Inter- and Intraspecific Variation in the Host Defence Portfolios of *Temnothorax* Ants

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SUMMARY

A central theme in evolutionary biology is to explain why some species readily adapt to their environment while others apparently do not. Regarding the coevolutionary arms race between hosts and parasites, this question is fuelled by a large variety of host defence strategies against parasites, even between hosts experiencing very similar selection pressures. Recent theory predicts that the effectiveness of one defensive strategy modifies the selective regime for further lines of defence, which may affect the complexity of host defence portfolios. The evolution of host defences thus not only depend on attributes of parasites, such as parasite pressure or host preference, but also on the efficacy of other traits in the adaptive portfolios of the host.

Here, we investigate the interplay between the different behavioural and chemical strategies in host defence portfolios and parasite prevalence. We focus on *Temnothorax* ant colonies, whose workforce is parasitically exploited by the social parasite and slavemaker ant, *Protomognathus americanus*. We assessed the level of mobilization in the hosts' collective fight and flight defences, their resistance to behavioural manipulation by the slavemaker, their chemical adaptions involved in enemy recognition and their flexibility to respond to slavemaker attack. We studied these responses in up to 17 populations of two *Temnothorax* species, covering most of their geographic range.

The first part of this thesis correlatively investigates the causes and consequences of variation in colony aggression. Despite the known advantages of aggressive defences, chapter 2 shows that *Temnothorax* populations exposed to high slavemaker prevalence were less aggressive towards the slavemaker than populations where the slavemaker was rare or absent. Instead, severely parasitized populations resorted to an alternative defence strategy in the form of nest evacuation. Chapter 3 shows that the decrease in host aggression with increasing slavemaker prevalence was not driven by variation in the aggressive potential of *Temnothorax* colonies. Instead, chapter 4 demonstrates that more aggressive populations were more resistant to manipulation of their aggressive responses by the slavemaker. Slavemakers benefitted from manipulating their hosts aggressive defences, as both the likelihood that slavemakers survived host encounter and slavemaker prevalence in ant communities increased with slavemaker-induced changes in host aggression. In addition, we show differences between host species in their expression of the different defence strategies, which had important implications for their level of exploitation by the slavemaker. *Temnothorax longispinosus* colonies were more susceptible to manipulation than *T. curvispinosus* colonies, which 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*.

The second part of this thesis focusses on two novel defence strategies against slavemakers: the host's chemical adaptions involved in enemy recognition and their flexibility to respond to slavemaker attack. Specifically, chapter 5 investigates whether slavemakers can drive diversification in the host's nestmate recognition cues. We show that *T. longispinosus* populations that co-occur with slavemakers are more variable in their chemical recognition cues. Larger variation between, but not within colonies, enables hosts

to distinguish friend from foe and makes it impossible for slavemakers to adapt to any one recognition profile in their host population.

In chapter 6, we experimentally investigate the fitness consequences of behavioural specialisation by *T. longispinosus* workers during slave raids. While specialized workers may be more efficient in the tasks they perform than generalists, they may also lack the flexibility to respond to rapid shifts in task needs. Such rigidity could impose fitness costs when societies face dynamic and unpredictable events, such as an attack by slavemaker ants. We indeed find that strict specialisation is disadvantageous for a colony's annual reproduction and growth during slave raids. These fitness costs may favour generalist strategies in dynamic environments, as we also demonstrate that societies exposed to slavemakers in the field show a lower degree of specialisation than those originating from slavemaker-free populations. Our findings thus provide an explanation for the ubiquity of generalists and highlight their importance for the flexibility and functional robustness of entire societies.

In conclusion, this thesis demonstrates that ant colonies use a variety of strategies to cope with social parasites. The level of expression of these strategies varies across populations and species, depending not only on parasite prevalence but also on other strategies in the host's defence portfolios. Hosts with efficient front line defences suffered lower parasite prevalence in the field. Nonetheless, hosts whose frontline defences were breached by the parasite were found to mount further lines of defence, providing empirical support that host defence portfolios can reach remarkable depths.

CHAPTER 1

General Introduction

Evelien Jongepier

HOST-PARASITE COEVOLUTION

Antagonistic interactions between parasites and their hosts are among the strongest forces in evolution, driving a great diversity of defence strategies in hosts, as well as counter-strategies in parasites (Thompson 1994; Vermeij 1994; Paterson *et al.* 2010). Parasitic life styles are found in all taxa and every level of biological organisation, ranging from selfish genetic elements, viruses and microbes, to brood and social parasites that exploit the behaviour of host parents or even entire host societies (Davies *et al.* 1989; D'Ettorre and Heinze 2001; Kilner and Langmore 2011; Schmid-Hempel 2011).

Theory predicts a plethora of coevolutionary trajectories and outcomes of host-parasite interactions. For instance, coevolutionary dynamics can lead to an escalating arms race driven by directional selection for reciprocal adaptations in parasites and hosts; or they can promote diversity through frequency-dependent selection (Dawkins and Krebs 1979; Lively 2001). Interactions can continue indefinitely when hosts and parasites become locked in coevolutionary cycles; or they may seize entirely if one defeats the other, forcing parasites to switch hosts or driving either party to extinction (Thompson 2005; Nuismer and Thompson 2006; Soler 2014). Since few systems are accessible to track coevolutionary changes through time, most experimental support stems from microbial systems (Buckling and Raine 2002; Decaestecker *et al.* 2007; Paterson *et al.* 2010). Recent evidence however suggests that coevolution may lead to distinctly different trajectories and outcomes in other host and parasite taxa (e.g. brood parasites; Soler 2014).

Coevolution in depth

One particular outcome of host-parasite coevolution that has recently received considerable interest is the evolution of in-depth strategies (Planqué *et al.* 2002; Britton *et al.* 2007; Welbergen and Davies 2009; Langmore and Kilner 2010; Svennungsen and Holen 2010; Kilner and Langmore 2011; Feeney *et al.* 2014; Soler 2014). Host defence and parasite offence strategies can evolve at all stages of the host-parasite interaction, including frontline strategies employed prior to parasitic exploitation (Feeney *et al.* 2012; Curtis 2014). For instance, host species may avoid contact to parasites in the first place or prevent infection through resistance. Once infected, hosts may fight-off infection or tolerate the parasite by mitigating its adverse effect on host fitness. The emerging view is that once one line of defence is breached by the parasite, hosts are selected to mount further defences. As a result, hosts and parasites can deploy a whole battery of strategies to cope with their antagonist, where each successive line of defence in the adaptive portfolio of the host corresponds to a strategy in the offence portfolio of the parasite (Planqué *et al.* 2002; Welbergen and Davies 2009).

Hosts have a unique advantage when coevolution involves multiple traits in host and parasites (Gilman *et al.* 2012); yet, there are numerous examples where hosts fail to mount defences that are evolutionary attainable to others. For instance, many host species of brood parasites such as cuckoos have evolved the ability to recognize and

reject parasitic eggs. None of these egg rejecting hosts however show frequent discrimination against cuckoo chicks, despite their obvious aberrant appearance (Davies 2000; Davies 2011; Soler 2014). Nonetheless, chick rejection has evolved in several host species which accept cuckoo eggs into their nests (Langmore and Kilner 2010; Feeney *et al.* 2014; Soler 2014). Recent theory suggests that hosts lacking particular defences are not necessarily lagging behind in the coevolutionary arms race with a parasite (i.e. evolutionary lag hypothesis), but rather emphasises the importance of other strategies in the defence portfolio of hosts (Planqué *et al.* 2002; Britton *et al.* 2007). The rationale is that once an effective defence is in place, parasites become such a rare enemy that selection on further lines of defence is diminished, a concept known as strategy-blocking (Britton *et al.* 2007). Alternatively, one strategy may actually increase rather than decrease the fitness benefits of further lines of defence, in which case the evolution of one strategy may facilitate further adaptations (i.e. strategy facilitation; Kilner and Langmore 2011). Whether a particular strategy has evolved in one party thus not only depends on attributes of their antagonist, but also on the efficacy of other traits in their adaptive portfolio.

Coevolution in space

One of the biggest challenges in coevolutionary research is to understand how coevolution operates over the geographic range of species (Thompson 1994; Thompson 1999; Thompson 2005). Parasites rarely occur along the entire distribution of their host species and parasite prevalence often differs markedly across space (Wilson 1971; Soler 2014). Such geographic variation can affect the intensity of reciprocal selection pressures, and hence the strength of coevolutionary arms races between hosts and parasites. Geographic isolation may lead to unique adaptations and counter-adaptations at different locales (Paterson *et al.* 2010), or populations may converge on the same limited set of strategies to cope with their antagonist (Bull *et al.* 1997; Wichman *et al.* 1999). Evidence on brood and socially parasitic interactions indeed suggests that host and parasite adaptations are repeatable across different populations (Foitzik *et al.* 2001; Soler 2014), although their level of mobilisation may vary between coevolutionary hotspots (populations with intense host-parasite interactions and reciprocal adaptations and counter-adaptations) and cold spots (populations where host-parasite interactions are rare or absent; Thompson 1994; Thompson and Cunningham 2002; Thompson 2005).

On the other hand, there is increasing awareness that evolution in general, and coevolution in particular, can affect the ecological dynamics of interacting species (Johnson and Stinchcombe 2007; Schoener 2011; Lion and Gandon 2015). Theory and microcosm experiments show that the evolution of resistance can induce population cycles, where the party currently leading the coevolutionary arms race increases in abundance at the expense of the other (Abrams and Matsuda 1997; Yoshida *et al.* 2003). Evolutionary dynamics in host parasite-interactions may thus result in the local extinction of the parasites when hosts have evolved highly efficient defences, or

increase parasite prevalence when host defences are breached. Hence, parasite pressure can be both a cause and consequence of coevolution between hosts and parasites.

SOCIAL PARASITISM

Like cuckoos and cowbirds that lay their egg in a foreign nest to let their chicks be raised by their avian host, social parasites avoid the cost of brood care by relying on the workforce of another social species (Davies 2000; Kilner and Langmore 2011). It has been defined as the coexistence in the same nest of two species of social insects, one of which is parasitically dependent on the other (Hölldobler and Wilson 1990). Although social parasitism occurs in several Hymenopteran families, it is most often found within the ants. Nonetheless, only 230 out of the ca 12,500 described ant species have adopted a socially parasitic life style (Buschinger 2009), especially within the Myrmicinae and the Formicinae (Bolton 2003).

