danger during previous enemy encounter (Kleeberg et al. 2014, Chapter 1) and hence were able to recognize the threat. As the two hosts, *T. ambiguus* and *T. longispinosus* from Michigan are highly aggressive towards the allopatric slavemakers *P. americanus* and *T. duloticus*, the reduced aggression towards *T. pilagens* is not due to a low aggressive potential itself, but due to a recognition evasion of the parasite. Conflict escalations are not only costly for losers (here the hosts), but can also harm winners (Neat et al. 1998). Escalated raids not only resulted in more defenders ending up dead, but in the enslavement of fewer host workers.

One of the most intriguing findings of our study is the fact that *T. pilagens* manages to integrate unrelated adult host workers into its slave workforce in two-thirds of the raids. In raids where eudulosis occurred, slavemakers gained a tenfold of slave workforce ($\sim$ 69.4% of host brood and adult workers) compared to raids without eudulosis ($\sim$ 6.3% of host brood). Social acceptance by adult host workers is more commonly observed during colony founding of young slavemaker queens. For example, queens of the European slavemaker *Myrmoxenus ravouxi* invade host colonies and gain acceptance by adult host workers over the duration of a few weeks, whereas slavemaker workers get detected and attacked during raids (Buschinger 1989). Slavemaker queens only have to outwit the hosts´ recognition system of a single host colony. Slavemaker workers however raid several host colonies per year, facing different host colony odors. Based on our chemical analyses *T. pilagens* mimics its preferred host´s profile (*T. ambiguus*) by either actively biosynthesizing the CHCs - chemical mimicry - or by obtaining them through physical contact - chemical camouflage. Chemical insignificance - a third possible strategy - is unlikely as we detected quantitatively more instead of less hydrocarbons on the slavemaker *T. pilagens*. However, admittedly our absolute quantification is hampered by the usage of only a single internal standard. Coming back to the two indicated strategies chemical mimicry and camouflage, the latter most likely explains the high similarity in the slavemakers' profile (Lenoir et al. 2001), as we found that the chemical profile of slavemakers enslaving *T. ambiguus*

slaves differed from those with *T. longispinosus* slaves. Moreover *T. pilagens* often groom themselves during search for host colonies and during raids (personal observations), possibly a way to update the CHC profile, similar to the grooming behavior of the xenobiotic "shampoo-ants" *Formicoxenus provancheri* (Lenoir et al. 1997). Although, we cannot exclude chemical mimicry our results and observations during raids more likely point to chemical camouflage.

Next to the aggressive potential of the host colony, the presence of a host queen at the time of the host worker integration explains raiding outcomes. Host queen absence facilitates eudulosis, which could be caused by two possibly interrelated effects: changes in the CHC profile of the colony or lower worker aggression. Indeed, we could show that queenless host colonies are less aggressive. A reduction in worker aggression following queen death is a common phenomenon in ants and has been attributed to the lack of the queens' primer pheromone (Vander Meer and Alonso 2002). Moreover, it was hypothesized that the loss of a queen has a substantial influence on the colony Gestalt odor, weakening the recognition system of ant workers (Schneirla 1971). Here, non-aggressive host colonies did not attack the slavemaker and initiate escalated raids, suggesting that low aggressive ant societies might be more tolerant towards CHC variation than colonies with high aggressive potential. Even in the highly aggressive army ants, queen removal facilitates the fusion of unrelated neighboring colonies (Kronauer et al. 2010). Why slavemakers can only behaviorally manipulate workers from queenless colonies to join their colonies will be the objective of future studies. However, we could show that the aggression of slaves is reduced if a slavemaker queen is present. As *T. pilagens* queens have a much larger Dufour´s gland than other slavemaker queens (unpublished data), it is possible that they use glandular secretions to avoid aggressive escalations between already established and newly integrated slaves.

Besides using aggression, attacked host colonies could flee from their nest-site to save a fraction of their brood and the queen, giving them a chance to start over (Jongepier et al. 2014, Chapter 3). Only 14.0% of the attacked host colonies partially evacuated their nest, in contrast to 62.1 % of *T. longispinosus* colonies raided by *P. americanus* colonies (Kleeberg et al. 2014, Chapter 1). *T. pilagens* apparently circumvents nest evacuation effectively, presumably because they manage to enter host nests undetected. In standardized evacuation experiments we could demonstrate that indeed both hosts of *T. pilagens* show no evacuation when confronted with a living *T. pilagens* worker inside their colony. *T. ambiguus* never evacuated their nest-site and *T. longispinosus* only once out of 20 trials, whereas they evacuated more often when facing an allopatric *P. americanus* slavemaker worker inside their colony. Thus *T. pilagens* seems to be highly successful, by either killing the host colony when getting continuously attacked or by peacefully integrating adult hosts into its slave workforce. Both strategies are highly costly for the hosts, as colony fitness can be reduced to zero. This might result in the local

overexploitation of host colonies (Frank 1996). Indeed, we found a rather patchy distribution of the slavemaker within its evenly distributed host population, which could be indicative of a local meta-population dynamic. However, other parameters, such as differences in the microclimate could also explain the patchy distribution of slavemaker colonies (Wiens 1976). With its high virulence, *T. pilagens* resembles *T. duloticus*, which are both evolutionary young slavemakers (Beibl et al. 2005). Our behavioral observations do not indicate effective host defenses against *T. pilagens*, indicating that the slavemaker might lead the co-evolutionary arms race, possibly because the hosts did not have time to evolve a more fine-tuned recognition system.

Conflicts between social groups, especially between groups of antagonistic species, often escalate, for instance during territorial fights (Hölldobler and Lumsden 1980). In ants the outcome of group conflicts is often resolved through recognition of deviant phenotypes and communication. As ants rely on their chemical recognition system to identify opponents and also utilize chemical cues for (indirect) communication (Jackson and Morgan 1993), *T. pilagens* circumvents detection and hence aggressive conflicts during raids through chemical similarity, contrary to related slavemakers, which whom *T. pilagens* even shares its hosts. Avoiding escalation of the raiding interactions apparently allows the slavemaker to manipulate adult host workers into enslavement. Aggression, an effective defense in other host-parasite systems, is counter-productive for its hosts as it leads to raid escalation, only resulting in a high death toll among defenders. Our study further shows a strong impact of the host queens' presence on raiding outcomes, which apparently severely influences host behavior and recognition abilities. The apparent differences in raiding behavior in the three closely related slavemaker species point to divergent co-evolutionary outcomes of slavery in ants, which evolved convergently in these species.

SUPPORTING INFORMATION

Table S6.1 Collection sites and number of collected colonies per species in the field.

US State	Location	Coordinates	Species	No. of collected colonies	Year of collection
Michigan	Sleeping Bear Dunes National Lakeshore	44°50'35.4''N 86°3'36.9''W	*T. pilagens* (**slavemaker**) *T. ambiguus* (**host**) *T. longispinosus* (**host**)	44 200 200	2013
Michigan	Sleeping Bear Dunes National Lakeshore	44°50'35.4''N 86°3'36.9''W	*T. pilagens* (**slavemaker**) *T. ambiguus* (**host**)	231 200	2014
New York	Edmund Niles Huyck Preserve	42°31'57.0''N 74°08'45.2''W	*P. americanus* (**slavemaker**) *T. longispinosus* (**host**)	153 200	2013
Ohio	Columbus Metro Parks	40°14'13.8''N 82°59'06.5''W	*P. americanus* (**slavemaker**) *T. duloticus* (**slavemaker**) *T. curvispinosus* (**host**)	33 27 200	2013

Table S6.2 Sample sizes for chemical analyses

Population	Species	N of workers analyzed
Michigan	*T. ambiguus* (free living) *T. longispinosus* (free living) *T. pilagens*	25 22 26
New York	*T. longispinosus* (free living) *P. americanus*	18 18
Ohio	*T. curvispinosus* (free living) *P. americanus* *T. duloticus*	13 13 23

S6.1: *Details on the chemical analyses and temperature program*

Ant CHCs were extracted by individually immersing freshly frozen ants in approximately 0.5 ml hexane for 10 min. The ants have been stored at -20°C before the extractions. The extracts were analyzed using a GC-MS (Agilent Technologies, GC: Agilent 7890A; MS: Agilent 5975) equipped with a HP5-MS column (30 m x 0.25 mm; coating: 0.25 µm). Sample injection (5µl) was splitless over 2 min at 250°C. Helium was used as carrier gas at a constant flow of 1.2 ml/min. Oven temperature started at 150°C for 3 min, followed by a temperature increase to 300°C in 2 steps (150–250°C with 30°C/min and 250–300°C with 2°C/min). The final temperature of 300°C was held constant for 2 min. After an initial solvent delay of 5 min, a mass range of 40–500 amu was scanned with an ionization voltage of 70 eV. The transfer line was held constant at 320 °C.

T. pilagens mainly parasitizes *T. ambiguus* hosts (82.14% of all collected slavemaker colonies contained only *T. ambiguus* slaves; *N* = 252; based on collections in 2013 and 2014), but also takes *T. longispinosus* (7.94%) or both species (9.92%), but at much lower rates. *T. pilagens* workers originating from colonies containing *T. ambiguus* slaves were chemically different from those containing *T. longispinosus* slaves (F_1 = 2.61, *P* = 0.039). Hence, we only compared slavemakers from nests with *T. ambiguus* slaves with free-living *T. ambiguus* workers and slavemakers with *T. longispinosus* slaves with free-living *T. longispinosus* workers. Slavemakers from colonies with both slave species were included in both comparisons.

To test for chemical insignificance we calculated the total amount of CHC on ant cuticles and controlled for interspecific body size differences. Therefore we divided the total amounts of CHCs by the mean head width per species. We used measurement data done on a different set of individuals, but from the same populations, not used in the chemical analyses. We measured the head width of between 17 and 62 individuals and calculated a mean head size per species.

Table S6.3 Multivariate Analyses of Michigan host ants and two allopatric slavemaker species *P. americanus* from New York (NY) or Ohio (OH) and *T. duloticus* from Ohio (pairwise comparisons).

Groups	*t*	*P*
T. ambiguus and *P. americanus* (NY)	2.476	< 0.001
T. longispinosus and *P. americanus* (NY)	2.655	< 0.001
T. ambiguus and *P. americanus* (OH)	2.503	< 0.001
T. longispinosus and *P. americanus* (OH)	2.655	< 0.001
T. ambiguus and *T. duloticus* (OH)	2.420	< 0.001
T. longispinosus and *T. duloticus* (OH)	2.742	< 0.001

Table S6.4 Model selection results from the generalized linear model analyzing the proportion of enslaved adult host ants in relation to the number of fights between slavemakers and hosts, the proportion of captured brood and the number of killed slavemaking and host ants and whether or not the host queen was present/ still alive after a raid (queenless vs. queenright). Statistics indicated in bold were retained in the final model. All Δd.f. = 1.

	The proportion of enslaved adult host ants	
Predictors	*F*	*P*
Proportion of killed host ants	9.767	**0.004**
Proportion of captured brood	21.354	**<0.001**
Presence of host queen after raid	8.186	**0.008**
Number of fights between slavemakers and hosts	0.004	0.949
Proportion of killed slavemakers	2.682	0.115

Table S6.5 Model selection results from the generalized linear model analyzing the proportion of killed host ants during a raid in relation to host colony, slave colony and slavemaker aggression. Statistics indicated in bold were retained in the final model. All Δd.f. = 1.

	The proportion of killed host ants	
Predictors	*F*	*P*
Host colony aggression	5.269	**0.031**
Slave colony aggression	2.543	0.124
Slavemaker individual aggression	0 807	0.379

S6.2*: Additional results for the raiding trials: Effects of aggression on the outcome of raid*

Aggression levels of hosts, slaves and slavemakers had no influence on the proportion of integrated host ants (host aggression: F_1= 0.906, P = 0.351; slave aggression: F_1= 0.016, P = 0.899; slavemaker aggression: F_1= 0.058, P = 0.812), on the proportion of brood saved by the host (host aggression: F_1= 0.527, P = 0.476; slave aggression: $F1$= 2.195, P = 0.152; slavemaker aggression: F_1= 0.546, P = 0.468) or stolen by the slavemaker (host aggression: F_1= 0.242, P = 0.628; slave aggression: F_1= 4.022, P = 0.057; slavemaker aggression: F_1= 0.576, P = 0.456). We moreover found no effect of aggression on the number of fights (host aggression: F_1= 0.083, P = 0.777; slave aggression: F_1= 0.079, P = 0.781; slavemaker aggression: F_1= 3.324, P = 0.083) or the proportion of dead slavemakers (host aggression: χ^2_1= 2.583, P = 0.108; slave aggression: χ^2_1= 2.147, P = 0.143; slavemaker aggression: χ^2_1= 2.256, P = 0.133).

S6.3: *Analyses of aggression index*

Aggression behavior can be quantified in different ways. An alternative aggression measure to the one used in our study (*relative aggression*), is the *aggression index* (Errard and Hefetz 1997), which gives different aggression behaviors such as biting or dragging a different score. We repeated all analyses using this aggression measure. We calculated the *aggression index* as follows:

AI = 0*a + 1*m + 2*(d + h) + 3*(s + b) / (a + m + d + h + s + b)

with *a* being the number of antennating (no aggression with a score of 0), *m* being the number of threats represented by mandible spreading (low aggression with a score of 1), *d* and *h* being the number of dragging and holding (medium aggression with a score of 2) and *s* and *b* being the number of stinging and biting (high aggression with a score of 3).

Statistics for colony aggression tests

We first assessed whether the aggressive responses of host species from Michigan were related to the opponent species and/or host species, using a linear mixed model (LME; lme function implemented in the nlme for R package). Normality was tested using the One-sample Kolmogorov-Smirnov test (type = pearson; D = 0.089, P = 0.108). We fitted the aggression index as dependent variable. Opponent species (conspecific, *T. pilagens, T. duloticus and P. americanus* from OH / NY) and host species (*T. ambiguus* or *T. longispinosus*) as well as their interaction were included as fixed predictors. We fitted host colony identity as a random factor to account for the repeated measure design and to avoid

pseudo-replication. For model selection, we used a backwards, stepwise selection procedure (α = 0.05). Therefore we removed non-significant terms, starting with the least significant interaction. Terms that significantly reduce the explanatory power of the model after removal, were retained in the minimal model.

In addition, to compare aggression of the three host species towards their respective social parasites with the aggression towards a non-nestmate conspecific (constitutive aggression), we assessed whether the aggressive responses of all three host species (*T. ambiguus* from MI, *T. longispinosus* from MI, *T. longispinosus* from NY and *T. curvispinosus* from OH) were related to the opponent type (conspecific or respective slavemaker species) and host species (*T. ambiguus*, *T. longispinosus* and *T. curvispinosus*), using a linear mixed model (Kolmogorov-Smirnov test: D = 0.061, *P* = 0.573). *T. curvispinosus* was tested against two slavemaker species, *T. duloticus* and *P. americanus*. As we did not find any differences in the aggressive responses towards those two slavemaker species (lme: χ^2_1= 1.37, *P* = 0.242), we pooled the aggression data as being on factor ("slavemaker" now including aggression towards *T. duloticus* and *P. americanus*). We fitted the aggression index as dependent variable. Opponent type (slavemaker vs. conspecific) and host species as well as their interaction were included as fixed predictors. Host colony was included as random factors to account for repeated measures and to avoid pseudo-replication. To correct for multiple testing (the use of the same data set in two models), the significance level for the analyses of aggression of host species from Michigan (*T. longispinosus* and *T. ambiguus*) was adjusted using the MFDR (mean false discovery rate) approach to αc = 0.0375 according to αc = (n+1)/(n*2)*0.05 where n denotes the number of times a data set was used. All analyses were performed in R version 3.0.2 (R Core Team 2013).

Statistics for raiding experiments

We assessed whether the hosts´, the slaves´ or the slavemakers´ aggressive potential (assessed one day prior to the raiding trials) was related to the outcome of a raid. Therefore we separately analyzed the proportion of brood saved, brood captured, dead hosts and dead slavemakers and the proportion of integrated adult hosts using a set of generalized linear models (GLMs) with quasi-binomial error distribution and logit link function. We included host colony and slave colony aggression index as well as the mean slavemaker aggression index per colony as predictors. Similarly we analyzed the number of fights between slaves and hosts and between slavemakers and hosts using generalized linear models with quasi-Poisson error distribution and log link function. Again, we included host, slave and slavemaker aggression index as predictors. Additionally we tested associations between adult enslavement and other raiding outcomes and hence analyzed the proportion of enslaved adult host ants using a generalized linear model with quasi-binomial error distribution and log link function. We included the number of fights between slavemakers and hosts, the proportion of captured brood and of killed slavemaking and host ants and whether or not the host queen was present/ still alive after a raid (queenless vs. queenright) as predictors.