There are three distinct life-history strategies among social parasites: temporal parasitism, inquilism and dulosis or slavery (Hölldobler and Wilson 1990; Buschinger 2009). Temporary social parasites only rely on their hosts during the initial phases of colony establishment, when a newly mated queen invades a host colony, kills the resident queen and appropriates the work force to care for her and her brood. During colony ontogeny, the host workers are not replenished but gradually replaced by the parasite's own workers, resulting in a single species colony comparable in social structure and ecology to their free-living host. Inquiline species, on the other hand, have completely lost their worker caste (but see Sumner *et al.* 2003) and thus remain obligatorily dependent on their host throughout their life. To ensure a steady production of host workers, most inquilines peacefully coexist with the host queen(s). Hence, inquilines can be considered as true endo-parasites which drain resources from their host colony without jeopardizing host survival. In slavemakers, queens do produce their own workers, but instead of taking care of her brood, these workers are specifically adapted to pillage the brood of neighbouring host nests, as detailed below.

Slavery

Slavery or dulosis is a form of permanent social parasitism that is only known in ants (Hölldobler and Wilson 1990; D'Ettorre and Heinze 2001). During colony foundation, a newly mated slavemaker queen penetrates a host nest, eliminates the host queen and appropriates the workers and brood. In some genera (*Protomognathus*, *Harpagoxenus*, *Chalepoxenus*), the parasitic queen also kills or evicts adult workers, leaving her with the brood that will develop into her first generation of slaves. In the absence of a host queen to replenish the slave work force, slavemakers perform raids on neighbouring host colonies. During these slave raids they capture brood which they transport back to their own colony. The host workers developing from the captured brood become new slaves which forage, nurse the parasitic brood and perform all other colony maintenance tasks. Because slavemaker workers are usually unable to perform such

tasks themselves, slavemaking is, with few exceptions, obligatory (Wheeler 1910). For an example of the life cycle of a slavemaker see Box 1.

Slavery has been described in approximately 50 ant species, in particular among the myrmicine tribe Formicoxenini and the formicine tribe Formicini (D'Ettorre and Heinze 2001). It is thought to have evolved independently more than 10 times, with 6 origins among the Formicoxenini alone (Beibl *et al.* 2005); although a recent genome-based phylogeny suggests that two of these actually represent a single origin; D. Elsner, A. Alleman, S. Foitzik & B. Feldmeyer, unpublished data).

Slavemakers and their hosts as coevolutionary models

Over recent years, slavemakers and their hosts have become increasingly popular for the study of coevolutionary arms races (e.g. Davies *et al.* 1989; Foitzik *et al.* 2001; Hare and Alloway 2001; Blatrix and Herbers 2003; Foitzik *et al.* 2003; Brandt and Foitzik 2004; Brandt *et al.* 2005; Bauer *et al.* 2009a; Bauer *et al.* 2009b; Foitzik *et al.* 2009; Pamminger *et al.* 2012), which can be attributed to the following three aspects of slavemaker-host coevolution:

i) Similarity in evolutionary potential

The evolutionary potential of populations is determined by migration, mutation, population size and generation time, which dictate the outcome of coevolutionary dynamics (Lively 1999; Gandon and Michalakis 2002). Parasites that have a higher evolutionary potential than their hosts can quickly evolve counter-measures in response to host adaptations for resistance, whereas hosts are more slowly to reciprocate when novel parasite offences evolve. Although most parasites have such an advantage over their host due to their shorter generation times and larger effective population sizes, slavemakers and their hosts are exceptionally similar in this respect (Brandt *et al.* 2007; Foitzik *et al.* 2009; Pennings *et al.* 2011). This is mainly due to the fact that slavemakers are not only closely dependent on, but often also closely related to their hosts (i.e. "Emery's rule"; Emery 1909). Hence, hosts can match the evolutionary potential of slavemakers (Brandt *et al.* 2007; Foitzik *et al.* 2009; Pennings *et al.* 2011), which predicts that the coevolutionary arms race proceeds through stepwise reciprocal adaptations that can be tracked through space or time.

ii) Strong reciprocal selection pressures

There is no doubt that the obligate dependency of slavemakers on their host imposes strong selection for morphological, behavioural and chemical adaptations to the slavemaker life-style. Such adaptations have been intensely investigated and show remarkable functional convergence between independently evolved slavemaker species (D'Ettorre and Heinze 2001). Whether slavemakers exert sufficiently strong selection pressure for the evolution of defences in the host has however been subject to debate, because social parasites were thought to be too rare to drive counter-adaptations in their host species *de toto* (Dawkins 1982; Davies *et al.* 1989). Evidence however suggests that slavemakers can be highly abundant in ant communities (D'Ettorre and Heinze 2001; see also this thesis, e.g. chapter 2). Moreover, the numerical abundance of

slavemakers underestimates the actual costs they impose on their host populations, because established slavemaker colonies can raid numerous host colonies every year (D'Ettorre and Heinze 2001). Given that such raids decimate reproduction and growth in the targeted host nests and can lead to the death of a colony, slavemakers do exert a substantial selective force on their hosts. It is therefore not surprising that numerous behavioural, chemical and life-history studies have found evidence for anti-slavemaker adaptations in hosts, placing slavery squarely in the framework of host-parasite coevolution.

iii) Coevolution at the front line

Studying host defence portfolios can be challenging, especially in long-lived hosts such as ants (Keller 1998). The reason is that hosts usually have numerous possibilities to avoid or reduce the cost of parasitism, which can be manifested throughout their life (Feeney *et al.* 2014; Soler 2014). For instance, hosts of brood parasites may prevent parasitation by mobbing, or remove parasitic young in the egg or nestling phase of the breeding circle. And even if their current reproductive investment is entirely lost on parasitic young, they may still raise offspring of their own during future breeding attempts. Contrastingly, hosts of slavemakers cannot gain direct fitness benefits from resisting the parasite once enslaved (Gladstone 1981). Slave rebellion may bring indirect fitness benefits through reduced raiding pressure on related host colonies (Achenbach and Foitzik 2009; Pamminger *et al.* 2012); yet these are likely marginal in comparison to the benefits of avoiding slavemaker exploitation altogether. Hence, anti-slavemaker defences are mostly confined to front-line defences (Feeney *et al.* 2012), which narrows down the time frame relevant for coevolutionary dynamics and facilitates the study of the multiple strategies in the defence portfolio of hosts.

STUDY SYSTEM

Protomognathus americanus (Emery) is an obligatory slavemaker ant which exploits three closely related host species of the genus *Temnothorax* (formerly *Leptothorax*; (Bolton 2003): *T. longispinosus* (Roger), *T. curvispinosus* (Mayr) and *T. ambiguus* (Emery). It was formally assigned to the genus *Harpagoxenus* and it was recently proposed to be synonymised with its host genus *Temnothorax* (Ward *et al.* 2015), but in this thesis I will continue using *Protomognathus*. It is a phylogenetically old parasites, which clusters as a distant sister taxon to its *Temnothorax* hosts (Beibl *et al.* 2005; D. Elsner, A. Alleman, S. Foitzik & B. Feldmeyer, unpublished data). Hence, *P. americanus* is equally closely related to each of its three host species, two of which are the subject of this thesis (*T. longispinosus* and *T. curvispinosus*).

A slavemaker colony is founded by a newly mated *P. americanus* queen that usurps a *Temnothorax* colony. After colony establishment, slavemakers replenish their slave workforce by conducting slave raids on neighbouring hosts. The life cycle of *P. americanus* is detailed in Box 1. Enslaved *Temnothorax* workers perform all regular tasks within the colony, including foraging, brood care and nest defence (Wesson 1939; Alloway and Del Rio Pesado 1983). Outside the two month raiding season in summer, *Protomognathus* workers do not leave their nest, and their activity consists primarily of

LIFE CYCLE OF THE SLAVEMAKER

BOX 1

Colony establishment - Immediately after the mating flight, a dealate *Protomognathus americanus* queen localizes and invades a free-living *Temnothorax* host colony (Figure 1.1, phase 1), kills or expels all adult host workers and queen(s) and appropriates the host brood (Figure 1.1, phase 2). This brood develops in her first generation of slaves, caring for the slavemaker queen and her brood, and performing all other colony maintenance tasks (Figure 1.1, phase 3; Sturtevant 1927; Wesson 1939).

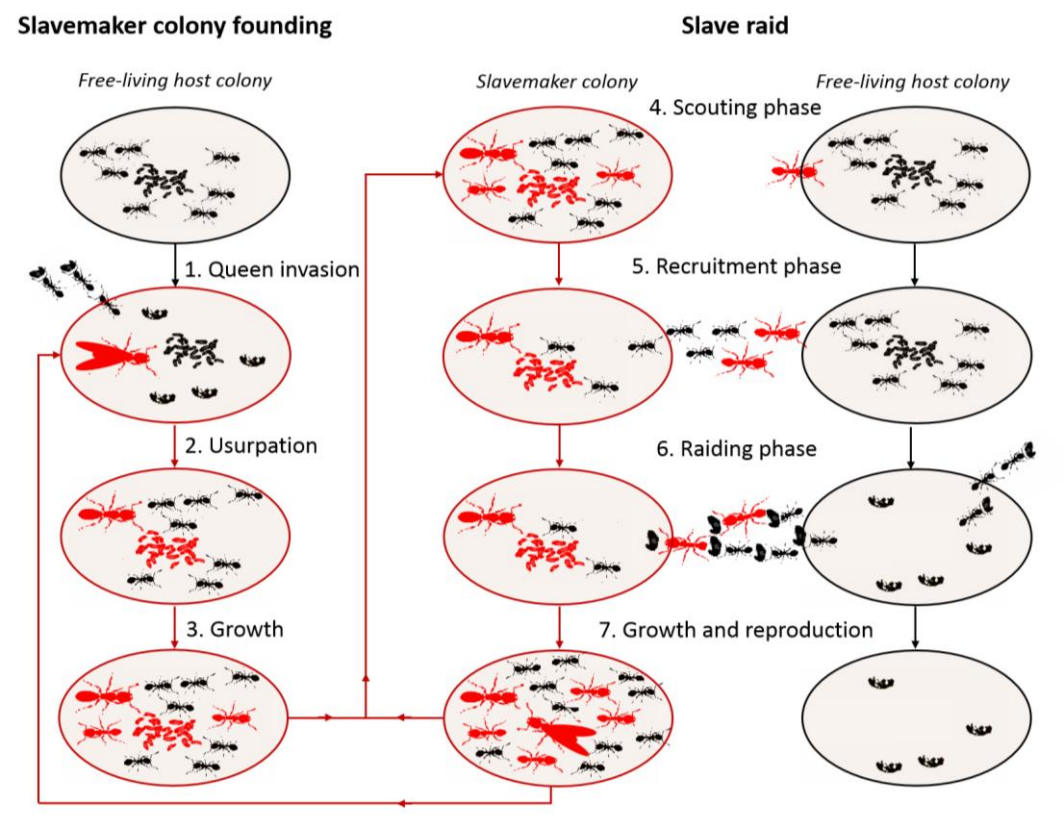

Figure 1.1. The life-cycle of *Protomognathus americanus* slavemakers. Black circles and icons represent *Temnothorax* colonies and *Temnothorax* ants/brood; red circles and icons represent *P. americanus* colonies and *P. americanus* ants/brood. Large *P. americanus* ant icons represent queens, and winged ant icons young queens. The different phases of slavemaker colony establishment (left) and slave raids (centre and right) are numbered. For further details see the text of Box 1.

Slave raids – Once established, slavemaker colonies raid the brood from neighbouring *Temnothorax* colonies to replenish their slave work force. Such slave raids are preceded by a scouting phase (Figure 1.1, phase 4), during which one or several slavemaker workers leave their nest site "in search for" a host target colony. After the discovery and inspection of a target colony the scout returns to its own colony to recruit a raiding party (Figure 1.1, phase 5), which can consist of both slavemakers and enslaved *Temnothorax* workers. The

raiding phase is initiated by the scout leading the raiding party back to the target colony, were the raiders force their way into the nest, kill or drive away adult host workers and queens and transport the captured brood back to the slavemaker colony (Figure 1.1, phase 6; Creighton 1927; Wesson 1939; Alloway 1979; Alloway and Del Rio Pesado 1983). A mature slavemaker colony conducts several slave raids per raiding season.

With the help of the newly enslaved workers, the slavemaker colony can raise new workers, males and queens (Figure 1.1, phase 7). After the mating flight, dealate queens will try to establish a new colony (Figure 1.1, phase 1), whereas the original slavemaker colony again conducts slave raids (Figure 1.1, phase 4-6).

No two raids are the same - Although most slave raids roughly follow the different phases depicted in Figure 1.1, they can be remarkably unpredictable events (Wesson 1939; Alloway 1979; Foitzik *et al.* 2001): The scouting phase may involve a single or multiple scouts, which may or may not warn the target colony by entering their nest site for inspection. Scouts can either recruit a raiding party; they can immediately attack and permanently take over a poorly defended target; or they can first kill or expel all adult hosts before recruiting nestmates for brood transport. During the recruitment phase, scouts may be able to initiate a slave raid within minutes or take several hours to lead the raiding party back to the target colony, and this raiding party may consist of few to several dozen slavemaker workers and slaves. Once the slave raid is initiated, slavemakers may repeatedly return back to their colony to recruit further reinforcements, they may or may not pose a guard in the target nest entrance to prevent hosts from evacuating their brood, and they may carry the captured brood back to their original colony or start an auxiliary nest in the targeted nest site. Hence, no two raids are the same and there is not one single host defence strategy that best protects host colonies that are targeted by slavemakers.

grooming each other and begging for food (Wesson 1939). Indeed, the first investigators to observe the generally inactive behaviour of workers inside the nest concluded that it is a "degenerate or evanescent slavemaker", whose worker caste was no longer necessary (Wheeler 1910; Creighton 1927). Later studies however showed that *P. americanus* workers are far from degenerate or redundant: They play a crucial role during slave raids, to which they are well adapted (Wesson 1939; Alloway 1979; Alloway and Del Rio Pesado 1983; Foitzik *et al.* 2001).

Although *Temnothorax* colonies can grow up to several hundreds of individuals, a typical colony contains only a few dozen workers. Slavemaker colonies can consist of up to 50 slavemakers, but the median number generally does not exceed 2 slavemaker workers (chapter 2) and 20 to 50 slaves. Both hosts and slavemaker colonies frequently occupy several separate nest sites at a time (i.e. polydomy; Alloway *et al.* 1982; Alloway and Del Rio Pesado 1983; Herbers 1986; Foitzik and Herbers 2001a; Foitzik and Herbers 2001b). Polydomy allows *P. americanus* colonies to extend their raiding radius over a larger area, causing a broader impact on the host population than monodomous colonies (Foitzik and Herbers 2001a). Polydomous subunits are autonomous, as they independently conduct raids and rarely exchange slaves (Foitzik and Herbers 2001a).

In this thesis, I therefore do not distinguish between monodomous colonies and polydomous nest units. Unlike the slavemaker, which is invariably monogynous (Buschinger & Alloway 1977), *Temnothorax* host are facultatively polygynous (Alloway *et al.* 1982; Foitzik and Herbers 2001a) due to the acceptance of newly mated queens by their natal nest (Herbers and Stuart 1996). However, nest defence by *Temnothorax* colonies, the main focus of this thesis, is unrelated to queen number (Stuart 1991).

Impact of slavemakers on host fitness

Every year, newly mated slavemaker queens can successfully usurp 21% of the local *Temnothorax* colonies (Pamminger *et al.* 2012). Although host colonies can survive such an event, they do lose about half of their annual brood production (Pamminger *et al.* 2012). These losses are however marginal in comparison to the impact of a mature slavemaker colony in the vicinity. Slave raids by *P. americanus* destroy the reproduction and growth of the targeted host and few host colonies survive such an attack (Foitzik and Herbers 2001a; Blatrix and Herbers 2003). Moreover, a single slavemaker colony conducts between two and ten successful raids on host colonies every year (Foitzik *et al.* 2001; Foitzik and Herbers 2001a; Blatrix and Herbers 2003). In some populations, host colonies therefore have a larger than 50% annual risk of being successfully raided, which may locally approach unity over a colony's life time (Foitzik and Herbers 2001b). Hence, the impact of *P. americanus* on its *Temnothorax* hosts is thought to be severe enough to drive an escalating coevolutionary arms race.

THIS THESIS

Although the specific focus and research questions addressed in each of the chapters of this thesis are diverse, the overarching aim is to investigate how geographic variation in host defence portfolios relates to slavemaker pressure. Few studies to date have focussed on multiple host defence strategies, and studies covering more than one or few host populations are rare. We therefore assessed host defence strategies in up to 17 populations of two *Temnothorax* ant species (Box 2), covering nearly the entire geographic range of the slavemaker *P. americanus*, as well as populations where the slavemaker was absent (e.g. chapter 2).

Slavemakers often rely on a combination of overt aggression, stealth, manipulation and surprise to gain access to the workforce of the host, and severely parasitized host populations may be expected to come with an evolutionary answer to each of these offensive strategies. The level of mobilisation in each line of defence may however not only depend on parasite pressure, but also on the efficacy of other traits in the host's adaptive portfolio. This is why this thesis focusses on six potentially interrelated defence strategies; Collective fight and flight strategies (chapter 2), increased aggressive potential (chapter 3), resistance to chemical manipulation by the slavemaker (chapter 4), chemical adaptions involved in enemy recognition (chapter 5) and flexibility to respond to slavemaker attack (chapter 6).

In the first part of this thesis, we focus on frontline host defences that likely yield the highest fitness benefits to the host as they can prevent a slavemaker attack altogether.

In chapter 2, we investigate the relative contribution of collective fight and flight strategies to the defence portfolio of *Temnothorax* ants. Chapter 3 more closely examines the aggressive potential of *Temnothorax* hosts towards conspecific and heterospecific opponents, including the slavemaker *P. americanus*. As these two chapters demonstrate that host populations that are most aggressive towards an intruding slavemaker do not necessarily show a higher aggressive potential, we investigate the role of manipulation of host defences by the slavemaker in chapter 4. Social parasites can alter their host's behaviour using offensive chemicals, which protects them during host invasions from aggression by the more numerous host defenders (Lenoir et al. 2001). Not all hosts may be equally susceptible to manipulation, which could have important implications for the parasite's host preference and prevalence. We therefore investigate inter- and intra-specific variation in host resistance to manipulation, its consequences for the intensity and the efficiency of aggressive colony defences by the host, as well as its effect on host preference and prevalence of the slavemaker.

The second part of this thesis focusses on two novel defence mechanisms: chemical adaptions involved in enemy recognition and flexibility to respond to slavemaker attack.

Although the ability to distinguish friend from foe is an important line of defence in social insects, the evolution and maintenance of enemy recognition cues it still poorly understood. To evade host aggression and become established within their hosts' nest, social parasites often obtain chemical congruence with the host's nestmate recognition cues. Selection likely favours parasites that match hosts with common colony signatures, providing an advantage to hosts with rare recognition profiles. Hence, slavemakers are predicted to drive recognition cue diversification in their host through negative frequency dependent selection, which we investigate in chapter 5.

Finally, chapter 6 experimentally investigates the fitness consequences of behavioural specialisation by *T. longispinosus* workers during slave raids. Specialized workers may be more efficient in the tasks they perform than generalists; yet, they may also lack the flexibility to respond to rapid shifts in task needs. Such rigidity could constrain a society's ability to cope with dynamic and unpredictable events, such as a slave raid (box 1). Hence, generalist workers are predicted to convey the largest fitness benefits to host colonies that are under slavemaker attack.

GEOGRAPHIC DISTRIBUTION AND COLLECTION SITES

BOX 2

Temnothorax longispinosus, *T. curvispinosus* and *P. americanus* are widely distributed throughout the deciduous forests of the north eastern United States and Canada, where they are among the most abundant species of ant communities (Herbers 1989). These tiny ants lead an inconspicuous life in the leaf litter, inhabiting hollow acorns, nuts, sticks and other preformed cavities. Ant communities can differ substantially in the presence and abundance of slavemaker colonies, although *T. longispinosus* is generally more heavily exploited than *T. curvispinosus* (Creighton 1927; Brandt and Foitzik 2004). Within host populations, *P. americanus* nests are patchily distributed, with local slavemaker prevalence ranging from 0 to over 60% (Herbers 1989; Herbers and Foitzik 2002). Hence, this common slavemaker (Sturtevant 1927; Wesson 1939) can locally exert an exceptionally strong selection pressure on its hosts.