Finally we studied demographic effects on colony aggression of hosts and slaves. We analyzed the aggression index of either the host colony or the slave colony (with slavemakers removed) in relation to colony size using generalized linear models with

quasi-binomial error distribution and logit link function. Here we included the number of workers, queens (as binary factor) and brood as predictors. For all model selections we used a backwards stepwise selection procedure based on the F-statistics (α = 0.05). All analyses were performed using R version 3.0.2 (R Core Team 2013).

Results

Colony aggression of Michigan hosts towards the three slavemaker species

Colonies of the two host species *T. ambiguus* and *T. longispinosus* from Michigan did not differ in their aggressive responses (L.Ratio = 8.760, DF = 1, *P* = 0.567), but the aggressive responses of both host species differed depending on the opponent species (L.Ratio = 16.894, DF = 4, *P* = 0. 002, Fig. S6.1). Both hosts were more aggressive towards *P. americanus* (OH) than towards a conspecific non-nestmate (*t* = 2.346, *P* = 0.020) but were not more aggressive towards *T. duloticus* and *P. americanus* (NY) (towards *T. duloticus*: *t* = 1.397, *P* = 0.165; towards *P. americanus* (NY): *t* = 1.544, *P* = 0.125). In contrast, they were slightly less aggressive towards their sympatric slavemaker, *T. pilagens* than towards non-nestmate conspecifics, albeit non-significantly (*t* = -1.796, *P* = 0.075). Moreover, *Temnothorax* hosts were more aggressive towards all other slavemaker species than towards their sympatric slavemaker *T. pilagens* (towards *P. americanus* (NY): *t* = 3.111, *P* = 0.002; towards *P. americanus* (OH): *t* = 3.794, *P* < 0.001; towards *T. duloticus* (OH): t = 2.986, *P* = 0.003). The aggressive responses of MI hosts did not differ between *P. americanus* from Ohio, New York and *T. duloticus* from Ohio (*T. duloticus* compared to *P. americanus* (OH): *t* = 0.950, *P* = 0.344 and to *P. americanus* (NY): *t* = 0.148, *P* = 0.882; *P. americanus* (OH) compared to *P. americanus* (NY): *t* = -0.802, *P* = 0.424).

Colony aggression towards conspecifics and sympatric slavemakers

A comparison of all hosts revealed an interaction between host species and opponent type (slavemaker vs. conspecific x host species - interaction: L.Ratio = 12.471, DF = 3, *P* = 0. 005, Fig. S6.1). While both *T. pilagens* hosts were about as aggressive towards a conspecific than towards their sympatric slavemaker, with *T. ambiguus* being slightly less aggressive (*T. longispinosus*: *t* = -0.698, *P* = 0.487; *T. ambiguus*: *t* = - 1.843, *P* = 0.069), the two host populations, *T. longispinosus* NY and *T. curvispinosus* OH, showed higher aggression towards their respective slavemaker species than towards conspecifics (*T. longispinosus* - *P. americanus* (NY): *t* = 2.398, *P* = 0.018; *T. curvispinosus* - *T. duloticus* (OH) and *P. americanus* (OH): *t* = 2.276, *P* = 0.026), supporting earlier findings of enemy recognition (Alloway 1990). All three host species did not differ in their constitutive aggression, tested towards a non-nestmate conspecific ant (χ^2_3 = 6.235, *P* = 0.101).

Raids of *T. pilagens*

More aggressive host colonies were unable to save more brood (F_1= 0.239, *P* = 0.630), in contrast to hosts of *P. americanus,* where colony aggression can be beneficial (Kleeberg et al. 2014; Pamminger et al. 2012). Moreover, aggression levels of hosts, slaves and slavemakers had no influence on the proportion of integrated host ants (host aggression: χ^2_1= 0.678, *P* = 0.410; slave aggression: χ^2_1= 0.477, *P* = 0.490; slavemaker aggression: χ^2_1 = 0.083, *P* = 0.774), on the proportion of brood saved by the host (host aggression: χ^2_1 = 0.239, *P* = 0.625; slave aggression: χ^2_1 = 2.799, *P* = 0.094.; slavemaker

aggression: $\chi^2_1 = 1.026$, $P = 0.311$) or stolen by the slavemaker (host aggression: $\chi^2_1 = 0.430$, $P = 0.512$; slave aggression: $\chi^2_1 = 2.442$, $P = 0.118$; slavemaker aggression: $\chi^2_1 = 1.163$, $P = 0.280$). We moreover found no effect of aggression on the number of fights (host aggression: $\chi^2_1 = 1.888$, $P = 0.169$; slave aggression: $\chi^2_1 = 0.609$, $P = 0.435$; slavemaker aggression: $\chi^2_1 = 0.369$, $P = 0.543$) or the proportion of dead slavemakers (host aggression: $\chi^2_1 = 1.366$, $P = 0.242$; slave aggression: $\chi^2_1 = 1.029$, $P = 0.311$; slavemaker aggression: $\chi^2_1 = 1.462$, $P = 0.227$).

Demographic effects on host and slave colony aggression

Host colonies were more aggressive if they contained more workers ($t = 2.652$, $P = 0.008$) and if they were queenright ($t = 3.488$, $P < 0.001$). The aggression of slaves however was elevated if they contained more brood ($t = 5.655$, $P < 0.001$) and more slavemakers ($t = 2.041$, $P = 0.043$).
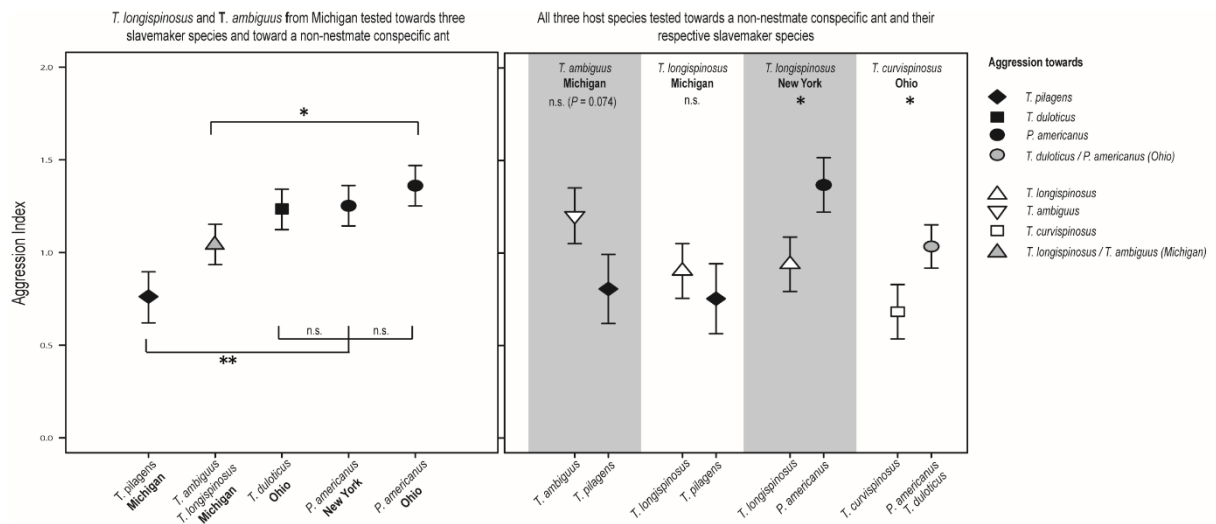
Figure S6.1 Host colony aggression, measured as the *aggression index* according to Errard and Hefetz (1979), towards different opponent species. (left figure) Colony aggression of *T. ambiguus* and *T. longispinosus* hosts from Michigan towards the sympatric slavemaker *T. pilagens*, a non-nestmate conspecific ant and the allopatric slavemakers *T. duloticus* from Ohio and *P. americanus* from either New York or Ohio. (right figure) Colony aggression of *T. ambiguus* and *T. longispinosus* from Michigan, *T. longispinosus* from New York and *T. curvispinosus* from Ohio towards their respective sympatric slavemaking species and towards a non-nestmate conspecific ant. Solid symbols represent aggression towards slavemaking species and hollow symbols towards non-nestmate conspecifics. Different symbols represent different opponent species. Symbols moreover represent the back-transformed logit-mean ± s.e. Significance levels: ✶ < 0.05; ✶✶ < 0.01; ✶✶✶ < 0.001.

Chapter **7**

The evolution of cuticular hydrocarbons in ants: The influence of parasitic lifestyle, caste and sex on chemical profiles

Isabelle Kleeberg, Florian Menzel and Susanne Foitzik

ABSTRACT

During evolution, chemical communication became increasingly important for the formation and maintenance of insect societies. Altruism only increases an actors' fitness when directed towards relatives, which are recognized chemically. Social parasites exploit insect societies and are selected to develop strategies to avoid recognition. We studied cuticular hydrocarbons of three socially parasitic slavemaking ant species and their three related hosts to investigate whether the parasitic lifestyle selects for specific chemical traits. Slavemaker chemical profiles were characterized by shorter-chained hydrocarbons and a shift from methyl-branched alkanes to n-alkanes. As nestmates are commonly recognized by the former, parasites exhibit fewer recognition cues. These chemical shifts were consistent across-species with independent origins of slavery. Thus, the production of n-alkanes is a convergent adaptation to the parasitic lifestyle, revealing that similar selection pressures can lead to parallel evolution of chemical traits. We also detected caste-specific signals, with workers, for which nestmate recognition is particularly important, carrying more and longer-chained hydrocarbons. Males showed no across-species chemical signal, despite their consistently different lifestyle from female ants. This first comprehensive study of cuticular hydrocarbons across castes and species reveals how lifestyle-specific selection pressures are reflected in ant chemical traits.

Keywords: host-parasite coevolution, odor, chemical strategies, social parasites, cuticular hydrocarbons

INTRODUCTION

Recognition of group members is vital for the evolution and maintenance of sociality. Animals only benefit from altruistic behaviors if these are selectively directed towards related group members (Hamilton 1987). In insects and especially in social ones, recognition and subsequent discrimination occurs through olfaction or contact reception of chemical cues (Hölldobler and Michener 1980; Breed 1998; Lahav 1999). Besides their role as desiccation barriers the little to non-volatile hydrocarbons on the insects' cuticle mediate chemical communication. They form the basis for nestmate, species and gender recognition and function as sex pheromones and fertility cues (Blomquist and Bagnères 2010).

Nestmate recognition in ants is based on a comparison between the cuticular hydrocarbon (CHC) profile of another individual and a neuronal template of the colony odor. Depending on profile similarity or dissimilarity, a conspecific will be accepted or rejected (Hölldobler and Michener 1980; Vander Meer and Morel 1998). Cuticular hydrocarbon profiles of conspecific individuals typically include the same set of substances (Nowbahari et al. 1990), so that social insects use quantitative hydrocarbon variation to recognize friends and foes (Bonavita-Cougourdan et al. 1987; Vander Meer et al. 1989; Espelie et al. 1990; Martin et al. 2008a). The mean template model states that the average quantity of each recognition cue is learned and that individuals will be rejected when their profiles deviate more than a certain threshold from the colony mean (Breed and Bennett 1987; Blomquist and Bagnères 2010). The colony mean is influenced by heritable cues of the individuals in a nest, but also by substances acquired from the environment, such as food or nest material or by cues derived from the queen (Hölldobler and Michener 1980; Carlin and Hölldobler 1983, 1986; Blomquist and Bagnères 2010). Recognition cues are blended within a colony through trophallaxis and allogrooming, combined with the use of the postpharyngeal gland, leading to a continuous renewal of the colony odor (Soroker et al. 1994, 1995). Recognition errors can occur, if for instance differences in labels are too small for the sensory systems to be detected. There are two types of recognition errors. Either a foreign individual, from another colony or species, will accidentally be accepted and integrated into the colony (acceptance errors), thereby minimizing false rejection (rejection of colony members) or nestmates will be rejected from their own colony if the recognition system is too stringent (Reeve 1989).

Not all cuticular hydrocarbons are used in nestmate recognition. Straight-chained alkanes are especially effective in preventing cuticular water loss and their production is influenced by environmental factors, including temperature, humidity and task (Wagner et al. 2001; Martin and Drijfhout 2009). Insertion of double bonds or methyl-branching in turn decreases the anti-desiccation capacity of the hydrocarbons (Gibbs 1998, 2002; Hefetz 2007; Blomquist and Bagnères 2010). However, these alkenes and methyl-branched alkanes are often used in

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communication (Dani et al. 2001; Lorenzi et al. 2011). Increasing the relative amounts of such recognition cues in the profile leads to a higher efficacy of chemical communication (Steiger et al. 2011). The CHC profile of insects is therefore likely to be under varying selection pressures. CHC profiles can differ between castes (Ayasse et al. 1999; Tentschert et al. 2002), tasks (Greene and Gordon 2003) and fertility states (Liebig et al. 2000), and depend on the dominance hierarchy within a colony (Ayasse et al. 1995; Heinze et al. 2002; Monnin et al. 2002), but are also influenced by environmental conditions (Blomquist and Bagnères 2010).

In social insects males and young queens are subject to different selective forces compared to workers. Sex-specific compounds are important to facilitate mate choice and sex discrimination (Thomas and Simmon 2008). While species-specific compounds allow species recognition, colony-specific profiles are important for inbreeding avoidance (Martin et al. 2009). Queens communicate their fertility status through pheromones implemented in the CHC profile or through the use of glands (Blomquist and Bagnères 2010). Ant workers specialize on certain tasks, such as foraging or brood care, and their behavioral caste is encoded in the CHC profile (Wagner et al. 1998, 2001; Martin and Drijfhout 2009). Although usually not involved in reproduction, dominant workers can become fertile when the queen dies, which will in turn influence their profile to communicate fertility and dominance status to nestmates (Blomquist and Bagnères 2010). Besides their role in colony communication, CHCs are under selection by social parasites (Jongepier and Foitzik 2016; Martin et al. 2011), which are thought to be a major driver of recognition cue diversity (Gardner and West 2007; Crozier 1986).

Despite their sophisticated recognition system, ants often fall victim to social parasites, such as inquilines or slavemaking ants, who exploit their social behavior (Thomas et al. 2005). Indeed, a third of all European ants are socially parasitic in at least a part of their life cycle (Seifert 2007). Social parasites need to invade host nests to exploit resources, e.g. food, protection or the slave work force (Brandt et al. 2005a). Parasite threat has led to the evolution of an effective recognition system in the hosts (e.g. Jongepier et al. 2014, Chapter 3; Kleeberg et al. 2014, 2015, Chapter 1-2), and social parasites have to circumvent these defenses to successfully invade the fortress of an ant colony. Slavemaking ants conduct several raids per year, during which they attack host colonies to steal their brood. Slave workers emerging from the stolen brood accept the slavemakers as masters and will work for them. Typical strategies of slavemaking ants to avoid host recognition include chemical insignificance, where the social parasite carries almost no cues on their cuticle to be "invisible" for the host ants (D´Ettorre and Errard 1998; Lenoir et al. 2001), chemical mimicry where the social parasite actively biosynthesizes the hosts´ CHC profile (Dettner and Liepert 1994; Lenoir et al. 1997) or chemical camouflage where the social parasite acquires the hosts´ odor through allogrooming, trophallaxis, rubbing or nest material (Dettner and Liepert 1994; Lenoir et al. 2001; D´Ettorre et al. 2002; Tsuneoka and Akino 2012). In addition, some parasites use offensive

chemicals to disrupt the host defense system - chemical manipulation – released from the Dufour´s, the Pygidial or the Poison gland (Lenoir et al. 2001; Mori et al. 2000; Brandt et al. 2006; Jongepier et al. 2015, Chapter 4). Despite these sophisticated strategies, colony invasion remains a risky task for slavemakers, resulting in injury or death (Foitzik et al. 2001), as hosts are under selection to counter parasite adaptations. For example, parasite-mediated selection has led to the diversification of chemical profiles in host populations in the presence of slavemaking ants, making it more difficult for social parasites to match host profiles (Jongepier and Foitzik 2016; Martin et al. 2011). Albeit there are a number of fascinating case studies, the influence of the parasitic lifestyle per se on the chemical profile in comparison to related non-parasitic species has never been systematically studied.