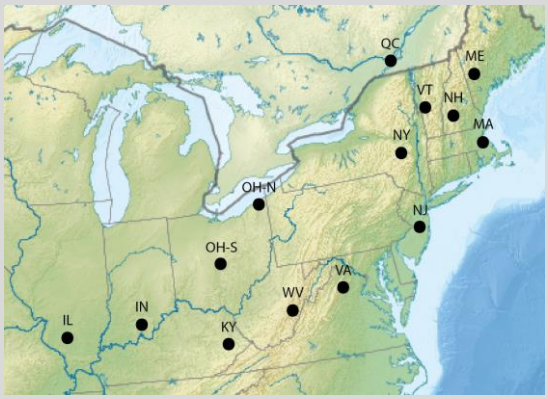

Figure 1.2. Collection sites of the *Temnothorax longispinosus*, *T. curvispinosus* and / or *Protomognathus americanus* colonies, used to assess the geographic distribution of host defence portfolios described in this thesis. Sites include Kentucky (KY), Illinois (IL), Indiana (IN), Massachusetts (MA), Maine (ME), New Hampshire (NH), New Jersey (NJ), New York (NY), Ohio-North (OH-N), Ohio-South (OH-S), Quebec (QC), Virginia (VA), Vermont (VT) and West Virginia (WV). For further details see appendix chapter 2.

CHAPTER 2

Collective Defence Portfolios of Ant Hosts Shift with Social Parasite Pressure

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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 [7]. 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 coevolutionary 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.* 2005; 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.* 2005; 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 (figure 2.1; table S2.1). 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.

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.*

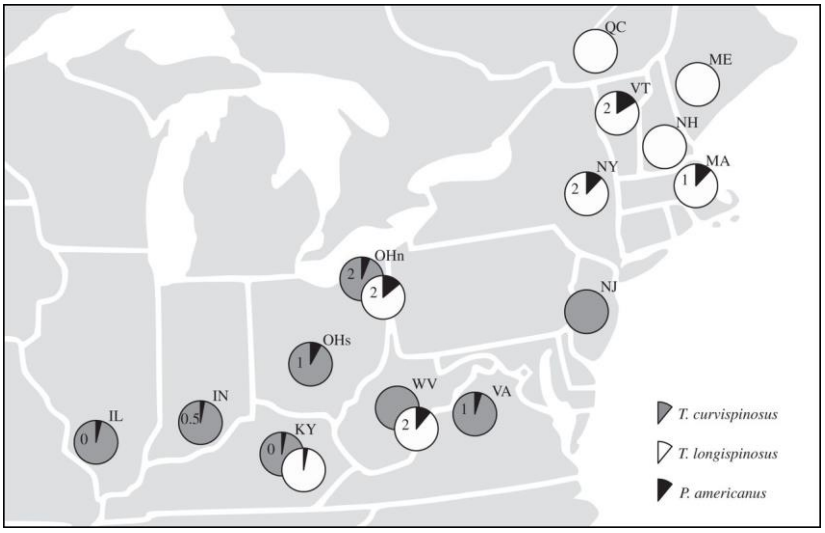

Figure 2.1. Distribution of experimental *Temnothorax* host populations and parasite pressure by the slavemaker ant *P. americanus*. Pie diagrams and numbers represent parasite prevalence and median slavemaker colony sizes (i.e. the median number of slavemaker workers), respectively, in Illinois (IL), 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 supplementary table S2.1.

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 the supplementary table S2.1.

Behavioural experiments

From each population, 32.0 ± 3.3 (mean ± s.d; supplementary table S2.1), 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: $F = 1.37$, $\Delta d.f. = 15$, $p = 0.157$). 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 25º 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: $\Delta deviance$ = -1.87, $\Delta d.f.$ = 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 (figure 2.2). 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 (figure 2.2a, p = 0.004) decreased with parasite prevalence. Contrastingly, the evacuation probability of host populations increased with parasite prevalence (figure 2.2c; 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, $\Delta d.f.$ = 1, p = 0.324). Parasite prevalence was not associated with the likelihood that the slavemaker escaped (χ^2 = 0.08, $\Delta d.f.$ = 1, p = 0.780).

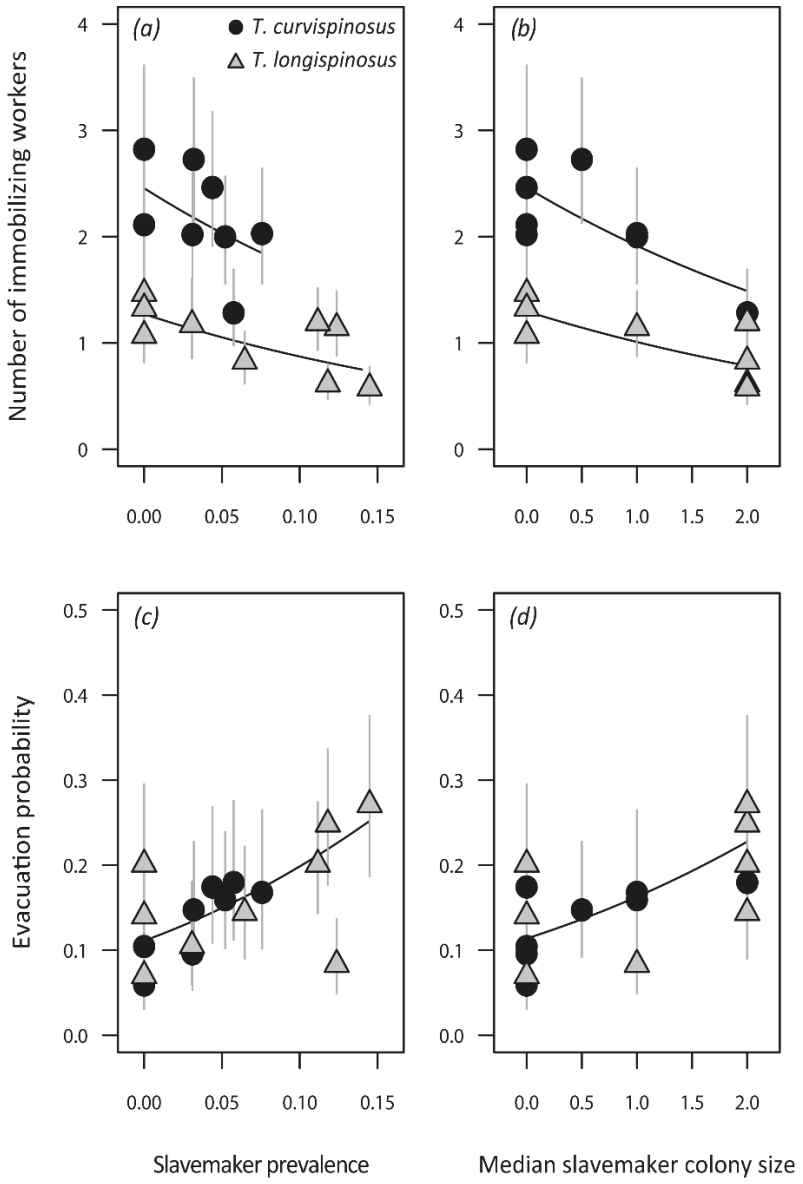

Figure 2.2. Collective host defences in relation to social parasite pressure. Parasite pressure is represented by the parasite prevalence *(a,c)* and the median slavemaker colony size *(b,d)*. Symbols represent the estimate ± s.e. per population, standardized for average host colony size (i.e. population 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 (figure 2.3a-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* (figure 2.3a; $p < 0.0001$). Contrastingly, *T. longispinosus* colonies primarily showed individual attack (figure 2.3b; $p < 0.0001$). 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 (figure 2.3c; $p < 0.0001$). Host species did not differ in evacuation probability ($\chi^2 = 0.05$, $\Delta d.f. = 1$, $p = 0.816$).

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 (figure 2.2d; $p = 0.003$), were less likely to show collective immobilization (-0.324 ± 0.116, $z = -2.80$, $p = 0.005$) and fewer workers immobilized the slavemaker (figure 2.2b, $p < 0.001$). The number of workers showing individual attack was unrelated to colony sizes of the host ($\chi^2 = 1.02$, $\Delta d.f. = 1$, $p = 0.312$) 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*

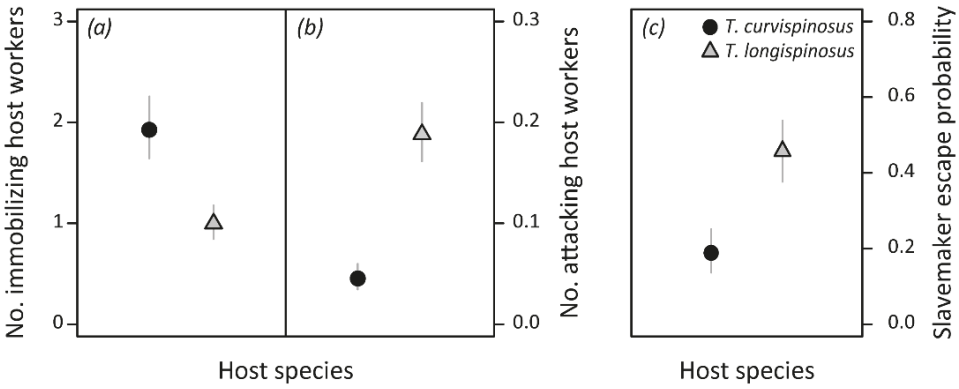

Figure 3. Differences in aggressive defence strategies and efficiency between host species. Symbols represent the GLMM estimates ± s.e. *(a)* $z = -5.45$, $p < 0.0001$; *(b)* $z = 4.54$, $p < 0.0001$; *(c)* $z = 5.30$, $p < 0.0001$.

(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.* 1991; 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 3). 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; 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 1991; 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 & 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.

All authors designed the study and contributed to the manuscript. EJ, IK and SJ collected the data and EJ analysed it. Ant collection permits were 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.

ACKNOWLEDGEMENTS

[removed for privacy purposes]

SUPPLEMENTARY MATERIAL

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, $\Delta d.f.$ = 1, p = 0.311; number of workers: χ^2 = 1.80, $\Delta d.f.$ = 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 S2.1). This may suggest that *T. longispinosus* defence strategies in West Virginia have been selected for under different conditions that those experienced at present.

SUPPLEMENTARY TABLES

Table S2.1. 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		Collected 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 S2.2. 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 $\Delta d.f.$ = 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 S2.3. 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 $\Delta d.f.$ = 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 3

Geographic Variation in Social Parasite Pressure Predicts Intra- but not Interspecific Aggressive Responses in Hosts of a Slavemaking Ant

Isabelle Kleeberg, Evelien Jongepier, Sylwester Job & Susanne Foitzik

Based on
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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 behavioral host defenses. 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; Coevolution; Collective defense; 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 coadaptation 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 behavioral traits (Dobson 1988; Barber *et al.* 2000). Hence, coadaptation 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 behavioral adaptations that increase their resistance or tolerance (Wakelin 1984; Gross 1993; Hart 1997; Sorci 2013). If parasites are common and their impact severe, hosts are expected to evolve effective defenses, which in turn selects for counter-adaptations in the parasite. If trait expression is costly, more effective defensive traits are expected only in severely parasitized host populations (coevolutionary 'hot spots'; Thompson 1999). The local coevolutionary process is furthermore affected by the co-occurrence of alternative hosts in a community (Brandt and Foitzik 2004). Parasites often favor 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 defenses, 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 behavioral 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 behavior. 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.* 2015), 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 2). 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). 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 2), which suggests that different ecological conditions favor different levels of trait expression. *Temnothorax* hosts show fine-tuned aggression behavior, where the intensity and type depend on opponent species and the perceived threat that they pose (Scharf *et al.* 2011): 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). 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 behavioral adaptations in two of the three host species of *P. americanus*. We investigated whether parasite pressure covaries 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). 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).

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 (figure 3.1; supplementary table S3.1; Jongepier *et al.* 2014, chapter 2). Ants of these species harbor 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 and Del 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 2); however, there is no behavioral data overlap between the two studies. As parasite-induced short-term behavioral 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 (figure 3.1; supplementary table S3.1). 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 (67 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 2). 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 (supplementary table S3.2). 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 (supplementary table S3.3).

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 ± s.d. = 30.17 ± 3.29; supplementary table S3.1) similar-sized host colonies. The colony sizes did not differ between 16 out of 17 populations (mean no. workers ± s.d. = 30.64 ± 12.86; quasi-Poisson GLM: $\chi^2_{12} = 16.97$, $p = 0.151$) with the exception of New Hampshire, which had smaller colony sizes (mean ± s.d. = 20.53 ± 11.32; all $p < 0.01$). However, by excluding New Hampshire from the following analyses the results remained

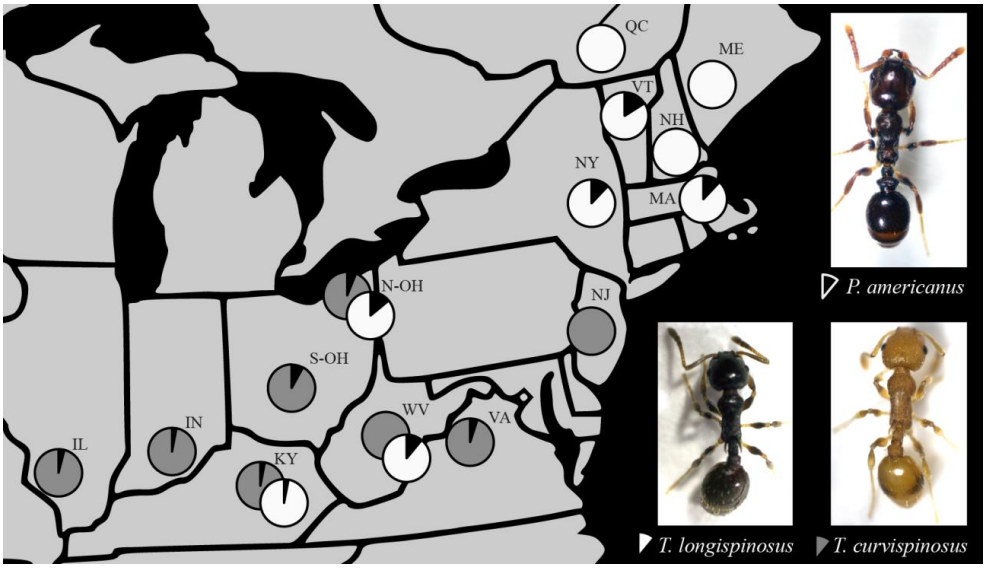

Figure 3.1. Geographic distribution of *Temnothorax longispinosus* (lower left picture) and *T. curvispinosus* (lower right picture) host populations and parasite pressure by the slavemaker ant, *Protomognathus americanus* (upper picture). Host worker ants are only 2-3mm in lengths, the slavemaker is 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 2.

qualitatively the same. Moreover, colony sizes did not differ between host species (Poisson GLMM with colony identity, nested in population identity as random factor: $\chi^2_1 = 1.70$, $p = 0.192$).

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 (supplementary table S3.1). For trial rounds (1) and (2) we used approximately 30 colonies (round 1: mean ± s.d. = 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' behavior (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.

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: $z = -0.82$, $p = 0.41$). *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 ($z = 0.84$, $p = 0.401$), 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: $z = -0.90$, $p = 0.367$). The origin of the competing *Temnothorax* ant was also standardized, as most ant communities did not harbor 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 (supplementary tables S3.2, S3.3). For model selection, we used a backwards, stepwise selection procedure ($\alpha = 0.05$). 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: $\chi^2_1 = 19.35$, $p < 0.001$). Host aggression against a conspecific opponent increased with parasite pressure ($z = 4.45$, $p < 0.001$; figure 3.2a) and this positive relationship was independent of population density (supplementary table S3.2). 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 ($z = 0.59$, $p = 0.555$; figure 3.2b) or a competing *Temnothorax* species ($z = -1.34$, $p = 0.182$; figure 3.2c) 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 (supplementary table S3.3).

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: $\chi^2_2 = 4.39$, $p = 0.112$; host species – parasite pressure interaction: $\chi^2_2 < 0.01$, $p > 0.999$). Nonetheless, the two host species differed in their aggressive responses in the absence of parasites (i.e. the intercepts in figure 3.2). *Temnothorax longispinosus* was more aggressive than *T. curvispinosus*, regardless of the opponent species (host species difference in aggression towards a conspecific: $z = 4.61$, $p < 0.001$; a parasite: $z = 10.34$, $p < 0.001$; and a competing *Temnothorax* species: $z = 7.82$, $p < 0.001$). Moreover, host species differed in their aggressive responses towards the different opponent species (host species - opponent species interaction: $\chi^2_2 = 19.67$, $p < 0.001$). *Temnothorax longispinosus* was as aggressive towards a worker of the competing *Temnothorax* species as towards the social parasite ($z = 1.83$, $p = 0.854$), whereas *T. curvispinosus* was more aggressive towards the latter ($z = -2.64$, $p = 0.008$). Both host species showed less aggression towards a conspecific host than towards a parasite (*T. longispinosus*: $z = 10.71$, $p < 0.001$; *T. curvispinosus*: $z = 7.43$, $p < 0.001$) or a competing

Temnothorax species (*T. longispinosus*: $z = 6.60$, $p < 0.001$; *T. curvispinosus*: $z = 2.19$, $p = 0.028$).

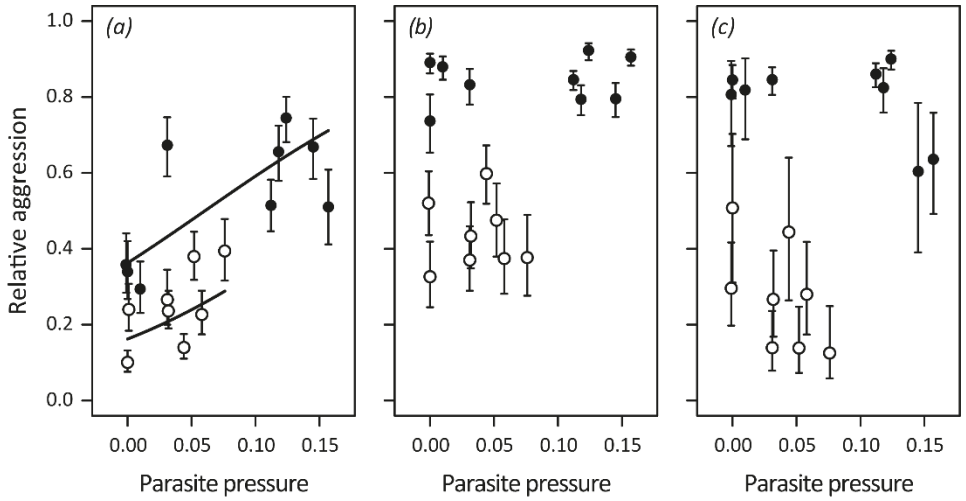

Figure 3.2 *Temnothorax* host colony aggression (proportion of aggressive interactions) in relation to parasite pressure (proportion of slavemaker colonies per host colony) exerted by the social parasite *Protomognathus americanus* across 17 host populations. a) Colony aggression towards a non-nestmate 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*: estimate ± s.e. = 9.73 ± 2.11, $z = 4.61$, $p < 0.001$; *T. longispinosus*: 9.34 ± 2.10, $z = 4.45$, $p < 0.001$.

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 behavior with parasite pressure, which is indicative of convergent evolution in host behavioral 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). Why is aggression against conspecifics an important anti-parasite

defense? 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 (Fürst *et al.* 2011; Delattre *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.* 2005), 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 (figure 3.1). 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 center 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 2). 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 defenses. Rather, convergence in behavioral defense 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 defense (Pamminger *et al.* 2011; Kleeberg *et al.* 2014). 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.* 2011), 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 2). This explains why the slavemaker prefers *T. longispinosus* with its less coordinated behavioral defenses (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 2), 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 *et al.* submitted, 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.* 2011), yet this response is unrelated to parasite pressure? One possible explanation could be the absence of costs of this defense 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.* 2004).

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 defense behavior (Jongepier *et al.* 2014, chapter 2), 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.* 2011). 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 behavioral 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 coevolution between hosts and parasites, does not affect behavioral responses towards heterospecific competitors, but can shape intraspecific interactions.

IK, EJ and SF designed the study. IK wrote the manuscript, and EJ and SJ contributed to the revisions. IK, EJ and SJ collected the data and IK and EJ analysed it. Ant collection permits were

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.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY TABLES

Table S3.1. 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, chapter 2).

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

* First number refers to sympatric and second to allopatric parasite

Table S3.2: 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 $\Delta d.f.$ = 1.