Here we compare the chemical profiles of three North American slavemaking species and their three host species of the same genus - *Temnothorax*. The three slavemaking species are of different evolutionary age and origin (Beibl et al. 2005) and show diverse behavioral and chemical raiding strategies (Alloway 1979; Foitzik and Herbers 2001; Kleeberg et al. 2014, Chapter 1; Kleeberg and Foitzik 2016, Chapter 6). *Temnothorax americanus* (formerly known as *Protomognathus americanus*, Ward et al. 2015), the evolutionary oldest slavemaker species in this taxon, shows little evidence of chemical mimicry (Brandt et al. 2005b), but uses the Dufour´s gland secretion to divert host aggression (Brandt et al. 2006; Jongepier et al. 2015, Chapter 4). Hosts recognize this parasite as enemy (Alloway 1990) and its raids are openly aggressive, often resulting in casualties among both attackers and defenders (Foitzik et al. 2001; Brandt and Foitzik 2004). *Temnothorax duloticus* shows a similar raiding behavior, however fighting strategies differ. *Temnothorax duloticus* responds with biting and stinging when attacked by targeted host workers during a raid, whereas *T. americanus* never uses its stinger. Hence, *T. duloticus* raids more often escalate, resulting in large numbers of host workers being stung to death (Alloway 1979). However, both slavemakers are frequently recognized as threats by their hosts, suggesting that the chemical profile of *T. duloticus* is as distinct from that of its hosts than that of *T. americanus* (Kleeberg and Foitzik 2016, Chapter 6; Pamminger et al. 2011). A third slavemaking species, *T. pilagens* shows a very different raiding strategy. During raids these ants are often not detected and attacked by their hosts *T. longispinosus* and *T. ambiguus*, facilitating the enslavement of adult host workers. *Temnothorax pilagens* appears to employ chemical camouflage to circumvent host recognition (Kleeberg and Foitzik 2016, Chapter 6).

Albeit our behavioral and chemical analyses indicate that the strategies of the North American slavemakers of the genus *Temnothorax* vary (Alloway 1979; Kleeberg et al. 2014, Chapter 1; Kleeberg and Foitzik 2016, Chapter 6), we hypothesize that due to similar selection pressures on their profiles compared to their hosts, these profiles should have certain characteristics in common. Firstly, we

predict that slavemaking species should show a reduction in the proportion of recognition substances, namely methyl-branched alkanes and alkenes. Secondly, they could exhibit a reduced total amount of CHCs and / or higher proportions of *n*-alkanes to achieve chemical insignificance (Nehring et al. 2015). Using a novel statistical approach Jongepier and Foitzik (2016a) could identify nine hydrocarbons that are associated with nestmate recognition in *T. longispinosus*. Hence, we would predict that slavemaking species should carry less of those putative recognition substances, to decrease the probability of host aggression. In our study we were additionally interested in differences between castes of slavemakers and non-parasitic hosts. Due to their different life histories and ecology, we would expect to find chemical differences between workers, males and virgin queens of both lifestyles. As slavemaker queens in contrast to host queens need to invade a host colony for colony foundation (Pamminger et al. 2012), they are expected to show chemical adaptations to circumvent host aggression (e.g. chemical insignificance; Nehring et al. 2015; D´Ettorre and Errard 1998). Males of slavemaking ants are not directly interacting with their host: They live only for a few days after leaving the maternal nest to participate in a mating flight. Male profiles should therefore mainly underlie sexual selection, but not selection stemming from host-parasite coevolution. However, signaling colony and species affiliation could be important for males to avoid inbreeding or hybridization.

Here we take a comprehensive look at chemical profile composition in all sexes and castes of an ant taxon including both parasitic and non-parasitic species. We are especially interested how the diverse selection pressures in these groups are reflected in their cuticular hydrocarbon profiles. We investigated changes in the abundance of specific substance classes, which are known to have certain functions in the ants´ communication system.

MATERIAL AND METHODS

Study system, collection and colony maintenance

From May to July 2013 we collected colonies of three slavemaker species, *Temnothorax americanus*, *T. duloticus* and *T. pilagens* from collection sites in the States of New York, Ohio, West Virginia and Michigan, USA (Table S7.1). We also gathered colonies of their three host species, *T. longispinosus*, *T. curvispinosus* and *T. ambiguus* from the same sites. Host colonies were not parasitized at the time of collection and are henceforth termed `non-parasitic species`. We collected colonies of *T. americanus* with *T. longispinosus* slaves in New York (*N* = 100) and West Virginia (*N* = 56), and with T. curvispinosus slaves in Ohio (*N* = 30). In Southern Ohio we also collected 30 colonies of a second slavemaker species *T. duloticus* with *T. curvispinosus* slaves. 42 colonies of a third slavemaker species *T. pilagens* with

either *T. longispinosus* (N = 19) or *T. ambiguus* (*N* = 8) slaves or both slave species (*N* = 15) were collected in Michigan. We collected on average 47.38 ± 32.62 colonies of the non-parasitic species from the same locations as the slavemaker species (Table S7.1). At the NY study site, *T. americanus* occurs with the host *T. longispinosus* (95%) and *T. ambiguus* (5 %; Table S7.1), but mainly parasitizes *T. longispinosus* (Herbers and Foitzik 2002; Brandt and Foitzik 2004; Foitzik et al. 2009). In West Virginia, *T. longispinosus* and *T. curvispinosus* are found, but again *T. americanus* focuses on *T. longispinosus* (Herbers and Foitzik 2002; Foitzik et al. 2009). In Southern Ohio, two slavemaker species, *T. duloticus* and *T. americanus*, co-occur, and both of them mainly parasitize *T. curvispinosus* (Johnson and Herbers 2006), although occasionally *T. longispinosus* colonies are found. *Temnothorax pilagens* at our Michigan study site preferentially uses *T. ambiguus* as its host (Kleeberg and Foitzik 2016), although *T. longispinosus* is abundant and occasionally exploited.

Ant colonies were transferred to Ziploc bags and stored at 7°C during the field trip. Back in our laboratory in Germany, colonies were moved to artificial glass nest-sites inside three chamber plastic boxes. All colonies were carefully counted and fed once a week with crickets and honey.

Collection of ants for chemical analyses

To exclude potential effects of fertility (e.g. Oettler et al. 2008; Cuvillier-Hot et al. 2001; Denis et al. 2006; D`Ettorre et al. 2004b; Dietermann et al. 2003), age (e.g. Cuvillier-Hot et al. 2001; Kaib et al. 2000; Kohlmeier et al. unpublished data) or environmental conditions on the CHC profile, we analyzed individuals that emerged in our laboratory under standardized conditions. Hence, all ants were young (three to five days old) and infertile.

We chose from each species and community (New York, West Virginia, Ohio and Michigan) on average 14.8 (± 2.44) colonies (with the exception of *T. ambiguus* from New York where we only had 5 colonies available) and observed brood development. We noted the emergence of ants from the pupae and then waited 3 to 5 days for the chemical profile to fully develop. Whenever possible we froze two individuals per caste per colony (total sample sizes: Table S7.1). Ants were individually frozen at -20°C in a glass vial until chemical analyses. Freshly emerged ants are distinguishable from old individuals by their lighter colored cuticle.

To exclude potential effects of nestmates and / or slaves on ant hydrocarbon profiles through trophallaxis or grooming (Lenoir et al. 2001; D´Ettorre et al. 2002), we additionally let ant pupae emerge in isolation. To this end we removed on average 16.88 (± 10.03) pupae of each species and caste (worker pupae: *N* = 23.58 ± 7.91; virgin queen pupae: *N* = 10.75 ± 9.23; male pupae: *N* = 16.33 ± 9.05) from their original colony and placed them into an empty glass slide-nest with watered cotton balls for humidity and provided them with honey and crickets. Pupae from the same colony and caste were placed together in a single glass slide. After five

days we froze the adult ants (Table S7.1). Social isolation is stressful for ants and particularly so for slavemaking ants, which are unable to feed themselves. Moreover, adult workers often help ants to emerge from the pupae, so that it is not surprising, that only 33.05% of the isolated slavemaker pupae and 45.16% of the non-parasitic pupae emerged and survived until sampling. All ants (from original colony and isolated individuals) were kept at room temperature. In total we analyzed the profiles of $N = 372$ ants from the original colony and $N = 246$ ants that emerged in isolation.

GC-MS analyses

We extracted and analyzed the CHCs of all samples using a GC-MS (Agilent Technologies, GC: Agilent 7890A; MS: Agilent 5975). The chemical data were processed by integrating peak areas with the software MSD ChemStation E.02.02 (Agilent). Chemical substances were identified based on the diagnostic ions and the retention index. We included all hydrocarbons larger than C20 with a mean abundance of at least 0.1% in any species (and caste) and which occurred at least in 20% of the samples per species and caste.

Ant CHCs were extracted by individually immersing freshly frozen ants in approximately 0.5 ml hexane for 10 min. The extracts were analyzed using the GC-MS equipped with a HP5-MS column (30 m x 0.25 mm; coating: 0.25 μm). Sample injection (5μl) was splitless over 2 min at 250°C. Helium was used as carrier gas at a constant flow of 1.2 ml/min. Oven temperature started at 150°C for 3 min, followed by a temperature increase to 300°C in 2 steps (150–250°C with 30°C/min and 250–300°C with 2°C/min). The final temperature of 300°C was held constant for 2 min. After an initial solvent delay of 5 min, a mass range of 40–500 amu was scanned with an ionization voltage of 70 eV. The transfer line was held constant at 320 °C.

Statistical Analyses

Using a permutational multivariate analysis of variance based on the Bray-Curtis Similarity Index (PERMANOVA) with the software Primer 6.0 (Primer-E Ltd), we constructed models for the chemical profiles of slavemakers and non-parasitic species (number of permutations: 999). For those analyses we only chose one individual per colony, as individuals from the same colony are likely to be more similar to each other than to conspecifics from different colonies. To test for local chemical adaptation, we compared similarities between slavemakers and their respective host species of either the same community or a different community. We expected slavemaker species to be more similar to their sympatric host than towards allopatric host species. We predicted that slavemakers are more similar to their preferred host species than towards co-occurring but less preferred host species. We additionally tested, whether slavemakers exhibit a characteristic profile, which is fundamentally different from that of non-parasitic species. For this

purpose we constructed a model where we included caste, lifestyle (whether the species is a slavemaker or a non-parasitic species) as well as species nested in lifestyle as factors, with interactions allowed. The community was included as a random factor.

The following analyses are based on two individuals per colony. We compared specific substance classes, to elucidate characteristic CHC compositions of slavemakers and non-parasitic species that might lead to other chemical strategies of social parasites than mimicry. The relative amount of specific substance classes, recognition substances (Jongepier and Foitzik 2016) and the mean chain length as well as the total amounts of CHCs were analyzed using linear models. We fitted the respective substance class, mean chain length or total amounts of CHCs as dependent variable and included lifestyle and caste as fixed predictors, with the interaction allowed. We included colony ID nested in species, nested in community as random factors to account for pseudo-replication. For model selection we used a backward stepwise selection procedure ($\alpha = 0.05$). To this end, we removed non-significant terms, starting with the least significant interaction. Terms that significantly reduced the explanatory power of the model after removal were retained in the final model. All analyses were performed in R version 3.0.2 (R Core Team 2013).

To analyze whether non-parasitic species show higher variance between colonies to hinder slavemakers to match host profiles, we compared the Bray-Curtis Similarity of individuals between and within colonies. Moreover we tested whether males show higher within-colony, but lower between-colony variance than workers or virgin queens. Males should not be under selection to exhibit colony-specific odors (except for inbreeding avoidance), but should rather need distinct species-specific profiles for sexual communication and mating. Therefore we calculated the Bray-Curtis Similarity of two castes of each species per colony, using Primer 6.0. We randomly chose the Bray-Curtis Similarity of one caste of each species per colony towards the same caste and species of another colony from the same community, to calculate between-colony similarity. Chemical similarities were analyzed using a linear model with lifestyle, caste and colony origin (whether the Bray-Curtis Similarity is within or between colonies) as fixed factors, with interactions allowed. Species nested in community were included as random factors.

RESULTS

Chemical composition and the absence of parasite mimicry

Thirty-six identified hydrocarbons were included in the analyses, which are shared among workers of all species. Only a few of them were absent in virgin queens and males (Table 7.1).

As social parasites often mimic host odors, we firstly investigated whether *Temnothorax* slavemakers chemically resemble their sympatric hosts. A slavemaker species per community was compared to all possible host species originating from the same or different communities. In all three comparisons (*T. americanus* from NY, *T. americanus* from WV, *T. duloticus* from OH and *T. pilagens* from MI) slavemakers were chemically distinct from their host species independent of their community of origin (Table 7.2; pairwise comparisons: Table S7.2). Hence, we found little evidence for chemical resemblance of host odors, and turn to investigate whether slavemakers use other chemical strategies.

Chemical differences between slavemakers and non-parasitic species

We analyzed whether slavemaker species exhibit common features in their chemical profile, which sets them apart from the non-parasitic species. Indeed, the composition of cuticular chemicals differed between the two lifestyles (PERMANOVA: slavemaker vs. non-parasitic species; *Pseudo-F* = 3.51, *P* = 0.027; Fig. 7.1) and among the six species (nested in lifestyle: *Pseudo-F* = 8.23, *P* = 0.001). Caste differences were species-specific (caste: *Pseudo-F* = 1.68, *P* = 0.152; species (nested in lifestyle) x caste: *Pseudo-F* = 6.07, *P* = 0.001; Table S7.3; pairwise comparisons: Table S7.4, S7.5). A repetition of the analyses including sexuals vs. workers instead of caste showed across-species chemical differences associated with being a sexual (male or virgin queen) or a worker (*Pseudo-F* = 9.51, *P* = 0.001; Table S7.6; pairwise comparisons: Table S7.7, S7.8, S7.9), next to the lifestyle (*Pseudo-F* = 19.73, *P* = 0.001) and species differences (*Pseudo-F* = 5.69, *P* = 0.001).
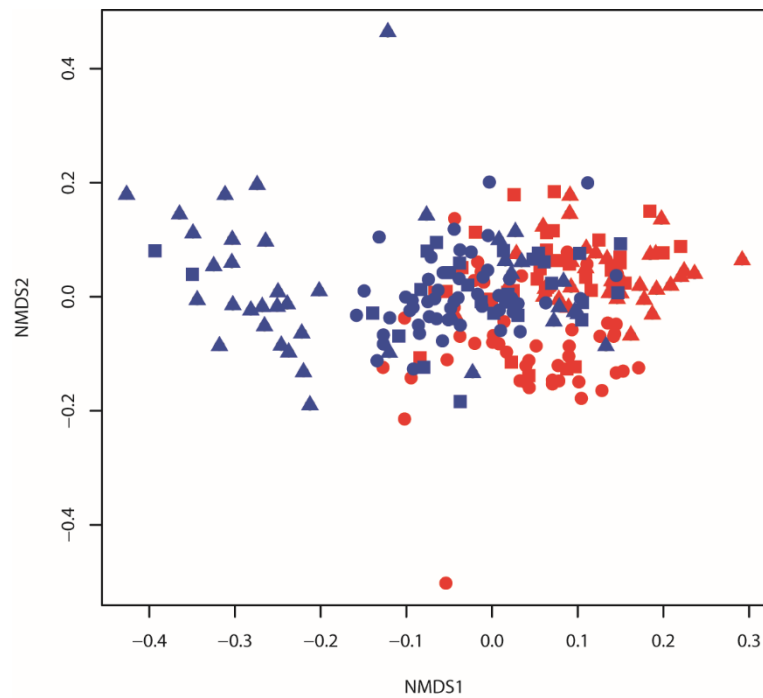
Figure 1: NMDS Plots showing lifestyle differences. Red symbols represent slavemakers and blue symbols non-parasitic hosts of all three castes. Different symbols represent different castes (workers = circles, virgin queens = squares and males = triangles). Stress value: 0.185.

Identification of characteristic substance classes for lifestyle and caste

In the following, we compared the effect of lifestyle and caste on mean chain length, the amount of hydrocarbons and the different CHC classes to identify characteristic substances first for slavemakers versus non-parasitic species and then for the different castes within each lifestyle. The statistical results for the following passages are given in Table 7.3 (model selection results) and 7.4 (model summaries).

The chemical profile of all species included *n*-alkanes, monomethyl- and dimethyl-alkanes. We identified only a single alkene, which was present in 65.86 % of all individuals. On average slavemakers carried 0.007% ± 0.009% and non-parasitic species carried 0.088% ± 0.157% alkenes on their cuticle. Hence, the alkene data were zero-inflated and omitted from further analyses (Table 7.1; Fig. 7.2).
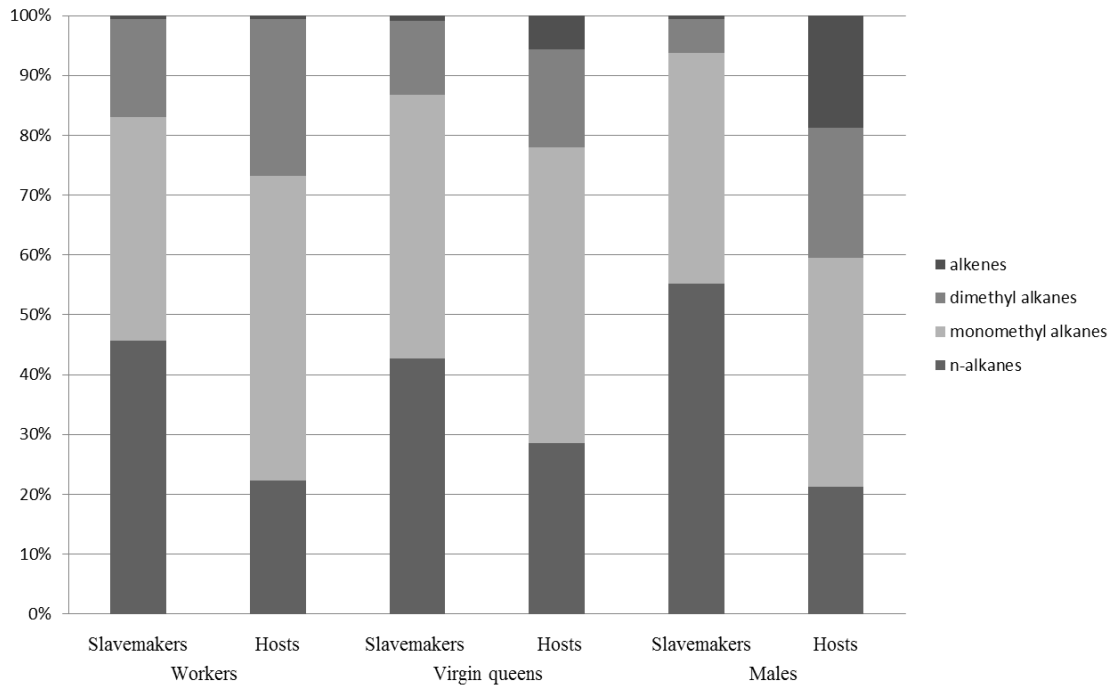
Figure 7.2 Composition of substance classes per lifestyle and caste. Shown are the average amounts of *n*-alkanes, monomethyl-alkanes, dimethyl-alkanes and alkenes.

Effect of lifestyle

The chemical profile of slavemakers was composed of shorter-chained hydrocarbons than that of the non-parasitic hosts and this was true for all three castes (Fig. 7.3a). The total amounts of CHCs did not depend on lifestyle, that is, slavemakers were not chemically insignificant (Fig. 7.3b). However, across all three castes, slavemakers carried proportionally more *n*-alkanes on their cuticle than non-parasitic species (Fig. 7.3c). Methyl-branched alkanes were often less abundant in slavemakers than in non-parasitic species, but these effects did not occur in all castes. Slavemaker workers had proportionally less monomethyl-alkanes than non-parasitic workers, whereas slavemaker sexuals did not carry less on their cuticle than virgin queens and males of non-parasitic species (Fig. 7.3d). Additionally, slavemaker workers and males exhibited less dimethyl-alkanes in their profile than non-parasitic species, whereas this effect of lifestyle was absent in virgin queens (Fig. 7.3e). Finally, we compared putative recognition substances (Jongepier and Foitzik 2016) and expected to find lower abundances in slavemakers than in non-parasitic species, to avoid host aggression during attacks. Indeed, slavemaker workers carried proportionally less of those recognition substances than host workers, whereas slavemaker sexuals did not differ from non-parasitic species (Fig. 7.3f).
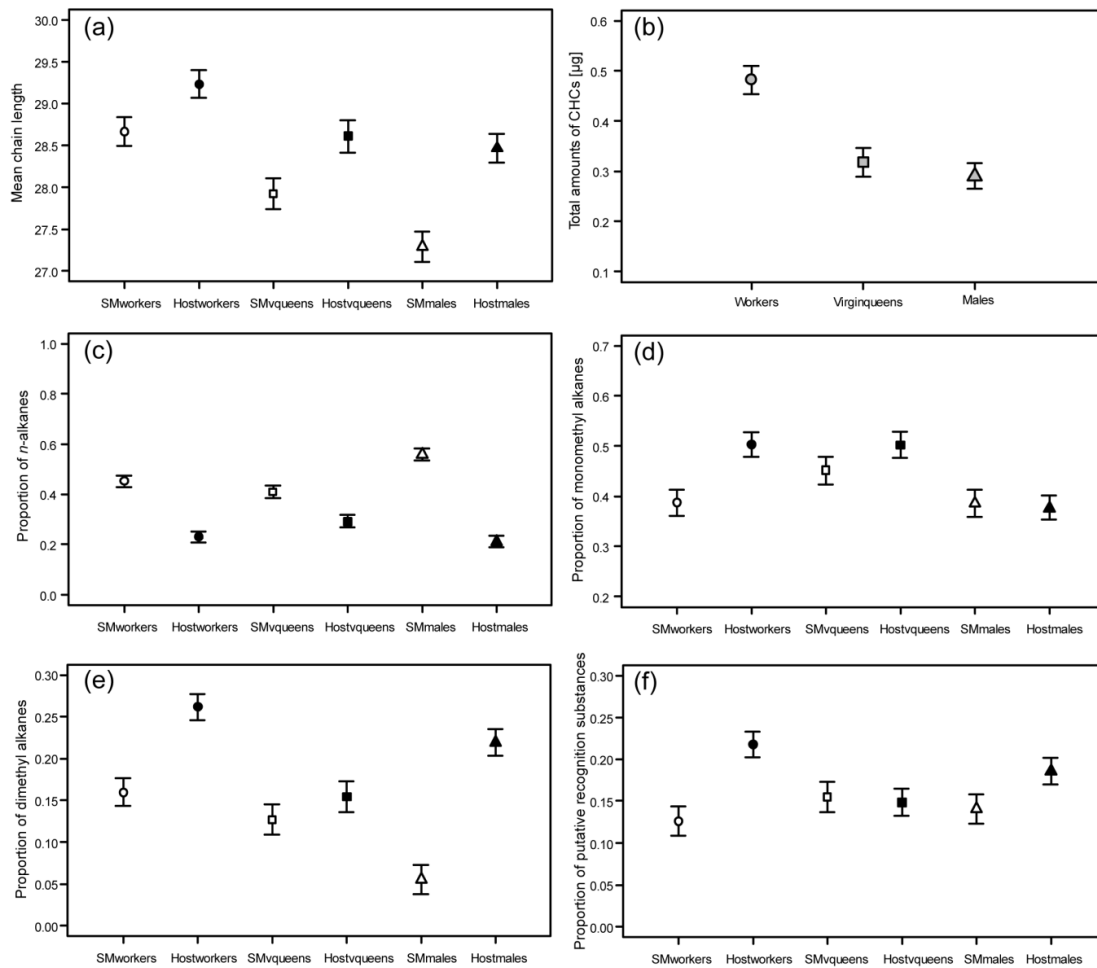
Figure 7.3 Influence of lifestyle and caste on different substances classes, including the mean chain length (a), the total amount of hydrocarbons on the cuticle (b), the relative amounts of n-alkanes (c), monomethyl -alkanes (d), dimethyl- alkanes (e) as well as the putative recognition substances (f). Slavemakers are shown with white symbols whereas non-parasitic host species are shown in black symbols. Different symbols represent different castes (workers = circles, virgin queens = squares and males = triangles). Symbols in grey for the total amounts of hydrocarbons on the cuticle represent both slavemakers and non-parasitic species, as we found no effect of lifestyle (L.Ratio = 0.02, P = 0.887).

Effect of caste within lifestyle

Workers of both lifestyles exhibited longer hydrocarbons than males and virgin queens. Moreover, virgin queens showed longer hydrocarbons than males among slavemakers, whereas this was not true for non-parasitic species (Fig. 7.3a). Workers generally carried more hydrocarbons than sexuals, whereas virgin queens and males did not differ in their total amounts (Fig. 7.3b). In addition, slavemaker males had more *n*-alkanes than workers and virgin queens, whereas in the non-parasitic species, virgin queens carried more *n*-alkanes than males and workers

(Fig. 7.3c). Moreover, slavemaker queens carried proportionally more monomethyl-alkanes than workers and males, whereas in non-parasitic species, virgin queens and workers did not differ. However, workers and virgin queens of non-parasitic species carried proportionally more monomethyl-alkanes than males (Fig. 7.3d). Workers and virgin queens of slavemakers did not differ in their proportion of dimethyl-alkanes, but both carried more than males. Non-parasitic workers showed higher relative amounts of dimethyl alkanes than virgin queens and males, and males exhibited higher relative amounts than virgin queens (Fig. 7.3e). Within the slavemakers, virgin queens carried more recognition substances than workers, but they did not differ from males neither did the latter differ from workers. Within the non-parasitic species, workers carried proportionally more of these recognition substances than virgin queens and males, and males carried more than virgin queens (Fig. 7.3f).

The effect of social environment

The previous analyses are based on young ants, which were taken from their original colony up to five days after emergence. By that time, slavemakers could potentially have obtained hydrocarbons from their heterospecific slaves by grooming. The only way to avoid this hydrocarbon transfer is to keep the ants in isolation after emergence. Hence, we repeated all analyses including samples from isolated individuals and adding "social environment" as a factor to our models. We were thus able to study the effects of isolation – a stressful situation for a social insect - on the CHC profile. The effects of lifestyle and caste on the chemical profiles of isolated ants were similar to those of individuals that emerged in their original colony (Supplement S7.1). Hence, we present only the influence of isolation on the chemical profile (model selection Table S7.10, model summaries Table S7.11).

The chemical profile of ants that emerged in isolation contained hydrocarbons of shorter chain lengths than those that emerged in their original colony with contact to nestmates (Fig. S7.1a). For the slavemakers, isolated virgin queens and males carried more CHCs on their cuticle than those that emerged in their mother nest. There was no influence of social environment on slavemaker workers. For the non-parasitic species, workers carried less CHCs when emerged in isolation, but isolated males and virgin queens carried more CHCs (Fig. S7.1b). Moreover, isolated ants had proportionally more *n*-alkanes than those emerged in their original colony (Fig. S7.1c). They carried less monomethyl-alkanes (Fig. S7.1d) and less dimethyl-alkanes (Fig. S7.1e) than individuals, that emerged in their original colony, with the exception of slavemaker males where the social environment had no effect on their proportion of dimethyl-alkanes. Isolated slavemaker workers did not differ in their proportion of recognition substances from those that emerged in their original nest (Fig. S7.1f). Isolated slavemaker males and virgin queens carried relatively less recognition substances than those that emerged in their original colony. Isolated non-parasitic workers and males

carried relatively less recognition substances than those that emerged with contact to their nestmates, whereas there was no influence of social environment on virgin queens.

Chemical diversity between and within colonies

Our chemical diversity analyses detected an effect of caste (*L.Ratio* = 8.74, *P* = 0.013) and colony (within or between colonies′ variance; *L.Ratio* = 21.01, *P* < 0.001), as well as an interaction of lifestyle and caste (*L.Ratio* = 10.79, *P* = 0.004) on the Bray-Curtis Similarity Index. As expected, the chemical similarity was always higher within colonies than between (*t* = -4.61, *P* < 0.001; Fig. 7.4). For slavemakers, the Bray Curtis Similarity Index, within as well as between colonies, did not differ between the castes (worker vs. males: *t* = 0.03, *P* = 0.980, workers vs. virgin queens: *t* = 0.52, *P* = 0.601; virgin queens vs. males *t* = -0.50, *P* = 0.614), yet it differed for the non-parasitic species. Males showed lower similarity, within and between colonies, than workers and virgin queens (vs. workers: *t* = 3.74, *P* < 0.001; vs. virgin queens: *t* = 3.40, *P* < 0.001), whereas the latter two did not differ (*t* = 0.24, *P* = 0.814; Fig. 7.4).
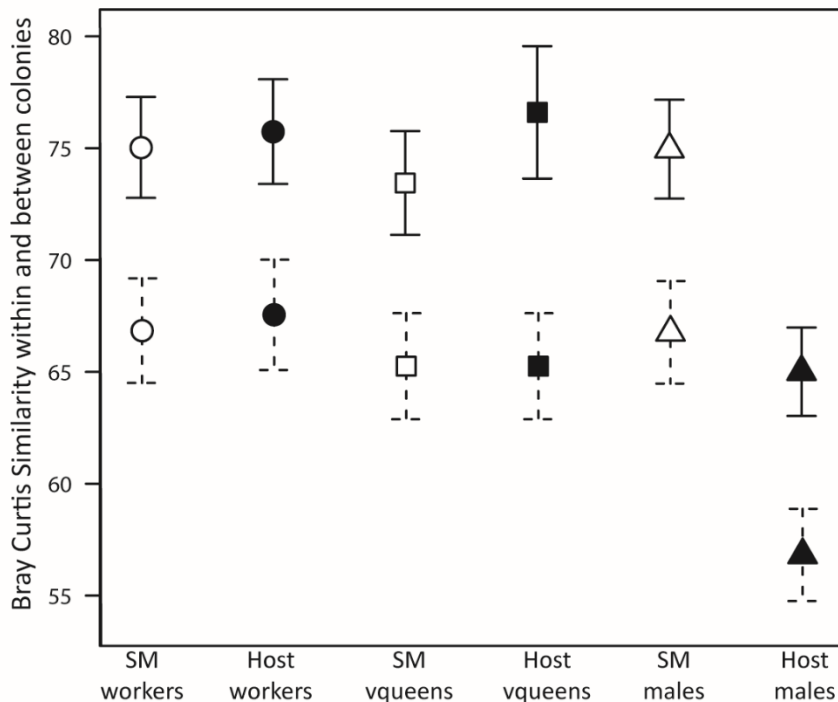
Figure 7.4 Bray Curtis Similarity Index within and between colonies. Dotted error bars show the chemical similarity between colonies and solid error bars within colonies. Slavemakers are shown with white symbols whereas non-parasitic host species are shown in black symbols. Different symbols represent different castes (workers = circles, virgin queens = squares and males = triangles).

DISCUSSION

Here, we demonstrate that slavemaking species exhibit a characteristic chemical profile that clearly differs from that of related non-parasitic species. Slavemaker ant profiles are characterized by shorter hydrocarbons, fewer methyl-branched alkanes and more n-alkanes. Hence, slavemakers carry less CHCs that are known to be important in nestmate recognition in general (Dani et al. 2001; Lorenzi et al. 2011), including the putative recognition substances described for Temnothorax longispinosus (Jongepier and Foitzik 2016). Instead, they possess more n-alkanes than hosts, which are thought to have little recognition value (Dani et al. 2001; Martin et al. 2008a; Blomquist and Bagnéres 2010; Kilner and Langmore 2011). Slavery arose several times independently among the species studied (Beibl et al. 2005), so that the chemical similarities in slavemaker profiles cannot be due to a common origin. Rather, similar selection pressures to avoid host recognition and counterattacks have apparently led to the convergent evolution of chemical traits in slavemakers. To our knowledge, such general strategies that are similar across multiple interaction systems, and yield similar effects on CHC composition across species, only have been shown in mutualistic ant-ant associations so far (Menzel and Schmitt 2011), but not for any other interaction type.

Myrmecophiles and social parasites frequently use chemical mimicry to overcome their hosts' nestmate recognition system (Lenoir et al. 2001). They can chemically mimic even hosts from a different order, such as lycaenid butterflies or spiders, both of which mimic ants (Schlick-Steiner et al. 2004; Allan et al. 2002). The slavemaker species studied here do not employ chemical mimicry, but have clearly distinct chemical profiles. Similar reports exist for other slavemaker species (Liu et al. 2000; Brandt et al. 2005b; Lambardi et al. 2007; Blomquist and Bagnéres 2010). This is also evidenced by the aggressive responses of their hosts (Alloway 1990; Pamminger et al. 2011; Kleeberg et al. 2014, Chapter 1), with the exception of *T. pilagens* (Kleeberg and Foitzik 2016, Chapter 6). Why do the slavemakers fail to mimic host profiles? We suggest that a lack of mimicry may be due to host defenses (Kleeberg et al. 2014, 2015, Chapter 1-2; Jongepier et al. 2015, Chapter 4). Indeed, there is evidence that the host *T. longispinosus* evolved counter-adaptations (Achenbach and Foitzik 2009; Pamminger et al. 2012; Jongepier et al. 2014, Chapter 3), such as a diversification of the chemical profile between colonies, disallowing the parasite to match all hosts (Jongepier and Foitzik 2016). Moreover, chemical mimicry only allows adaptation to one host species. In contrast, reduced recognition cues through larger abundances of *n*-alkanes might be a more suitable strategy, since it allows simultaneous exploitation of multiple host species. Slavemakers indeed frequently exploit several host species, and even live with multiple slave species in the same nest (Bauer et al. 2010; Kleeberg and Foitzik 2016, Chapter 6). Slavemakers may profit from the fact that compared to their hosts, they only differ in quantitative CHC composition, but do not possess

qualitatively different substances. Combined with the reduced recognition cue quantities, this may decrease the risk of being recognized and attacked by hosts. Although *T. americanus* and *T. duloticus* are frequently attacked by hosts (Alloway 1990; Kleeberg and Foitzik 2016, Chapter 6), attack rates can still vary over a wide range, thereby strongly affecting the success rate of raids.