	Conspecific	
Predictors	*χ2*	*p*
Parasite pressure	11.43	**<0.001**
Species	9.56	**0.001**
Population density	0.20	0.654
Species x Population density *	4.01	**0.045**
Species x Parasite pressure	<0.01	0.943

**T. curvispinosus*: estimate ± s.e. = -0.13 ± 0.06, z = -2.11, p = 0.030; *T. longispinosus*: 0.02 ± 0.03, z = 0.62, p = 0.534

Table S3.3: Model selection results from the generalized linear mixed model analysing relative colony aggression of *T. longispinosus* and *T. curvispinosus* against a heterospecific host ant in relation to parasite pressure and host sympatry. Statistics indicated in bold were retained in the final model All $\Delta d.f.$ = 1.

	Heterospecific	
Predictors	*χ2*	*p*
Parasite pressure	2.64	0.104
Species	28.46	**< 0.001**
Host sympatry	0.94	0.333
Species x Host sympatry	0.25	0.619
Species x Parasite pressure	0.78	0.376

CHAPTER 4

The Ecological Success of a Social Parasite Increases with Manipulation of Collective Host Behaviour

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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.* 2009; Blanchet, Thomas, *et al.* 2009). 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

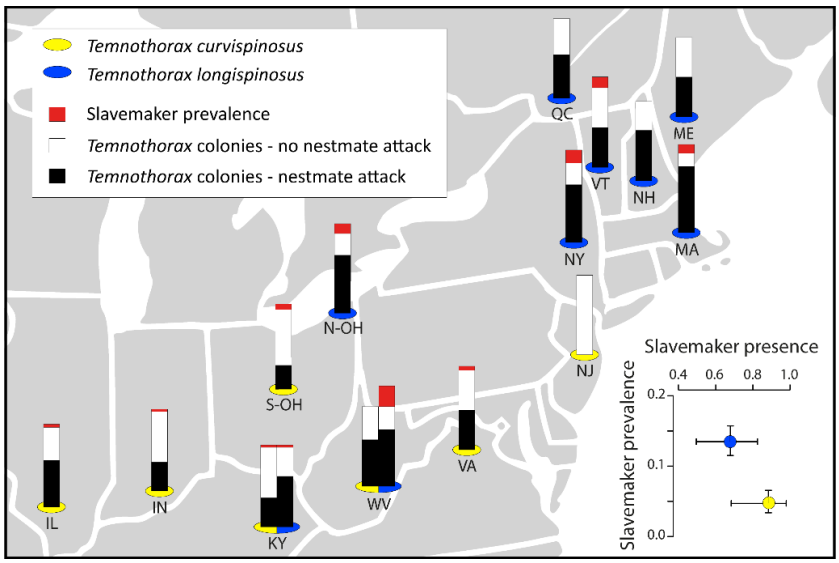

Figure 4.1. Slavemaker prevalence and the proportion of colonies attacking their Dufour-treated nestmate for each of the 16 *Temnothorax* populations. Ant communities represent Illinois (IL), Indiana (IN), Kentucky (KY), Maine (ME), Massachusetts (MA), New Hampshire (NH), New Jersey (NJ), New York (NY), 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: t_{14} = 2.90, p =0.012) although it did not co-occur more often with the slavemaker (binomial GLM: z_{14} = -0.85, p = 0.395).

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). 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 2). 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 absense of manipulation; Kleeberg et al. 2015, chapter 3). 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 2) and dead, non-manipulative slavemakers (Kleeberg *et al.* 2015, chapter 3). 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 2), 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 (figure 4.1; Jongepier *et al,* 2014, chapter 2). 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 2); 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 3). 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. Slavemaker 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 2). 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 3). 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 2), 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 3). 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 (figure 4.1; Jongepier *et al.* 2014, 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).

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 3), 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 2). 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 ($\alpha = 0.05$). 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: $\chi^2_1 = 29.70$, $p < 0.0001$). Colonies that attacked their Dufour-treated nestmate showed less aggression towards a live slavemaker than colonies that did not attack their Dufour-treated nestmate (figure 4.2a; $z = -2.27$, $p = 0.005$). Contrastingly, dead slavemakers

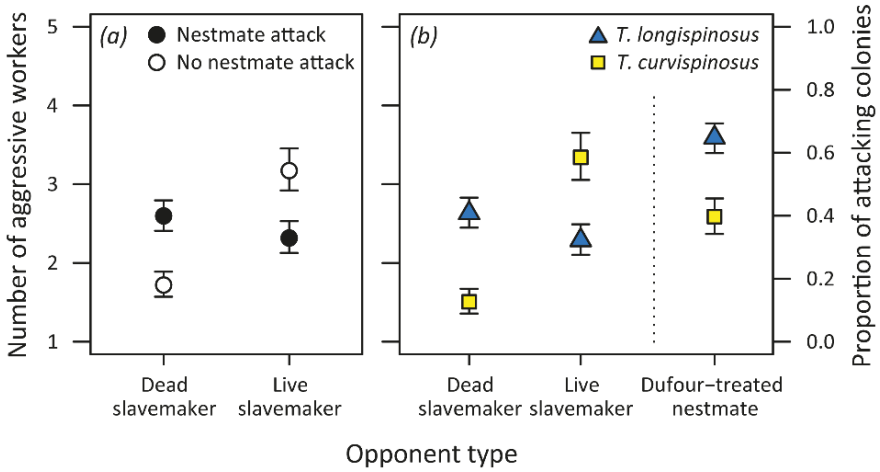

Figure 4.2. The relationship between aggression towards slavemakers, slavemaker-induced nestmate attack and *Temnothorax* ant species. *(a)* The number of *Temnothorax* workers showing aggression towards a dead and a live slavemaker, in relation to whether or not those colonies attacked their Dufour-treated 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.

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 (figure 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 2). 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 3).

Slavemaker-induced change in aggression and slavemaker survival

Slavemaker escape probability increased with the proportion of colonies that attacked their Dufour-treated nestmate (figure 4.3a; $t_{14} = 2.67$, $p_{corr} = 0.024$) and decreased with the average aggression towards live slavemakers (figure 4.3b; $t_{14} = -2.49$, $p_{corr} = 0.026$, also shown in Jongepier *et al.* 2014, chapter 2). 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 2), 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 (figure 4.3c; t_{14} = 3.63, p_{corr} = 0.009) and decreased with the average aggression towards live slavemakers (figure 4.3d; t_{14} = -3.18, p_{corr} = 0.014; also shown in Jongepier *et al.* 2014, chapter 2). 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 3).

The higher slavemaker prevalence in *T. longispinosus* compared to *T. curvispinosus* populations (figure 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).

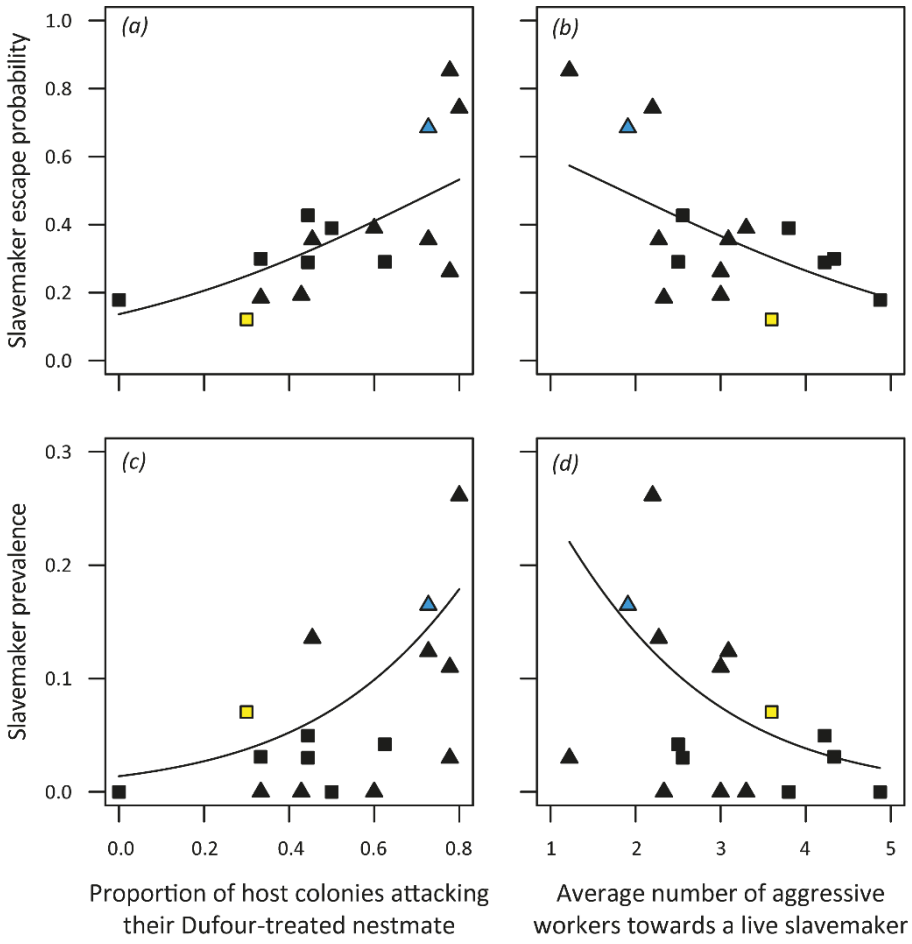

Figure 4.3. Slavemaker-induced nestmate attack and anti-slavemaker aggression of *Temnothorax* ant colonies in relation to *(a-b)* slavemaker escape and *(c-d)* slavemaker prevalence per population. Slavemaker-induced nestmate attack is represented by the proportion of colonies attacking their Dufour-treated 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 2), but not towards a non-manipulative parasite (this study; Kleeberg *et al.* 2015, chapter 3). 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 3). 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 a 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 2). 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; figure 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.

All authors designed the study and contributed to the manuscript. EJ and IK collected the data and EJ analysed it. Ant collection permits were 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.