High abundance of *n*-alkanes have previously been reported for social parasitic queens of leaf-cutting ants and eggs of the social parasite *Vespa dybowskii*, which was interpreted as "chemical transparency" facilitating host colony entry by the absence of recognition labels (Martin et al. 2008b; Nehring et al. 2015). We suggest that the n-alkanes 'dilute' the other substances, thus weakening the recognition label of an individual. Individuals with fewer recognition cues might be difficult to interpret by host workers (Cini et al. 2009) and could be more easily accepted by foreign colonies. Hosts may be forced to integrate such parasites, because discriminating against individuals deficient of recognition cues would lead to the rejection of young callow ants, which just start to build up a hydrocarbon signature (Lenoir et al. 2001). In turn, the higher abundance of methyl-alkanes in host species might be a counter-adaptation to enhance their discrimination ability against slavemakers (Steiger et al. 2011), next to more diverse chemical profiles in host populations (Jongepier and Foitzik 2016; Martin et al. 2011). Slavemakers possessed similar total CHC quantities as their hosts, and thus were not chemically insignificant sensu Lenoir et al. (2001).

The profiles of slavemaking ants were also characterized by shorter chained hydrocarbons, which was also found in hosts of the inquiline Acromyrmex insinuator (Nehring et al. 2015), but contrasts to an incipient leaf-cutting ant social parasite which is richer in long-chained hydrocarbons (Lambardi et al. 2007) or mutualistically associated (parabiotic) ants, which are characterized by significantly longer chains than non-associated species (Menzel et al. 2008; Menzel and Schmitt 2011). This might seem surprising, since long-chain hydrocarbons are harder to perceive and thus should be more appropriate to conceal one's chemical profile. However, a plausible explanation is that the large percentage of *n*-alkanes in slavemakers requires shorter chain lengths to maintain the fluidity of the cuticular hydrocarbon layer, since longer-chain *n*-alkanes would aggregate too tightly, thus reducing the CHC fluidity (Gibbs 1995; Gibbs and Pomonis 1995). This hypothesis is corroborated by the strong correspondence of n-alkane percentage and average chain length in the different castes and species (Fig. 7.1a,c).

As only slavemaker workers and queens interact with hosts, we expected a signal of the parasitic lifestyle to be predominantly present in the females, but not in the males. While the parasitic lifestyle of slavemaker queens and workers clearly differs from that of their non-parasitic host species, this is not true for the males. Parasites that exert stronger selection on one sex, were shown to select for sex-specific adaptations (Dargent et al. 2016). However, the characteristic chemical

composition of slavemakers was present to various degrees in all castes and sexes. We interpret this as a sex-specific atavism, i.e. male parasite profiles are dragged along due to strong selection on female profiles. There was however another trait, in which slavemaker males differed from their non-parasitic counterparts. Host males showed lower Bray Curtis chemical similarities (i.e. higher diversity) both between and within colonies, whereas workers or queens did not differ between slavemakers and hosts. As parasitized host populations are chemically more diverse compared to unparasitized ones (Jongepier and Foitzik 2016), we had predicted higher chemical diversity in host females compared to slavemakers. However, we found this effect only in the males. Since the haploid males lack allelic variation in their genome compared to the diploid females, chemical difference due to different alleles might be more pronounced in the males, while they are more likely to be balanced out in heterozygous females.

Our dataset enables us to systematically explore chemical differences between workers, queens, and males. While specific compounds that are characteristic for queens (queen pheromones) have been identified in various species (Brunner et al. 2011; Kocher and Grozinger 2011; Oi et al. 2015), we are the first to show chemical characteristics that, beyond queen signaling, differentiate sexuals from workers and are related to their different tasks. Throughout all species, workers possessed higher CHC quantities, and a higher average chain length than queens or males. Since workers are usually smaller than queens or males, the difference we found probably underestimates the real difference corrected for body surface. The higher CHC quantity is probably due to their need for waterproofing, since workers spend more time outside the nest than sexuals. Secondly, workers possessed more dimethyl-alkanes than queens and males, at least in non-parasitic species. Workers encounter non-nestmates much more frequently than queens and males. Thus, by exposing larger quantities of recognition cues (dimethyl-alkanes), they facilitate recognition by their nestmates, while this is less important for queens and males. However, the multivariate analyses on the entire CHC composition did not detect an overall caste signal. Within each species, castes were chemically distinct, with some exceptions in *T. ambiguus*, *T. curvispinosus* and *T. pilagens*, where virgin queens and males were indistinguishable. The absence of an overall caste signal indicates that species affiliation and recognition is more important than a caste signal. This agrees with earlier studies, where species differences were higher than sex differences, in ants and Drosophila flies (Billeter et al. 2009, Brunner et al. 2011). The profiles of males and queens are mainly under sexual selection and could be important for mate choice (Thomas and Simmon 2008). In contrast to Billeter et al. (2009), however, we did not find a sex-specific compound, which coincides with other studies on social insects that only found quantitative differences between the sexes (Beibl et al. 2007; Oppelt et al. 2008; Hojo et al. 2009; Chernenko et al. 2012). In ants, information on the sex seems to be encoded in the hydrocarbon blend rather than

by single compounds. Still, we found that some substances were missing in males or virgin queens. One compound can be enough to establish a species barrier, so that the absence or presence of a single compound might already function as a reproductive isolation barrier between different species (Grillet et al. 2006).

Most of our results obtained from individuals in functioning colonies were also found in individuals that emerged in isolation. This indicates that the chemical traits we analyzed are largely genetically determined, and do not depend on individual CHC acquisition from nestmates. Although the differences between lifestyles and castes were similar in functioning nests and in isolation, however, social isolation itself had a dramatic effect on the hydrocarbon profiles. Isolated ants possessed drastically increased proportions of n-alkanes, which may be a way to compensate for the lack of nestmate care. Newly emerged insects often increase their amount of *n*-alkanes within the first days, but also adjust it throughout their lifetime (de Renobales and Blomquist 1983).

Our results show that selection pressures due to differences in lifestyle between social parasites and hosts, but also between workers and sexuals result in characteristic signals in the chemical profile. Cuticular hydrocarbons are adapted to the different requirements of exposed cues (non-parasitic; workers), less exposed (sexuals) and concealed (social parasites) recognition cues. Cue exposition and waterproofing are adjusted through variation in hydrocarbon quantity, percentage of n-alkanes, and CHC chain length. To our knowledge, this is the first study to demonstrate how chemical profiles systematically respond to these diverse selective pressures, yielding similar differences between castes and lifestyles across multiple species.

Chapter 7

SUPPORTING INFORMATION

Table S7.1: Collection sites and details for the *Temnothorax* colonies and samples. Slavemaker species are given in bold.

Community and species	Coordinates	*N* of collected colonies	*N* of analyzed samples from original colonies			*N* of analyzed samples that emerged in isolation		
			Workers	Virgin queens	Males	Workers	Virgin queens	Males
T. americanus								
New York	42°31'57.0''N 74°08'45.2''W	100	17	20	19	2	16	3
West Virginia	38°06'28.8''N 80°07'52.9''W	56	14	3	9	9	1	0
Ohio	40°14'13.8''N 82°59'06.5''W	30	3	17	6	1	0	0
T. longispinosus								
New York	42°31'57.0''N 74°08'45.2''W	21	11	8	17	24	22	14
West Virginia	38°06'28.8''N 80°07'52.9''W	92	15	10	11	3	11	0
Ohio	40°14'13.8''N 82°59'06.5''W	19	6	1	4	20	1	2
Michigan	43°26'24.6''N 72°09'20.8''W	88	13	8	17	17	2	6
T. duloticus								
Ohio	40°14'13.8''N 82°59'06.5''W	30	11	9	9	16	17	10
T. curvispinosus								
Ohio	40°14'13.8''N 82°59'06.5''W	65	8	3	3	14	0	10
West Virginia	38°06'28.8''N 80°07'52.9''W	35	11	4	5	9	0	1
T. pilagens								
Michigan	43°26'24.6''N 72°09'20.8''W	42	27	6	16	2	0	0
T. ambiguus								
Michigan	43°26'24.6''N 72°09'20.8''W	54	5	9	13	3	1	5
New York	42°31'57.0''N 74°08'45.2''W	5	7	2	6	0	0	0

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Table S7.2: Pairwise comparisons of slavemakers toward their potential host species, either allopatric or sympatric, including the average chemical similarity between groups.

	t	P	Unique Perms	Average similarity
***T. americanus* from New York vs.**				
T. longispinosus NY	3.55	0.001	996	51.938
T. longispinosus WV	3.36	0.001	997	53.380
T. longispinosus OH	2.16	0.008	530	52.178
T. longispinosus MI	3.74	0.001	997	53.676
T. curvispinosus OH	3.91	0.001	964	42.069
T. curvispinosus WV	3.28	0.001	996	43.698
T. ambiguus NY	3.50	0.001	969	39.700
T. ambiguus MI	3.59	0.001	852	41.984
***T. americanus* from West Virginia vs.**				
T. longispinosus NY	1.88	0.007	917	61.292
T. longispinosus WV	1.93	0.006	968	60.417
T. longispinosus OH	1.24	0.211	165	59.441
T. longispinosus MI	2.64	0.001	977	58.002
T. curvispinosus OH	2.46	0.004	688	52.917
T. curvispinosus WV	1.73	0.017	918	53.085
T. ambiguus NY	1.69	0.005	695	54.407
T. ambiguus MI	2.63	0.005	427	48.566
***T. duloticus* from Ohio vs.**				
T. longispinosus NY	1.92	0.027	928	58.815
T. longispinosus WV	2.12	0.004	981	56.165
T. longispinosus OH	1.41	0.136	165	54.222
T. longispinosus MI	2.53	0.002	975	55.764
T. curvispinosus OH	2.07	0.011	689	53.826
T. curvispinosus WV	1.84	0.021	918	49.624
***T. pilagens* from Michigan vs.**				
T. longispinosus NY	2.46	0.001	999	50.595
T. longispinosus WV	2.58	0.001	999	49.652
T. longispinosus OH	1.58	0.030	680	48.472
T. longispinosus MI	2.45	0.002	998	53.295
T. ambiguus NY	2.49	0.001	977	40.324
T. ambiguus MI	2.23	0.003	906	46.073

Table S7.3: Differences in CHC composition depending on lifestyle (slavemaker vs. non-parasitic species), caste (workers, virgin queens and males) and species. The table shows PERMANOVA results based on Bray-Curtis similarities. Significant factors are given in bold.

Factors	DF	Pseudo-F	*P*
Lifestyle	**1**	**3.51**	**0.027**
Caste	2	1.68	0.152
Species (Lifestyle)	**4**	**8.23**	**0.001**
Lifestyle x caste	2	1.33	0.292
Species (Lifestyle) x Caste	**8**	**6.07**	**0.001**

Table S7.4: Pairwise comparisons following the species (lifestyle) x caste interaction for pairs of levels of factor species.

Comparisons	t	P	Unique Perms
Within level "non-parasitic" and "worker"			
T. longispinosus vs. *T. curvispinosus*	2.06	0.002	999
T. longispinosus vs. *T. ambiguus*	1.93	0.002	999
T. curvispinosus vs. *T. ambiguus*	1.43	0.051	998
Within level "non-parasitic" and "virginqueen"			
T. longispinosus vs. *T. curvispinosus*	3.61	0.006	189
T. longispinosus vs. *T. ambiguus*	2.01	0.003	996
T. curvispinosus vs. *T. ambiguus*	2.79	0.027	36
Within level "non-parasitic" and "male"			
T. longispinosus vs. *T. curvispinosus*	3.04	0.001	977
T. longispinosus vs. *T. ambiguus*	4.29	0.001	999
T. curvispinosus vs. *T. ambiguus*	1.86	0.002	633
Within level "slavemaker" and "worker"			
T. americanus vs. *T. pilagens*	2.60	0.001	999
T. americanus vs. *T. duloticus*	1.28	0.138	999
T. duloticus vs. *T. pilagens*	1.98	0.002	999
Within level "slavemaker" and "virginqueen"			
T. americanus vs. *T. pilagens*	3.07	0.001	973
T. americanus vs. *T. duloticus*	2.40	0.002	996
T. duloticus vs. *T. pilagens*	4.19	0.002	208
Within level "slavemaker" and "male"			
T. americanus vs. *T. pilagens*	1.84	0.004	998
T. americanus vs. *T. duloticus*	2.36	0.001	998
T. duloticus vs. *T. pilagens*	1.58	0.019	938

Table S7.5: Pairwise comparisons following the species (lifestyle) x caste interaction for pairs of levels of factor caste.

Comparisons	t	P	Unique Perms
Within level "non-parasitic" and "*T. longispinosus*"			
male vs. worker	6.09	0.001	999
male vs. virginqueen	5.88	0.001	997
worker vs. virginqueen	2.82	0.001	997
Within level "non-parasitic" and "*T. curvispinosus*"			
male vs. worker	1.71	0.010	703
male vs. virginqueen	3.13	0.075	15
worker vs. virginqueen	2.68	0.020	78
Within level "non-parasitic" and "*T. ambiguus*"			
male vs. worker	1.42	0.059	994
male vs. virginqueen	0.90	0.564	964
worker vs. virginqueen	1.06	0.345	969
Within level "slavemaker" and "*T. americanus*"			
male vs. worker	3.24	0.001	998
male vs. virginqueen	2.42	0.005	999
worker vs. virginqueen	3.26	0.001	999
Within level "slavemaker" and "*T. duloticus*"			
male vs. worker	2.01	0.005	838
male vs. virginqueen	1.32	0.115	405
worker vs. virginqueen	1.95	0.021	858
Within level "slavemaker" and "*T. pilagens*"			
male vs. worker	3.04	0.001	999
male vs. virginqueen	2.61	0.001	621
worker vs. virginqueen	1.22	0.206	920

Table S7.6: Differences in CHC composition depending on lifestyle (slavemaker vs. non-parasitic species), worker vs. sexuals and species. The table shows PERMANOVA results based on Bray-Curtis similarities.

Factors	DF	Pseudo-F	P
Lifestyle	1	19.73	0.001
Workers vs. sexuals	1	9.51	0.001
Species (Lifestyle)	4	5.69	0.001
Lifestyle x workers vs. sexuals	1	8.94	0.001
Species (Lifestyle) x workers vs. sexuals	4	3.00	0.001

Table S7.7: Pairwise comparisons following the species (lifestyle) x workers vs. sexuals interaction for pairs of levels of factor species (lifestyle).

Comparisons	t	P	Unique Perms
Within level "non-parasitic" and "*T. longispinosus*"			
Workers vs. sexuals	3.30	0.001	997
Within level "non-parasitic" and "*T. curvispinosus*"			
Workers vs. sexuals	1.65	0.001	969
Within level "non-parasitic" and "*T. ambiguus*"			
Workers vs. sexuals	1.39	0.077	998
Within level "slavemaker" and "*T. americanus*"			
Workers vs. sexuals	3.49	0.001	997
Within level "slavemaker" and "*T. duloticus*"			
Workers vs. sexuals	2.33	0.001	995
Within level "slavemaker" and "*T. pilagens*"			
Workers vs. sexuals	2.51	0.001	999

Table S7.8: Pairwise comparisons following the species (lifestyle) x workers vs. sexuals interaction for pairs of levels of factor.

Comparisons	t	P	Unique Perms
Within level "non-parasitic" and "workers"			
T. longispinosus vs. *T. curvispinosus*	2.08	0.001	998
T. longispinosus vs. *T. ambiguus*	1.98	0.001	999
T. curvispinosus vs. *T. ambiguus*	1.52	0.019	993
Within level "non-parasitic" and "sexuals"			
T. longispinosus vs. *T. curvispinosus*	0.95	0.430	999
T. longispinosus vs. *T. ambiguus*	2.94	0.001	996
T. curvispinosus vs. *T. ambiguus*	2.03	0.008	992
Within level "slavemaker" and "workers"			
T. americanus vs. *T. pilagens*	2.60	0.001	999
T. americanus vs. *T. duloticus*	1.28	0.132	999
T. duloticus vs. *T. pilagens*	1.98	0.001	999
Within level "slavemaker" and "sexuals"			
T. americanus vs. *T. pilagens*	2.47	0.001	998
T. americanus vs. *T. duloticus*	3.03	0.001	999
T. duloticus vs. *T. pilagens*	2.65	0.001	998

Table S7.9: Pairwise comparisons following the lifestyle x workers vs. sexuals interaction for pairs of levels of factor lifestyle, and following the lifestyle x workers vs. sexuals interaction for pairs of levels of factor workers vs. sexuals.

Comparisons	t	P	Unique Perms
Within level "sexuals"			
Slavemakers vs. non-parasitic species	3.76	0.001	998
Within level "workers"			
Slavemakers vs. non-parasitic species	3.93	0.001	999
Within level "non-parasitic"			
Workers vs. Sexuals	2.59	0.001	999
Within level "slavemakers"			
Workers vs. Sexuals	3.61	0.001	998

Table S7.10: Model selection results from the linear model analyzing the mean chain length, the total amounts of CHCs, the proportion of n-alkanes, monomethyl-alkanes, dimethyl-alkanes and the putative recognition substances (Jongepier and Foitzik 2016) in relation to lifestyle, caste and social environment of the individuals. Statistics indicated in bold retained in the final model.

Predictors	L.Ratio	P-Value
Mean chain length		
Social environment x Caste x Lifestyle	**12.26**	**0.002**
Caste x Lifestyle	**10.92**	**0.004**
Caste x Social environment	**29.99**	**< 0.001**
Social environment x Lifestyle	**21.20**	**< 0.001**
Caste	0.88	0.643
Lifestyle	0.73	0.392
Social environment	**244.00**	**< 0.001**
Total amounts of CHCs		
Social environment x Caste x Lifestyle	**8.14**	**0.017**
Caste x Lifestyle	3.47	0.177
Caste x Social environment	**75.97**	**< 0.001**
Social environment x Lifestyle	0.86	0.353
Caste	5.89	0.053
Lifestyle	0.65	0.421
Social environment	**29.66**	**< 0.001**
Proportion of n-Alkanes		
Social environment x Caste x Lifestyle	3.15	0.207
Caste x Lifestyle	**24.91**	**< 0.001**
Caste x Social environment	**8.40**	**0.015**
Social environment x Lifestyle	1.21	0.271
Caste	**9.08**	**0.011**
Lifestyle	**24.43**	**< 0.001**
Social environment	**136.63**	**< 0.001**
Proportion of monomethylated Alkanes		
Social environment x Caste x Lifestyle	**21.63**	**< 0.001**
Caste x Lifestyle	**9.91**	**0.007**
Caste x Social environment	**7.17**	**0.028**
Social environment x Lifestyle	0.72	0.398
Caste	**33.41**	**< 0.001**
Lifestyle	**6.91**	**0.009**
Social environment	**183.73**	**< 0.001**
Proportion of dimethylated Alkanes		
Social environment x Caste x Lifestyle	**10.17**	**0.006**
Caste x Lifestyle	**21.79**	**< 0.001**
Caste x Social environment	**8.09**	**0.018**
Social environment x Lifestyle	**6.74**	**0.009**
Caste	**38.05**	**< 0.001**
Lifestyle	**24.56**	**< 0.001**
Social environment	**134.68**	**< 0.001**
Proportion of putative recognition substances		
Social environment x Caste x Lifestyle	**35.90**	**< 0.001**
Caste x Lifestyle	5.49	0.064
Caste x Social environment	**6.67**	**0.036**
Social environment x Lifestyle	0.76	0.384
Caste	**8.02**	**0.018**
Lifestyle	**7.11**	**0.008**
Social environment	**102.81**	**< 0.001**

Table S7.11: Summaries after model selection of the linear models analyzing the effects of lifestyle, caste and social environment on the mean chain length, the total amounts of CHC and the different substance classes. The reference includes slavemakers or non-parasitic species (lifestyle), workers, virgin queens or males (caste) and original colony or isolation (social environment). The reference is always compared to the other lifestyle, castes and social environment. Abbreviations as follows: Slavemakers = SM, non-parasitic species = HOST, workers = w, virgin queens = vq, males = m, original colony = OC, isolated individuals = II. Significant P-Values are given in bold.

Reference	Comparison	Mean chain length		Total amounts of CHCs [μg]		Proportion of n-alkanes		Proportion of monomethyl alkanes		Proportion of dimethyl alkanes		Proportion of recognition substances	
		t	*P*	*t*	*P*	*t*	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Original colony Workers Slave-makers	SM w II	-5.211	**< 0.001**	-1.563	0.119	7.331	**< 0.001**	-3.042	**0.003**	-5.631	**< 0.001**	-1.114	0.266
	SM m OC	-3.163	**0.002**	-3.683	**< 0.001**	3.506	**< 0.001**	0.534	0.594	-6.498	**< 0.001**	1.251	0.211
	SM vq OC	-1.695	0.091	-3.077	**0.002**	-1.004	0.316	2.783	**0.006**	-1.662	0.097	1.932	0.054
	HOST w OC	1.461	0.178	-0.776	0.457	-5.798	**< 0.001**	4.642	**0.001**	6.104	**< 0.001**	4.855	**< 0.001**
Isolation Workers Slave-makers	SM w OC	5.211	**< 0.001**	1.563	0.119	-7.331	**< 0.001**	3.042	**0.003**	5.631	**< 0.001**	1.114	0.266
	SM m II	-0.760	0.448	4.874	**< 0.001**	3.477	**< 0.001**	-2.915	**0.004**	-0.392	0.695	-2.755	**0.006**
	SM vq II	-1.638	0.102	3.624	**< 0.001**	2.096	0.036	-3.593	**< 0.001**	-0.696	0.487	-2.773	**0.006**
	HOST w II	-5.480	**< 0.001**	-0.828	0.429	-5.798	**< 0.001**	0.312	0.762	2.671	**0.026**	0.767	0.463
Original colony Workers Hosts	HOST w II	-16.111	**< 0.001**	-2.158	**0.031**	7.331	**< 0.001**	-10.547	**< 0.001**	-10.908	**< 0.001**	-9.217	**< 0.001**
	HOST m II	-2.045	**0.041**	-2.826	**0.005**	-0.705	0.481	-6.389	**< 0.001**	-3.079	**0.002**	-3.148	**0.002**
	HOST vq II	-1.251	0.212	-1.911	0.057	1.354	0.176	-0.699	0.485	-5.756	**< 0.001**	-5.906	**< 0.001**
	SM w OC	-1.461	0.178	0.776	0.457	5.798	**< 0.001**	-4.642	**0.001**	-6.104	**< 0.001**	-4.855	**< 0.001**
Isolation Workers Hosts	HOST w OC	16.111	**< 0.001**	2.158	**0.031**	-7.331	**< 0.001**	10.547	**< 0.001**	10.908	**< 0.001**	9.217	**< 0.001**
	HOST m OC	5.219	**< 0.001**	3.872	**< 0.001**	0.617	0.537	-2.106	**0.036**	-1.380	0.168	-1.506	0.133
	HOST vq OC	5.079	**< 0.001**	6.881	**< 0.001**	4.625	**< 0.001**	0.385	0.701	-0.966	0.334	1.508	0.132
	SM w II	5.481	**< 0.001**	0.828	0.429	5.798	**< 0.001**	-0.312	0.762	-2.671	**0.026**	-0.767	0.463
Original colony Virgin queens Slave-makers	SM vq II	-5.751	**< 0.001**	5.267	**< 0.001**	9.957	**< 0.001**	-9.482	**< 0.001**	-5.095	**< 0.001**	-5.891	**< 0.001**
	SM m OC	-1.253	0.211	-0.412	0.681	4.221	**< 0.001**	-2.264	**0.024**	-4.378	**< 0.001**	-0.777	0.438
	SM w OC	1.695	0.091	3.077	**0.002**	1.004	0.316	-2.783	**0.006**	1.662	0.097	-1.932	0.054
	HOST vq OC	1.557	0.154	0.218	0.832	-3.540	**0.006**	1.937	**0.085**	1.740	0.116	0.366	0.723
Isolation Virgin queens Slave-makers	SM vq OC	5.751	**< 0.001**	-5.268	**< 0.001**	-9.957	**< 0.001**	9.482	**< 0.001**	5.095	**< 0.001**	5.891	**< 0.001**
	SM m II	0.496	0.620	2.188	**0.029**	1.562	0.119	-0.188	0.851	0.139	0.890	-0.664	0.507
	SM w II	1.638	0.102	-3.624	**< 0.001**	-2.096	**0.037**	3.593	**< 0.001**	0.696	0.487	2.773	**0.006**
	HOST vq II	0.477	0.645	1.104	0.298	-3.540	**0.006**	3.394	**0.008**	2.313	**0.046**	3.388	**0.008**
Original colony Virgin queens Hosts	HOST vq II	-6.580	**< 0.001**	5.973	**< 0.001**	9.957	**< 0.001**	-9.482	**< 0.001**	-3.989	**< 0.001**	-0.674	0.501
	HOST m OC	-0.549	0.583	-0.566	0.571	-1.981	**0.048**	-2.264	**0.024**	3.086	**0.002**	3.184	**0.002**
	HOST w OC	1.251	0.212	1.911	0.057	-1.354	0.176	-2.783	**0.006**	5.756	**< 0.001**	5.906	**< 0.001**
	SM vq OC	-1.557	0.154	-0.218	0.832	3.540	**0.006**	1.937	0.085	-1.740	0.116	-0.366	0.723
Isolation Virgin queens Hosts	HOST vq OC	6.580	**< 0.001**	-5.973	**< 0.001**	-9.957	**< 0.001**	6.970	**< 0.001**	3.989	**< 0.001**	0.674	0.501
	HOST m II	-0.230	0.818	-2.818	**0.005**	-3.553	**< 0.001**	-2.079	**0.038**	-0.286	0.775	-2.595	**0.010**
	HOST w II	-5.079	**< 0.001**	-6.881	**< 0.001**	-4.625	**< 0.001**	-0.385	0.701	0.966	0.334	-1.508	0.132
	SM vq II	-0.477	0.645	-1.104	0.298	3.540	**0.006**	-3.394	**0.008**	-2.313	**0.046**	-3.388	**0.008**
Original colony Males Slave-makers	SM m II	-2.760	**0.006**	6.297	**< 0.001**	7.190	**< 0.001**	-5.537	**< 0.001**	-0.799	0.425	-4.384	**< 0.001**
	SM vq OC	1.253	0.211	0.412	0.681	-4.221	**< 0.001**	2.264	**0.024**	4.378	**< 0.001**	0.777	0.438
	SM w OC	3.163	**0.002**	3.683	**< 0.001**	-3.506	**< 0.001**	-0.534	0.594	6.498	**< 0.001**	-1.251	0.211
	HOST m OC	2.451	**0.037**	0.075	0.942	-8.406	**< 0.001**	0.158	0.878	8.969	**< 0.001**	2.600	**0.028**
Isolation Males	SM m OC	2.760	**0.006**	-6.297	**< 0.001**	-7.190	**< 0.001**	5.537	**< 0.001**	0.799	0.425	4.384	**< 0.001**
	SM vq II	-0.496	0.620	-2.188	**0.029**	-1.562	0.119	0.188	0.851	-0.139	0.890	0.664	0.507
	SM w II	0.760	0.448	-4.874	**< 0.001**	-3.477	**< 0.001**	2.915	**0.004**	0.392	0.695	2.755	**0.006**

|													
Slave-makers	HOST vq II	-0.257	0.803	-3.246	**0.010**	-8.406	**< 0.001**	1.730	0.118	1.487	0.171	2.080	**0.067**
Original colony	HOST m II	-7.233	**< 0.001**	4.406	**< 0.001**	7.190	**< 0.001**	-5.691	**< 0.001**	-7.888	**< 0.001**	-6.810	**< 0.001**
Males	HOST vq OC	0.549	0.583	0.566	0.571	1.981	**0.048**	4.960	**< 0.001**	-3.086	**0.002**	-3.184	**0.002**
	HOST w OC	2.045	**0.041**	2.826	**0.005**	0.705	0.481	6.389	**< 0.001**	3.079	**0.002**	3.148	**0.002**
Hosts	SM m OC	-2.451	**0.037**	-0.075	0.942	8.406	**< 0.001**	-0.158	0.878	-8.969	**< 0.001**	-2.600	**0.029**
Isolation	HOST m OC	7.233	**< 0.001**	-4.406	**< 0.001**	-7.190	**< 0.001**	5.691	**< 0.001**	7.888	**< 0.001**	6.810	**< 0.001**
Males	HOST vq II	0.230	0.818	2.818	**0.005**	3.553	**< 0.001**	2.079	**0.038**	0.286	0.775	2.595	**0.010**
	HOST w II	-5.219	**< 0.001**	-3.872	**< 0.001**	-0.617	0.537	2.106	**0.036**	1.380	0.168	1.506	0.133
Hosts	SM m II	0.257	0.803	3.246	**0.010**	8.406	**< 0.001**	-1.730	0.118	-1.487	0.171	-2.080	0.067
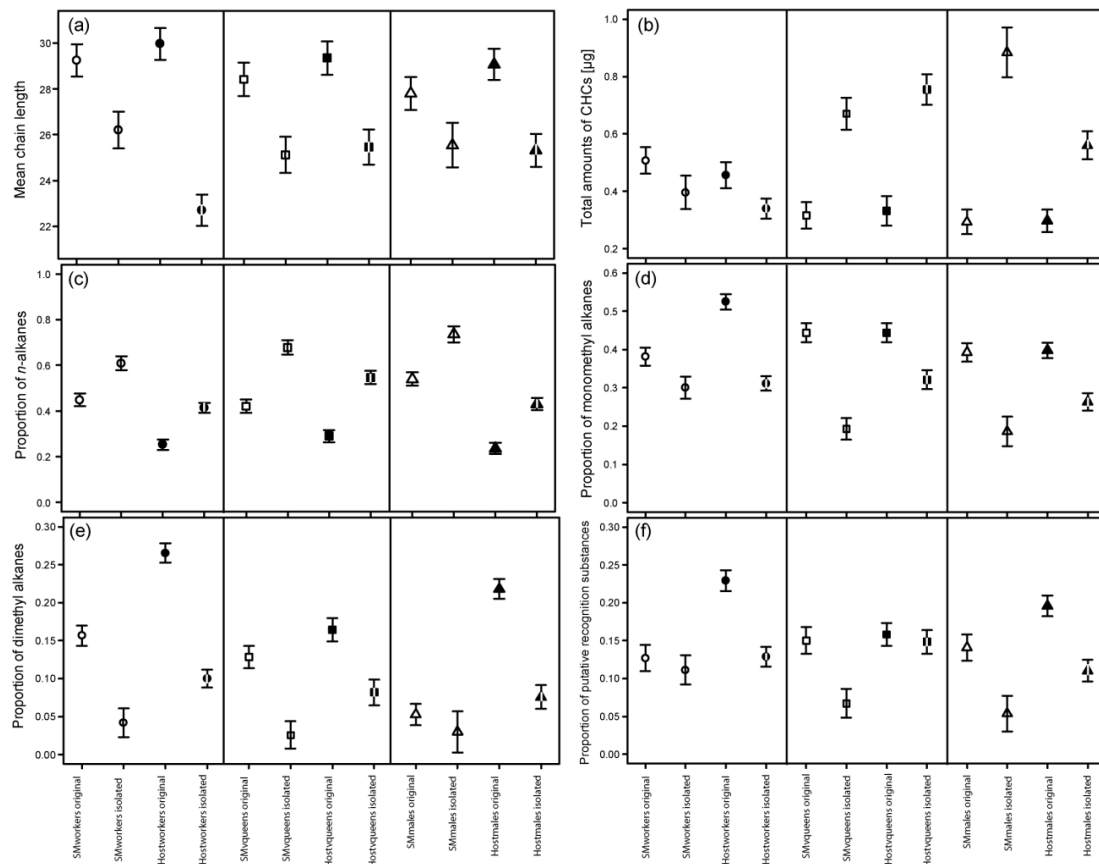
Figure S7.1: Differences between lifestyle, caste and social environment of different substances classes, including the mean chain lengths (a), the total amount of hydrocarbons on the cuticle (b), the relative amounts of n-alkanes (c), monomethyl-alkanes (d) and dimethyl-alkanes (e) as well as the putative recognition substances (f). Slavemakers are shown with white symbols whereas non-parasitic species are shown in black symbols. Different symbols represent different castes (workers = circles, virgin queens = squares, males = triangles). Symbols with a white or black line represent individuals that emerged in isolation and symbols without lines represent individuals that emerged in their original colony.