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CHAPTER 5

Ant Recognition Cue Diversity is Higher in the Presence of Slavemaker Ants

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Based on
Behavioral Ecology, in press

ABSTRACT

Social insect colonies defend themselves from intruders through nestmate recognition, yet the evolution and maintenance of recognition cue diversity is still poorly understood. We compared the recognition cue diversity of nine populations of *Temnothorax longispinosus* ant colonies, including populations that harbored the socially parasitic slavemaker ant, *Protomognathus americanus*. Although ants recognize friends from foe based on recognition cues encoded in their cuticular hydrocarbon profile, which specific compounds are involved in recognition is unknown for most species. We therefore started by statistically identifying nine putative recognition compounds involved in worker and colony aggression. We find that colonies that co-occur with slavemakers were more variable in these recognition compounds and hence less similar in their recognition profiles than unparasitized populations. Importantly, these differences appear to be regulated by processes that specifically act upon the level of the colony, which rules out potentially confounding effects altering chemical profiles of populations, such as differences in abiotic conditions or standing genetic variation. Instead, our findings indicate that slavemakers drive recognition cue diversity in their ant hosts, in much the same way that avian hosts diversify their egg appearance in response to brood parasite pressure. Such recognition cue diversification through negative frequency-dependent selection favors rare host phenotypes and renders it impossible for parasites to match the recognition profile of all potential hosts.

Keywords: Nestmate recognition; Cuticular hydrocarbons; Social parasites; Slavemaker ants; Negative frequency-dependent selection

INTRODUCTION

The Red Queen model of co-evolution predicts that parasites primarily target common host phenotypes in a population, which favors rare ones through negative frequency-dependent selection (Van Valen 1973; Bell 1982). Coevolutionary arms races can thus drive host diversification (Hamilton 1982), generating phenotypic polymorphisms that constrain coevolving parasites or pathogens to exploit any one potential host. This rare-advantage has been implicated in the evolution and maintenance of variation in host taxa as diverse as bacteria (Koskella and Brockhurst 2014), plants (Holt III *et al.* 2000) and animals (Koskella and Lively 2009), including hosts whose behavior, rather than physiology, is exploited by parasites (Kilner and Langmore 2011). For instance, polymorphism in avian egg appearance is believed to be the host's evolutionary response to egg mimicry by brood parasites (Davies and Brooke 1989; Øien *et al.* 1995; Spottiswoode and Stevens 2011), which renders it impossible for the parasite to match the egg recognition cues of all potential hosts. Similar dynamics have been suggested for social parasites that exploit the cooperative behavior of their ant, bee or wasp hosts (Kilner and Langmore 2011). Yet, empirical evidence that social parasites drive recognition cue diversity among host societies is scant (Martin *et al.* 2010; 2011).

Parasites that exploit the social behavior of their host are found in many social insects but are especially manifold in ants. Ant colonies protect themselves from intruders such as social parasites through nestmate recognition, which usually ensures that altruistic behavior is directed towards related recipients. Ants primarily rely on chemical cues for nestmate recognition, which are encoded in their cuticular hydrocarbon profile (Howard and Blomquist 2005). Nestmates share similar cuticular profiles due to genetic relatedness (Vander Meer and Morel 1998; Van Zweden *et al.* 2009; Van Zweden *et al.* 2010; Van Zweden and D'Ettorre 2010; but see Martin *et al.* 2009; Helanterä *et al.* 2011) as well as the exchange of compounds through trophallaxis and grooming (Stuart 1988; Soroker *et al.* 1995; Van Zweden and D'Ettorre 2010). To evade host aggression and become established within their hosts' nest, social parasites can obtain chemical congruence with the host's nestmate recognition cues (Lenoir *et al.* 2001). Social parasites that match hosts with common colony signatures are likely favored by selection, providing an advantage to hosts with rare recognition profiles in the coevolutionary arms race with parasites.

Among the social parasites, slavemakers may be a particular potent driver of recognition cue diversity in their hosts for three reasons. Firstly, selection pressures of slavemaking ants on their host populations are high and can lead to changes in nest density, social organization and reproductive strategies of their hosts (Foitzik and Herbers 2001; Foitzik *et al.* 2009). In contrast to workerless inquilines or temporal social parasites that are parasitic only during the nest founding phase, slavemaking ants can reduce the fitness of multiple host colonies per year through destructive slave raids. Secondly, slavemaker brood is reared by enslaved host workers and young queens may thus already acquire host-specific chemical compounds in their natal nest, providing camouflage when they invade a host nest during colony foundation. In this respect, slavemakers differ from temporary social parasites, where host workers are not

replenished but rather replaced by parasite workers. Without direct contact with host workers in their natal nest, founding queens of temporary social parasites may need to rely more on innate chemical strategies during colony usurpation. Thirdly, a host colony that is attacked by a slavemaker raiding party does not only face slavemakers but often also conspecific slaves, which capture the host's brood and are responsible for many of the host casualties (Wesson 1939; Stuart and Alloway 1983; Foitzik *et al.* 2001). Since these slaves originate from previously raided host colonies in the local population, colonies with a deviant recognition profile may be better able to distinguish nest mates from conspecific slaves.

The small acorn ant, *Temnothorax longispinosus* is the primary host of the slavemaking ant *Protomognathus americanus* (Herbers and Foitzik 2002), an obligate social parasite that relies entirely on its slave workforce for brood care, foraging and other colony maintenance tasks (Wesson 1939). During colony foundation, a slavemaker queen invades a *T. longispinosus* colony, kills or evicts the host queen and workers, and exploits the host brood after it develops in the first generation of slaves. Without a host queen to replenish the slave workforce, slavemaker colonies conduct regular slave raids to pillage the brood of nearby host colonies (Alloway 1979; Foitzik *et al.* 2001). Although *P. americanus* partially resembles its host's cuticular hydrocarbon profile (Brandt *et al.* 2005), *T. longispinosus* colonies are able to recognize slavemakers as enemies (Alloway 1990) and aggressively defend themselves against slavemakers and slaves (Foitzik *et al.* 2001). Comparing two ant communities, Brandt *et al.* (2005) showed that *P. americanus* populations differ in how closely they resemble the cuticular hydrocarbon profile of their local *T. longispinosus* host, and the population that better matched the host profile is more successful during raids (Brandt *et al.* 2005). Although slavemakers thus seem to benefit from a close chemical congruence with their hosts, little is known about the host's chemical anti-slavemaker defenses.

Although nestmates share similar cuticular profiles, not all substances encode nest affiliation. Some serve as desiccation barriers (Gibbs and Rajpurohit 2010), as a signal of caste affiliation (Greene and Gordon 2003) or fertility (Monnin 2006; Liebig 2010), and the chemical profile of workers can be affected by external factors (Heinze *et al.* 1996; Silverman and Liang 2000), masking the more stable recognition cues. Drawing biological inferences about nestmate recognition based on entire hydrocarbon profiles may therefore be misleading (Martin and Drijfhout 2009). With few exceptions (Akino *et al.* 2004; Martin, Helanterä, *et al.* 2008; Krasnec and Breed 2013), the specific compounds involved in recognition in social insects remain however unknown.

Presumably, recognition cues are detected and compared to a template, resulting in an aggressive reaction if the cues do not match the familiar template (Lacy and Sherman 1983; Guerrieri *et al.* 2009; Newey 2011). Aggression is thus considered one of the best proxies for the recognition ability of social insect workers (Martin *et al.* 2012), and the most compelling evidence for the role of cuticular hydrocarbons in nestmate recognition indeed comes from aggression bioassays (Lahav *et al.* 1999; Wagner *et al.* 2000; Dani *et al.* 2001; Akino *et al.* 2004; Dani *et al.* 2005; Greene and Gordon 2007; Martin, Vitikainen, *et al.* 2008; Guerrieri *et al.* 2009). The identification of specific compounds eliciting

aggression has however proven more challenging (Van Zweden and D'Ettorre 2010), as such experimental approaches are currently impaired by the lack of more complex synthetic hydrocarbons thought to be important in nestmate recognition. The identification of recognition cues therefore mainly relies on statistical methods, which aim to identify compounds that reliably predict nest affiliation. This approach has proven particularly powerful when followed up with aggression bioassays, confirming the role of colony-specific compounds in nestmate recognition (Akino *et al.* 2004; Martin, Vitikainen, *et al.* 2008) or even quantifying how differences in these compounds determines the likelihood of aggressive responses (Martin *et al.* 2012). We apply a similar statistical approach to directly identify compounds that reliably predict aggression in *T. longispinosus*, out of the complete cuticular hydrocarbon profiles.

Here, we test whether recognition cue diversity of *T. longispinosus* is higher in the presence of slavemakers. We compared the cuticular hydrocarbon profile of over 1000 workers from 110 colonies. These colonies originated from nine *T. longispinosus* populations, covering almost the entire geographic range of this host, including populations that co-occur with *P. americanus* slavemakers. Firstly, we identified nine putative recognition compounds, based on their involvement in individual and colony aggression. Secondly, we used generalized mixed models to assess the repeatability of these putative recognition compounds, based on variance decomposition into three levels of biological organization: within colonies, between colonies and between populations. At the level of the colony, a high repeatability in recognition cues among nest mates indicates consistency within colonies and distinctness between colonies, the two criteria for reliable recognition cues. Thirdly, we compared parasitized and unparasitized populations in their chemical similarity within and between colonies, based on the entire recognition profile (i.e. the profile of putative recognition compounds). If slavemakers indeed drive recognition cue diversity in their host, we predict that 1) parasitized populations are more consistent within colonies and more distinct between colonies (i.e. more repeatable) in their recognition compounds than unparasitized populations, and 2) colonies that occur in sympatry with slavemakers are less similar in their recognition profile than those originating from unparasitized populations.

METHODS

Colony collection and maintenance

From May to July 2012, we collected 2033 *Temnothorax longispinosus* and *Protomognathus americanus* ant colonies from nine ant communities in the North-Eastern United States and Canada (table 5.1). To assess slavemaker presence, we collected at least 100 colonies per community, with the exception of Kentucky due to low abundances of our study species at this site. Slavemaker colonies were present in six (KY, MA, NY, OH, VT and WV) and absent in three ant communities (ME, QC and NH). All colonies were transported to the laboratory in Ziploc bags, where they were counted, transferred to artificial nest sites (50 x 10 x 3 mm) and housed in plastered nest boxes (10 x 10 x 3 cm) to prevent desiccation. To control for the effect of nest material or diet

on the chemical profile of workers (Heinze *et al.* 1996; Silverman and Liang 2000), colonies were kept under common garden conditions for three months (constant 25°C - 12 h light: 12 h dark cycle - honey, cricket and water diet) before workers were subjected to the aggression bioassays and chemical analyses.