S7.1: The effect of the social environment – additional results

Within the non-parasitic hosts that emerged isolated, castes differed, with workers having shorter chain lengths than virgin queens (t = 5.08, P < 0.001) and males (t = 5.22, P < 0.001), whereas virgin queens and males did not differ (t = -0.23, P = 0.818). All castes of all slavemakers that emerged in isolation did not differ in their mean chain lengths (workers vs. males: t = -0.76, P = 0.448; workers vs. virgin queens: t = -1.64, P = 0.102; males vs. virgin queens: t = 0.49, P = 0.620). Slavemaker individuals that emerged isolated carried proportionally more n-alkanes than non-parasitic host individuals that emerged isolated (workers: t = -5.80, P < 0.001; virgin queens: t = -3.54, P = 0.006; males: t = -8.41, P < 0.001). Within the in isolation emerged slavemakers, workers carried relatively less n-alkanes than virgin queens (t = 2.10, P = 0.036) and males (t = 3.48, P < 0.001), whereas males and virgin queens did not differ (t = 1.56, P = 0.119). Within the in isolation emerged non-parasitic hosts, workers carried relatively less n-alkanes than virgin queens (t = 4.63, P < 0.001) but did not differ from the males (t = 0.62, P = 0.537). Moreover males carried proportionally less n-alkanes than virgin queens (t = -3.55, P < 0.001). Within the slavemakers that emerged in isolation workers carried relatively more monomethyl-alkanes than virgin queens (t = 3.59, P < 0.001) and males (t = 2.91, P = 0.003), whereas virgin queens and males did not differ (t = -0.19, P = 0.851). Within the non-parasitic species that emerged in isolation workers and virgin queens carried relatively more monomethyl-alkanes than males (workers: t = 2.11, P = 0.036; virgin queens: t = 2.08, P = 0.038), but workers were not different from virgin queens (t = 0.38, P = 0.701). Slavemaker workers and virgin queens that emerged isolated carried less dimethyl-alkanes than non-parasitic workers and virgin queens (workers: t = -2.67, P = 0.026; virgin queens: t = -2.31, P = 0.046), whereas slavemaker males did not differ from non-parasitic males, when emerged in isolation (t = -1.49, P = 0.171). Within the slavemakers that emerged isolated, castes did not differ in their relative amount of dimethyl-alkanes (workers vs. males: t = -0.39, P = 0.695; workers vs. virgin queens: t = -0.70, P = 0.487; males vs. virgin queens: t = 0.14, P = 0.890). Similarly within the non-parasitic species that emerged isolated, castes did not differ (workers vs. males: t = -1.38, P = 0.168; workers vs. virgin queens: t = -0.97, P = 0.334; males vs. virgin queens: t = -0.29, P = 0.0.775). Within the slavemakers that emerged isolated, workers carried relatively more recognition substances than males (t = 2.76, P = 0.006) and virgin queens (t = 2.77, P = 0.006), whereas the latter two did not differ (t = -0.66, P = 0.507).

GENERAL DISCUSSION

In my dissertation, I shed light on the diverse roles of recognition and aggression within *Temnothorax* slavemaker – host systems, and their convergent evolution in multiple species. Recognition plays a major role in the evolution of anti-parasite defense behavior, and often an elevation in aggression against opponents can help in reducing the costs of parasitism. However recognition can be evaded by slavemakers through chemical manipulation or through the use of co-adapted chemical profiles. Studying different host populations with differences in reciprocal selection allowed for the elucidation of the selective forces that shape host behavior. Comparing different slavemaker-host systems, each representing different origins of slavery and evolutionary age, within the same genus enables us to further investigate the basis of host defensive behavior, as we have empirical evidence for convergent evolution in all systems.

Summary of the results

Temnothorax hosts are able to recognize the slavemaker *T. americanus* as a threat and use the information of an upcoming attack to better defend themselves when being aggressive. But this was only true for aggression towards conspecifics – baseline aggression - which was moreover positively related to parasite abundance in a population (Kleeberg et al. 2015, Chapter 2). Aggression towards a living and potentially manipulative parasite decreased, instead host colonies switched to an alternative defense mechanism – evacuation – under high parasite pressure. Populations that were less aggressive towards the living parasite were also less resistant to chemical manipulation and slavemakers benefitted from it as the likelihood of survival and the parasites abundance increased. One of the three slavemaker species evolved a different strategy – chemical camouflage – to steal adult host ants, known as eudulosis. Hosts of this slavemaker do recognize the two slavemaker species *T. americanus* and *T. duloticus,* but are unable to identify their sympatric slavemaker *T. pilagens*. However, if they do recognize the slavemaker, aggression is costly as most workers die during raids. Evacuation, although utilized in confrontations with *T. americanus*, seems not to be employed during raids of *T. pilagens*. All three slavemaker species, although of different evolutionary origin, show convergent evolution of a specific chemical profile that may undermine host recognition.

Recognition and information use in *Temnothorax* ants

An important feature of eusocial species is the use of an elaborated recognition system that facilitates cooperation among group members. It helps to maintain the integrity of the society and at the same time reduces the negative impact caused by predators, competitors or social parasites (Crosland 1990; Fishwild & Gamboa 1992; Crowley et al. 1996; Wiley 2013). The correct identification of enemies can allow insect societies to respond with aggression to fend off parasites or predators. The first publication in my thesis aimed to investigate the benefits of high aggression in the ant *T. longispinosus*, host of the socially parasitic slavemaking ant *T. americanus*. Until now several studies shed light on the aggressive behavior of *T. longispinosus* colonies in different contexts (Scharf et al. 2011b; Pamminger et al. 2011) and it was suggested to have a genetic basis, which allows selection to act on this trait (Modlmeier et al. 2011). As *T. longispinosus* colonies increase their aggressive potential towards non-nestmate conspecifics after having contact with a slavemaking ant before, we predicted that host colonies are able to process information of an upcoming attack and communicate it throughout the colony. In contrast to our predictions, host colonies that have seen a slavemaker a day prior to the raiding experiments did not have any benefits, possibly because this information was already outdated. However, colonies that were more aggressive towards conspecifics, and that had seen a slavemaker scout during the raiding experiments – only a few hours before the actual raid occurred – could save more of their brood. We concluded that *T. longispinosus* colonies can make use of recent information, but outdated information might be costly to process (Dall et al. 2005). The mobilization that results from the processing of information might disrupt the regular colony functioning and it should only be maintained as long as the benefits of fight preparation outweigh the costs.

It is still unclear how the information is distributed among the members of a colony. Scout entries can be very diverse. A scout can enter the whole colony (Pohl et al. 2011), thereby having direct contact to a high number of host workers that will receive the information. Thus, information about an upcoming attack does not necessarily have to be distributed further, if enough workers are warned. However in other cases a scout enters the nest entrance, so that only a few host workers ("entrance-guards") will have direct contact and receive the information. In this case it is unclear how the other workers are informed about the upcoming raid. To respond with evacuation, not only defenders need to be informed but also the nurses that will relocate the brood and / or queen. It was suggested that large numbers of workers that congregate, such as on trails or in the nest, can trigger higher aggression in others (Sakata & Katayama 2001). In *T. longispinosus* confrontations with the slavemaker that occur at the entrance, cannot be communicated by a large number of workers, as the very small entrance sizes allow only one to three workers to guard the nest entrance. However, it was observed that individuals that had scented the slavemaker´s presence frequently antennate their

nestmates through rapidly drumming their antenna (personal observations). This led to frantic movements within the colony, possibly a way to alert the whole colony. Rapid antennation is commonly observed in many social insects and was often been attributed as antennal strikes exchanged between nestmates in conflict over reproduction, in the process of dominance hierarchy establishments (Heinze et al. 1994; Goyret & Farina 2003; Ratnieks et al. 2006), during food exchange (; Montagner & Pain 1971; Bonavita-Cougourdan 1984) or - in solitary insects species - in the process of mating (Perring & Symmes 2006; Da Silva et al. 2013). A recent study investigated antenna drumming in the context of aggression and found differences in the rates of rapid antennation (O´Fallon et al. 2016). This might suggest that different frequencies provide different information and that *Temnothorax* ants with a high aggressive potential are better able to convey this information to others, potentially because they engage in more interactions. The evolution of antenna drumming is an interesting and largely unexplored area of social insect biology, although being widespread and could be investigated in the process of information use and communication in *Temnothorax* ants.

Most studies on the use of information in social insects, concentrated on the use of social information in the context of foraging (Grüter & Leadbeater 2014). The best known example is the waggle dance in honey bees, which enables them to spread information about food quality throughout the colony (von Frisch 1968). In ants however, communication mainly occurs through the use of cuticular hydrocarbons or pheromones released by glands. The Dufour´s gland, which is also present in *Temnothorax* hosts and slavemakers, has various functions depending on the species (e.g. Dani et al. 1996; Katzav-Gozansky et al. 1997; Martin et al. 2002; Jongepier et al. 2015, Chapter 4). In *T. longispinosus* it is until now unclear for what purposes the secretion of the Dufour´s gland is used (Konrad et al. 2012), however it was shown that the secretion of fertile and infertile workers elicits different aggressive responses (Brandt et al. 2006). Thus, the Dufour´s gland secretion might be a good candidate to analyze its potential use as alarm pheromones in *Temnothorax* host ants, which has already been shown in other ant species (Ali et al. 1987; Billen & Morgan 1998).

Selection for anti-parasite defense behaviors in hosts

Host defenses at the frontline operate before infection, so that they are thought to provide the highest fitness benefits, if parasitism can be avoided entirely (Welbergen & Davies 2009; Feeney et al. 2012; Curtis 2014). These defenses include the avoidance of infected conspecifics, which can often lead to smaller group sizes (Altizer et al. 2003) or in extreme cases the adoption of a solitary lifestyle (Côté & Poulin 1995). Grouping however can have benefits, for instance fish that live in

groups benefit from information about parasites gained from members of that group to avoid habitats with high parasite abundance (Mikheev et al. 2013).

Such frontline defenses often depend on the ability to recognize parasites. In hosts of avian brood parasites it appears to be learned (Langmore et al. 2011; Feeney & Langmore 2013) and is associated with specific morphological cues of parasites. In ant hosts, recognition is associated with chemical traits and the recognition of specific CHC profiles seems to be partially inherited (Blomquist & Bagnères 2010; Wong et al. 2014). The recognition of parasites results in even higher aggression than recognition of other non-harmful species and has most likely evolved as a consequence of host-parasite coevolution (Pamminger et al. 2011; Scharf et al. 2011b; Kleeberg et al. 2015, Chapter 2). For example, *Temnothorax* hosts that are currently not occurring with their social parasite in a population, still respond towards the slavemaker with high aggression (Kleeberg et al. 2015, Chapter 2). This suggests that due to a potential parasite past, *Temnothorax* ants still have the ability to recognize and respond accordingly to the parasite. This was even apparent in *T. ambiguus* and *T. longispinosus* host populations, which are parasitized by the slavemaker *T. pilagens*. Colonies from these locales responded with high aggression towards *T. americanus* and *T. duloticus* although not being currently parasitized by them (Kleeberg & Foitzik 2016, Chapter 6). As recognition of and attacks on the slavemaker do not involve any costs if the parasite is absent, this trait can be maintained in a host population. Defensive traits that come with costs however, should disappear. *Temnothorax* hosts react aggressively towards non-nestmate conspecifics that can be of potential danger. During raids not only slavemaker workers but also enslaved *Temnothorax* workers participate in a raid. Hence, aggression towards potential slaves has an adaptive value as it could help fending off the raid (Kleeberg et al. 2014, Chapter 1). Indeed, we found that colony aggression towards conspecifics is higher in highly parasitized populations. As we were able to exclude other potential selective forces shaping host aggression, such as competition, we claim social parasites pressure to be a major selective force to shape host behavior (Kleeberg et al. 2015, Chapter 2). In the absence of parasites, high aggression towards non-nestmates that are usually not of great danger would lead to more fights und eventually more casualties.

Temnothorax hosts are characterized by not only a single defensive behavior at the frontline but inhabit a defense portfolio. Besides attacking the slavemaker, hosts evacuate the brood and / or the queen to a new nest site. Hence, *Temnothorax* hosts do not only rely on a single strategy – aggression – but on multiple defense traits. Aggression can be utilized during the scouting phase and the actual raid, however once a scout has targeted a host colony and returns to recruit more nestmates for the actual raid, *Temnothorax* ants can still evacuate the brood and / or queen before the raiding party returns. This can be a risky trait, as new nest sites are often rare (Herbers 1986). However, attacking the parasite can result in casualties among the defending host workers. With increasing parasite pressure

host defenses shifted from aggression to evacuation (Jongepier et al. 2014, Chapter 3). Attacking a slavemaker worker necessitates cooperative fighting as the slavemaker is larger and equipped with strong mandibles. The other two slavemaker species *T. duloticus* and *T. pilagens* moreover can make efficient use of their stinger. Hence multiple individuals are needed to immobilize or kill an invading slavemaking worker. Under low parasite pressure it might pay off to cooperatively immobilize thereby reducing the parasite´s chance to recruit for more help. If parasites are common, more attacks can be expected and higher casualties among the hosts are likely, so that switching to evacuation might be a more beneficial defense trait.

The complexity of the coevolutionary arms races between social parasites and their hosts has become apparent, not only in ants but more extensively in avian broodparasite-host systems (Feeney et al. 2014). Evidence of coevolved adaptations and counter-adaptations has been found at all stages of the avian host nesting cycle (Brooke & Davies 1988; Langmore et al. 2003; Welbergen & Davies 2009; De Mársico et al. 2012). Adaptations at one stage in the nesting cycle can influence evolution at other stages and that can lead to different coevolutionary trajectories in different host-parasite systems. The recognition of adult brood parasites is needed and hosts may assess the risk of parasitism to determine where to nest or to decide whether defenses should be deployed at either the frontline or at later stages (Feeney et al. 2014). Hosts of avian brood parasites have many opportunities to reduce the costs of parasitism during their nesting cycle. They can mob the adult parasite; they can reject the already laid parasitic eggs or even the hatching chicks. *Temnothorax* hosts however, need to employ defenses during raids of the slavemaker. Once enslaved, *Temnothorax* ants are not able to reproduce (Gladstone 1981), though killing the slavemaker brood might help nearby and often closely related host colonies, to reduce their chances of being exploited (Achenbach & Foitzik 2009; Pamminger et al. 2013; Pamminger et al. 2014; Metzler et al. 2016).

Natural selection tends to occur and favor the specialization of parasites to their hosts. *Temnothorax americanus* prefers *T. longispinosus* over its entire range (Brandt & Foitzik 2004), whereas *T. curvispinosus* is less frequently parasitized. Firstly *T. longispinosus* does not show collective aggression towards invading slavemakers in contrast to *T. curvispinosus*, so that *T. americanus* has an increased chance of escaping the attack (Jongepier et al. 2014, Chapter 3). Secondly *T. longispinosus* is more susceptible to manipulation through the use of the slavemaker´s Dufour´s gland (Jongepier et al. 2015, Chapter 4). This implies that *T. americanus* is specialized on *T. longispinosus* due to their inferior attack strategies compared to *T. curvispinosus,* which can be explained by their higher susceptibility to manipulation. This can moreover explain why we didn't find an association of parasite pressure and aggression towards a dead and thus non-manipulative slavemaker (Kleeberg et al. 2015, Chapter 2), but towards a living, potentially manipulative slavemaker (Jongepier et al. 2014, Chapter 3).

Selection for parasite counter-adaptations

As a consequence of host adaptations that can reduce or eliminate exploitation, parasites are likely to evolve counter-adaptations. In theory, parasites may have advantage in this evolutionary arms race, but only if parasite generation times are shorter, which is often the case in microparasites (e.g. Gandon & Michalakis 2002). Hosts of microparasites reproduce less quickly and hence have less time to evolve defenses. However, in social parasites, generation times are similar, given both counterparts the same "time" to adapt. Parasite adaptations can include various forms of morphological or chemical mimicry to avoid frontline defenses, such as the mobbing of adult brood parasites in host birds (Feeney et al. 2012). Avian brood parasites rely on deceptive or cryptic adaptations, such as plumage polymorphism, to decrease the likelihood of being identified (Honza et al. 2006; Krüger et al. 2007; Thorogood & Davies 2013; Trnka and Grim 2013). Similarly slavemaking ants have evolved various chemical strategies that allow prohibiting recognition by hosts, including chemical mimicry or camouflage and chemical insignificance (reviewed in Lenoir et al. 2001).

Some of these parasite strategies can only evolve if parasites infect and specialize on a single host species (e.g. Langmore et al. 2008; Langmore et al. 2011; Fossøy et al. 2011). *Temnothorax pilagens* (Seifert et al. 2014, Chapter 5) parasitizes two host species and is able to avoid recognition in both, possibly through chemical camouflage (Kleeberg & Foitzik 2016, Chapter 6), as chemical mimicry could be excluded (Kleeberg et al. *submitted*, Chapter 7). Those hosts are however able to recognize the other two slavemaker species, which do not occur in this population, so that aggression as a frontline defense potentially exists (Kleeberg & Foitzik 2016, Chapter 6). Moreover, they evacuate their colony after confrontations with *T. americanus* but do not so in the presence of *T. pilagens* (Kleeberg & Foitzik 2016, Chapter 6). These results suggest that the parasite *T. pilagens* is currently leading the evolutionary arms race and it moreover implies that this system does not share a long evolutionary history yet. In rare cases, hosts of *T. pilagens* seem to recognize the threat and respond with aggressive attacks, which doesn't provide any fitness benefits for the host colony. Instead, often all individuals get killed through the slavemaker´s stinger. Although parasites often inflict harm on their hosts, it is not in the best interest to kill the host entirely. This can lead to local overexploitation and parasites have to disperse further to find new host colonies. There is evidence for the high virulence of *T. pilagens* in our sampling site in New York. Several years back, *T. pilagens* and its host *T. ambiguus* were found in this habitat (personal communication, Susanne Foitzik), however recent sampling data suggest that *T. pilagens* was not able to sustain its host population. Less than 2% of the hosts found in New York are *T. ambiguus* colonies, and *T. pilagens* slavemaker colonies are

extremely rare. This indicates, that the high virulence of *T. pilagens* led to a dramatic reduction in host abundance as well as its own local extinction.

Altogether these results provide excellent opportunities to further investigate this system and track down the coevolutionary processes, especially in comparison to the other two slavemaker systems. As we have multiple indications for convergent evolution in all three systems (i.e. high aggression towards two slavemaker species in all three host species; similar patterns of aggressive potentials over the geographic range of two host species; same chemical adaptations in all three slavemakers), we might even be able to draw conclusions on the sequences of evolved host adaptations and parasite counter-adaptations in all three systems. *Temnothorax americanus* and its preferred host *T. longispinosus* share a long evolutionary history, whereas *T. pilagens* and *T. duloticus* are relatively young parasites (Beibl et al. 2005). Hence, by comparing all three systems, we might be able to gain a deeper understanding of the evolutionary and ecological consequences of host-parasite interactions.

Host manipulation can be achieved chemically, when the slavemaker alters the behavior of its host to their own advantage, through the use of glandular secretions. Slavemakers often make use of the Dufour´s gland to manipulate host workers into attacking nestmates instead of attacking the invading slavemaker (Brandt et al. 2006). Thus, slavemakers are able to disrupt the colony defense system and the success in manipulating a host colony is reflected in the abundance of parasites in a population (Jongepier et al. 2015, Chapter 4). *Temnothorax* host populations that were more susceptible to manipulation by *T. americanus* showed higher parasite pressures, indicating that this offensive slavemaker strategy is very effective. The use of chemical manipulation in *T. duloticus* and *T. pilagens* still needs to be investigated, but there is evidence for convergent evolution of Dufour´s gland secretions as a propaganda substance even in slavemakers of a different genus (Brandt et al. 2006).

Parasitism has often been used to explain the evolution of secondary sex characteristics in males (e.g. the plumage of male peacocks, Keyser & Hill 2000; Møller & Petrie 2002). Female hosts select males based on such characteristics that can indicate resistance to parasites. In turn, parasite females might select for male attributes that increase the success of exploiting hosts. We found that slavemaker CHC characteristics, that might improve raiding success through a reduction of host recognition, were not only present in females that are involved in raids, but in males as well (Kleeberg et al. *submitted*, Chapter 7). This could be a by-product from strong selection on female profiles. However, during mating, virgin queens might choose to mate with males that exhibit a typical slavemaker profile to ensure the raiding success of her future slavemaker colony.

Conclusions and future perspectives

We detected an intricate suite of adaptations and counter-adaptations in hosts and parasites, which are all related to recognition and aggression, and are mostly a result of convergent evolution. Convergent evolution refers to the independent origin of similar organismal traits and is often results from similar selective pressures operating in similar environments. Characterizing such examples of convergent evolution greatly improves our understanding of natural selection and macroevolution, as each example depicts a fundamental biological problem and its possible solutions (Conway-Morris 2006; Leander 2008). Parasites must solve similar problems associated with host exploitation. Despite their diverse origins even unrelated parasites converged towards similar extremeties in terms of their basic mode of parasitism (Poulin 2011). For example, the ability to alter host behavior in ways that benefit the parasite, has evolved independently in multiple lineages of parasitoids (e.g. Eberhard 2000; Thomas et al. 2002; Poulin & Latham 2002) as well as in slavemaking ants (Regnier & Wilson 1971; Brandt & Foitzik 2006). The evolution of resistance to common antibiotics occurred many times in parallel and highlights the adaptive potential and repeatability of parasite (bacterial) evolution (Didelot et al. 2016). Similar traits in unrelated organisms may be the product of different cellular and / or physiological processes and be the ultimate expressions of completely different genetic architectures. Selection can act on the same function but through different pools of genes (Clément et al. 2013). Hence, the convergent evolution at the phenotypic level is not necessarily reflected at the genomic level. Whether the same genes underlie the convergently evolved traits in *Temnothorax* ants, still needs to be investigated and the comparison of parasite genomes with those of their close free-living relatives could moreover help delineating clear trends in the evolution of parasitic lifestyles.

Host defenses in many taxa involve the recognition of parasites (e.g. Davies & Brooke 1988; Janeway 1992; Janeway & Medzhitov 2002; Spottiswoode & Stevens 2011) and has evolved in parallel due to host-parasite coevolution. Similarly the evasion of host recognition has evolved in a variety of parasite taxa (Alcami 2003; Moksnes and Roskraft 1995; Akino 2008). However the functionality of recognition and its evasion by parasites clearly differ between distantly related systems. The recognition through cuticular hydrocarbons has evolved multiple times and independently in ants, termites and many other social insects. Hence it seems likely that the exploitation of the chemical communication system in social insects is based on similar strategies. Chemical mimicry or insignificance has evolved in a number of slavemaking ants (Akino 2008) and the expression of similar CHC profiles was even shown to convergently evolve in three predators of ant-tended aphids, each from a different insect order (Lohman et al. 2006). *Temnothorax* slavemakers employ almost identical chemical profiles to avoid host recognition (Kleeberg et al. *submitted*, Chapter 7). Whether this is a convergent adaptation to similar exploitation strategies (namely *raiding behavior*) or whether

this is exclusively found in *Temnothorax* ants should be further tested by including more distantly related slavemaker species with similar lifestyles. Moreover, one should test which substances are involved in recognition and aggression in host species. We have identified almost all substances found on the cuticle of slavemakers and hosts (Kleeberg et al. *submitted*, Chapter 7), which gives us the opportunity to use synthesized CHCs in behavioral essays. This would allow to better understand the specific chemical profiles found on all slavemaker species, and whether this CHC composition indeed leads to recognition evasion in hosts.

The geographic range of coevolved adaptations has been studied in a variety of hosts species (this thesis; e.g. Soler et al. 1999; Kraaijeveld & Godfray 1999; Stachowicz & Hay 2000) however, to better understand the dynamics of coevolution, it is important to include differences in parasite counter-adaptations between different populations. Such as the comparison of the manipulative success of *T. americanus* between populations (Jongepier et al. 2015, Chapter 4), we could compare chemical adaptations as well as differences in raiding success. This might help to further understand the observed geographic variation in host defenses as well as the abundance of parasites. *Temnothorax pilagens* provides great opportunities to study a system where the parasite is clearly leading the co-evolutionary arms race and where host species have as yet not evolved any efficient anti-parasite defenses, in contrast to the other two *Temnothorax* systems. Due to recent advances in the study of the genomic basis of behavior, identification of the genes that are responsible for specific defensive or offensive behaviors in this system, provides excellent opportunities to further assess such strategies and understand the coevolutionary process.

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LEBENSLAUF

Isabelle Kleeberg / ███████████ / ██████████ / ████████████████████ / ██████████████████

PERSÖNLICHE DATEN

Geburtsdatum   02.06.1986

Geburtsort   Hildesheim / Niedersachsen / Deutschland

Familienstand   ledig

Staatsangehörigkeit   deutsch

BILDUNGSWEG

Seit 06/2012   Wissenschaftliche Mitarbeiterin mit dem Ziel der Promotion an der Johannes-Gutenberg Universität, Mainz, Abteilung Zoologie und Evolutionsbiologie bei ██████████████████████████ Forschungsschwerpunkte: Wirt-Parasit Koevolution, Verteidigungs- und Angriffsstrategien, Sozial-Parasitismus, soziale Insekten, Myrmekologie.

04/2006 bis 06/2012   Studium der Biologie an der Johannes Gutenberg Universität, Mainz. Schwerpunkte: Zoologie, Botanik und Humanphysiologie. Diplomarbeit in der Evolutionsbiologie bei ████████████████ ██████████ „Vorteile induzierbarer Verteidigungen bei der Abwehr von Sklavenraubzügen", Abschlussnote: 1.3.

1996 bis 2005   Jugenddorf Christophorus Schule, Elze, Niedersachsen. Abitur, Note: 3.0.

1992 bis 1996   Grundschule Wallensen, Niedersachsen

PRAKTISCHE ERFAHRUNGEN

Sommersemester 2015   Kursleitung und Vorlesungen für einen Bachelorkurs in der Abteilung Zoologie bei ██████ ██████ ██████████ „Tierbestimmung: Reptilia, Amphibia, Mammalia". Beinhaltet die Vorbereitung theoretischer Grundlagen von speziellen Tiergruppen in Form einer Vorlesung und die Betreuung der Studenten während der praktischen Übungen zur Bestimmung von Tieren.

4/2015 und 4/2016   Dozent in einer vom pädagogischen Landesinstitut Rheinland Pfalz organisierten Fortbildung für Lehrer (A.N.T.S. // www.Ameisen-in-Schulen.de).

Seit 2014   Vertreterin der Studentenschaft in der Abteilung Zoologie, Evolutionsbiologie.

06/2012 bis 04/2015   Praktische und theoretische Betreuung von 7 Bachelor Kandidaten (jeweils 16 Wochen)

Praktische und theoretische Betreuung von 2 Master Kandidaten (jeweils 32 Wochen)

Praktische Assistenz in einem Bachelorkurs: Evolution und Verhalten

Sommersemester 2011   Studentische Hilfskraft in der Abteilung Zoologie bei ██████ ██████ (Tierbestimmung) und in der Abteilung Botanik bei Prof. ██████ (Pflanzenbestimmung)

Wintersemester 2010/2011   Studentische Hilfskraft in der Abteilung Botanik bei ████ ████ ██████ (Botanische Anfängerübungen)

Sommersemester 2010   Studentische Hilfskraft in der Abteilung Zoologie bei ██████ ██████ (Tierbestimmung und Fortgeschrittenen Praktikum Artikulata)

02/2006 bis 04/2006   Praktikum in der Mikrobiologie bei Wülfing Pharma in 31028 Gronau

04/2001   Praktikum als Tierpflegerin im Zoo Hannover

QUALIFIKATIONEN

Fremdsprachen   Englisch – *verhandlungssicher*

Französisch – *Grundkenntnisse*

Software   Office Programme – *sehr gute Kenntnisse*

R, Primer 6 und STATISTICA (Statistik Programme) – *sehr gute Kenntnisse*

Leica Application Suite LAS V 3.7 (Foto und Vermessungsprogramm) – *sehr gute Kenntnisse*

Rasterelektronenmikroskopie - *Grundkenntnisse*

Laborerfahrungen   Design und Durchführung von diversen Verhaltensexperimenten – *sehr gute Kenntnisse*

GCMS Analysen (Gaschromatographie, Massenspektroskopie) – *sehr gute Kenntnisse*

Verwandtschaftsanalysen mittels Mikrosatelliten - *Grundkenntnisse*

Spezielle Kenntnisse   Wissenschaftliches Publizieren

Schreiben von Anträgen zur finanziellen Förderung von Forschungsprojekten

Statistische Analysen

Begutachtung wissenschaftlicher Publikationen (bei Animal Behaviour, Procceedings of the Royal Society and Behavioral Ecology, Insectes Sociaux)

EIGENSTÄNDIG ERWORBENE FÖRDERUNGEN

2014	Reiseförderung zur Teilnahme an der internationalen Konferenz der sozialen Insekten (IUSSI, International Union for the Study of Social Insects) in Cairns, Australien (850 Euro)
2013	Forschungsförderung des Huyck Preserve in New York, USA. „The proximate basis of slave rebellion" (3500 US$)
2012	Forschungsförderung des Huyck Preserve in New York, USA. „Reciprocal adaptations underlying the slave rebellion trait " (2850 US$)

TEILNAHME AN KONFERENZEN

2015	IUSSI Konferenz in Lichtenfels, Deutschland. Posterpräsentation: „The placid slavemaker: avoiding detection and conflict as an alternative, peaceful raiding strategy"
2015	DFG Forschergruppen Abschlusstreffen an der LMU in München. Posterpräsentation: „Fitness benefits of slave rebellion: the evolution of an altruistic trait"
2014	IUSSI Konferenz in Cairns, Australien. Vortrag: "Appeasement versus fighting: a new slavemaker employs alternative raiding strategies"
2013	BEHAVIOUR Konferenz in Newcastle, England. Vortrag: "Aggression as a behavioural defence trait against social parasites"
2013	Teilnahme an einem Internationalen Symposium organisiert von ▇▇▇ ▇▇ ▇▇▇▇▇ ▇▇▇▇ und gefördert durch die Volkswagenstiftung: "Personality: Causes and consequences of consistent behavioural variation".
2013	DFG Forschergruppentreffen an der LMU in München. Vortrag: „Geographic mosaic of host defences"
2012	IUSSI Konferenz in Montecatini Terme, Italien. Posterpräsentation: „Slavemakers select for high aggression in host colonies"

EXKURSIONEN UND FELDARBEIT

2011 - 2015	jährlich: Feldarbeit in den USA und Kanada. Sammeln von Ameisenkolonien und Kartierung. Forschungsaufenthalte am E. N. Huyck Preserve NY und an der South Western Research Station AZ.
2010	Meeresbotanische Exkursion in Frankreich (Bretagne); Projekt: "Einfluss der Gezeiten auf die Photosynthese von Algen"
2009	Meereszoologische Exkursion in Frankreich (Banyuls sur Mér); Projekt: „Chaetognatha und Tunicata; Morphologie und Lebensweise"

PUBLIKATIONEN

Kleeberg I, Menzel F and Foitzik. *The evolution of cuticular hydrocarbons: The influence of parasitic lifestyle, caste and sex on chemical profiles of ants.* Submitted to Evolution

Kleeberg I and Foitzik S. 2016. *The placid slavemaker: avoiding detection and conflict as an alternative, peaceful raiding strategy.* Behavioural Ecology and Sociobiology 70: 27 – 39..

Jongepier E, **Kleeberg I** and Foitzik S. 2015. *The ecological success of a social parasite increases with manipulation of collective host behaviour.* Journal of Evolutionary Biology 28: 2152 – 2162.

Kleeberg I, Jongepier E, Job S and Foitzik S. 2015. *Geographic variation in parasite pressure predicts intraspecific but not interspecific aggressive responses in hosts of a slavemaking ant.* Ethology 121: 694 – 702.

Kleeberg I, Pamminger T, Jongepier E, Papenhagen M and Foitzik S. 2014. *Forewarned is forearmed: aggression and information use determine fitness costs of slave raids.* Behavioral Ecology 25: 1058 – 1063.

Jongepier E, **Kleeberg I**, Job S and Foitzik S. 2014. *Collective defense portfolios of ant hosts shift with social parasite pressure.* In press. Proceedings of the Royal Society B 281: 20140225..

Seifert B, **Kleeberg I**, Feldmeyer B, Pamminger T, Jongepier E and Foitzik S. 2014. *Temnothorax pilagens sp. n. – a new slave-making species of the tribe Formicoxenini from North America (Hymenoptera, Formicidae).* ZooKeys 368: 65 – 77.

Pamminger T, Leingärtner A, Achenbach A, **Kleeberg I**, Pennings PS and Foitzik S. 2013. *Geographic distribution of the anti-parasite trait "slave rebellion".* Evolutionary Ecology 27: 39 – 49.