Colony characteristics and ant samples

From each of the nine *T. longispinosus* populations, we selected 10 (KY, MA, NH, OH and WV) to 15 colonies (ME, NY, QC and VT; table 5.1) with on average (± SD) 20.11 ± 3.36 workers, which is typical for *T. longispinosus* (Foitzik *et al.* 2009). Colony size did not differ between colonies originating from communities where slavemakers were present or absent (Poisson GLMM with population ID as random factor: $Z = 0.210$, $p = 0.834$). Out of the 110 experimental colonies, 80% had a queen(s). Populations that occurred in the absence or presence of slavemakers did not differ in queen presence (binary GLMM: $Z = 1.103$, $p = 0.270$), although colonies were more often polygynous in slavemakers-free populations (binary GLMM: estimate ± s.e.$\Delta = 2.684 \pm 0.560$, $Z = 4.792$, $p < 0.0001$), supporting earlier findings (Foitzik and Herbers 2001; Foitzik *et al.* 2009).

From each of the 110 colonies, we selected 10 focal workers for our aggression bioassays and/or chemical analyses (table 5.1). Since both aggression and cuticular profiles are known to differ between internal and external workers (e.g. Wagner *et al.* 1998), we tried to capture maximum variation by selecting focal individuals in the order in which they left the nest site upon opening it. Thus, for an average colony of 20 workers, every second worker leaving its nest site served as focal individual. Focal workers were isolated from their colony and either stored in separate Petri dishes for at least 30 min prior to the aggression bioassays (focal workers from MA, ME, NH, NY, QC and VT), or immediately separated in glass vials and frozen at -80° C for chemical analyses (focal workers from KY, OH and WV).

Aggression bioassays

To assess worker aggression we subjected each of the 800 focal workers from MA, ME, NH, NY, QC and VT that were selected for subsequent chemical analyses to a standardized aggression assay. Hereto, each focal worker was introduced into an 8 mm ⌀ circular arena containing a randomly chosen non-nestmate, conspecific worker, which originated from the same population as the focal worker. To rule out that variation in opponent aggression affected focal worker aggression, we used freshly frozen rather than live opponents. Because freshly frozen opponents still carry their nestmate recognition cues on their cuticle, worker aggression towards live and dead opponents are correlated in *T. longispinosus* (Modlmeier and Foitzik 2011). During the 3 min 20 s after introduction, we recorded at 10 s intervals the occurrence of aggressive (biting, holding, dragging, mandible spreading and stinging) and non-aggressive interactions (antennating), yielding 20 observations per focal worker. We used a new opponent for each aggression bioassay (i.e. for each focal worker). In between assays, the arenas were cleaned with ethanol which was allowed to evaporate. All aggression experiments were performed blindly (i.e. observers were unaware of the focal worker's population of origin).

For the analyses, we only considered workers that showed aggression at least once. The reason for excluding non-aggressive workers was that the occurrence of aggression not only depends on the worker's recognition ability but also on its internal motivational state, which differs between colony members depending on caste affiliation, age or physiological state. The absolute aggression intensity was defined as the total number of aggressive observations per focal worker, and the relative aggression intensity as the number of aggressive observations out of the total number of contacts between the focal worker and the opponent. After the aggression bioassays, workers were placed individually into glass vials and frozen at -80° C.

Additionally, we assessed the aggressive responses of colonies as a whole, based on data that was previously published but not analyzed in relation to chemical cue diversity (Kleeberg *et al.* 2015; chapter 3). Hereto, we introduced a freshly frozen, non-nestmate conspecific worker into the host nest, one cm away from the nest entrance. Every 30 s for 5 min, we recorded the number of workers that interacted with the non-nestmate, distinguishing between aggressive and non-aggressive interactions. The 273 colonies included in these aggression tests originated from the same populations as those used for the individual aggression and chemistry analyses (table 5.1).

Table 5.1. Collection sites and sample sizes for the nine *Temnothorax longispinosus* populations. Populations depicted in bold co-occur with slavemakers. The number of workers for which we collected aggression and chemistry deviates slightly from 10 x # *Experimental colonies* because 4 (0.5%) workers were injured when setting up the aggression bioassays and 27 (2.45 %) of the chemical samples could not be analyzed.

Population	County	Coordinates		# Collected colonies	# Experim. colonies	# Workers aggression	# Workers chemistry	# Colony aggression
KY - Kentucky	Estill	37°48'13"	083°41'83.3"	67	10	-	100	21
MA - Massachusetts	Middlesex	42°23'98''	071°11'67.9"	109	10	100	97	30
ME - Maine	Oxford	42°23'98''	070°51'35.1"	104	15	149	146	30
NH - New Hampshire	Sullivan	43°26'41"	072°09'34.6"	105	10	98	97	31
NY - New York	Albany	42°31'95"	074°08'75.3"	1153	15	150	145	30
OH - Ohio	Ashtabula	41°50'34"	080°57'55.7"	113	10	-	98	33
QC - Quebec	Montreal	45°30'37"	073°36'94.7"	111	15	150	146	31
VT - Vermont	Addison	43°58'25"	073°04'31.6"	118	15	149	145	31
WV - West Virginia	Pocahontas	38°06'48"	080°07'88.2"	153	10	-	99	36

Chemical methods

For the extraction of cuticular hydrocarbons we immersed each of the 1100 focal workers in ~0.5 ml hexane for 10 min, after which the worker was removed and vials were sealed and stored at -20°C. Samples were analyzed on 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). They were injected (5 µl) in splitless mode over 2 min at 250°C, using helium as carrier gas (flow rate =1.2 ml/min). Oven temperature was programmed at 150°C for 3 min, then increased via 250°C (+30°C/min) to 300°C (+2°C/min) and held constant for 2 min. After an initial solvent delay of 5 min, a mass range of 40-500 amu was recorded at an ionization voltage of 70 eV. To quantify the relative amounts of cuticular hydrocarbons, we integrated peak areas using MSD ChemStation E.02.02 (Agilent). For each population, we additionally pooled and analyzed five samples, originating from five randomly selected colonies. These pooled samples were used to characterize cuticular hydrocarbons based on their retention times (in comparison to known reference standards) and diagnostic ions. Only substances that were present at a relative abundance of >1% in at least one pooled population sample were included in our analyses.

Identification of recognition compounds

In total, we identified 31 hydrocarbons (or combinations of co-eluting compounds), consisting of *n*-alkanes, methyl-branched alkanes and two unidentified compounds. To assess which of these substances are involved in enemy recognition we evaluated their effect on the aggression intensity of *T. longispinosus* workers. We first calculated the Bray-Curtis similarity in cuticular hydrocarbon profiles between each pair of workers, originating from the same population but different colonies. For each worker, this score was then averaged across all comparisons, yielding one score per worker that represents its average chemical similarity to other colonies from its population of origin. The relationship between a worker's chemical similarity score and its aggression intensity was then analyzed using Generalized Linear Mixed Models with penalized quasi-likelihood parameter estimation (glmmPQL function, implemented in the MASS library; Venables and Ripley 2002). The aggression intensity was fitted as dependent variable, the chemical similarity score as fixed predictor and colony ID, nested in population ID as random factor. In the first step, we calculated the chemical similarity based on all 31 compounds and recorded the effect size of that chemical similarity score on aggression intensity. In subsequent steps, we sequentially removed each substance from the chemical similarity calculation and assessed the change in effect size on the aggression intensity. The substance whose removal yielded the strongest increase in effect size was excluded and the previous step was repeated, until the removal of any further substances decreased rather than increased the effect size on aggression. Using this backwards-stepwise selection procedure, we identified a subset of compounds that likely encode recognition cues given their involvement in aggression. Those substances that showed involvement in the analyses of both the relative (quasi-binomial GLMM with logit-link function) and the absolute aggression intensity (quasi-Poisson GLMM with logit-link function) were characterized as putative recognition cues and included in the

comparison of chemical variability between parasitized and unparasitized *T. longispinosus* populations.

The use of the same workers to test aggressive responses and the chemical similarity between non-nestmates allowed us to directly link behavior to cuticular hydrocarbon variability. However, contact with the opponent during the aggression assay could result in the transfer of hydrocarbons to the focal worker, potentially eroding the chemical similarity within colonies or homogenizing the profile between colonies. The chemical similarity between nestmates did however not decrease with the contact frequency between focal workers and opponents (Spearman's ρ = -0.020, $S = 7.3 \cdot 10^6$, p = 0.578), nor did the chemical similarity between non-nestmates increase with contact frequency (Spearman's ρ = -0.046, $S = 7.5 \cdot 10^6$, p = 0.207). More importantly, workers from unparasitized populations did not have more contact with the opponent (quasi-Poisson GLMM with colony ID, nested in population ID as random factor: t_4 = 0.678, p = 0.535), which makes a biased transfer of hydrocarbons to workers originating from unparasitized populations an unlikely explanation for the predicted lower chemical similarity between colonies in the presence of slavemakers.

To confirm that the putative recognition compounds identified based on the individual aggression tests were also involved in colony aggression, we assessed the relationship between a colony's aggressive responses and the average chemical similarity between colonies in their population of origin. For the chemical similarity calculation we either included all 31 hydrocarbons or the subset of putative recognition cues identified based on individual aggression. The total number of aggressive versus non-aggressive interactions was analyzed using quasi-binomial GLMMs with logit-link function (glmmPQL; Venables and Ripley 2002) and population ID as random factor.

Variability in recognition profiles and slavemaker presence

To test the hypothesis that *T. longispinosus* populations that occur in the presence of slavemakers show higher chemical cue diversity between, but not within colonies, we used two complementary approaches, based on 1) the putative recognition compounds and 2) the overall chemical similarity in recognition profiles. For the first approach, we decomposed the variance in each of the putative recognition compounds into variance explained by population ID, colony ID and individual ID. The rationale is that if recognition cues are consistent within colonies, most variation will be explained at the colony level and little variation at the individual level. Likewise, if recognition cues are distinct between colonies, most variation will be explained at the colony level and little variation at the population level. Hence, we predict that the colony-level repeatability (colony ID variance component / $\sum$ variance components) in parasitized populations is larger than that in unparasitized populations. Specifically, we fitted the logit-transformed, relative amount of each putative recognition compound as dependent variable, the intercept as fixed factor and individual ID, nested in colony ID, nested in population ID as random factors using Linear Mixed Models (LMM; lme function implemented in the nlme package; Pinheiro *et al.* 2013). We obtained separate variance components for populations that occurred in the presence or absence of slavemakers (syntax: random = ~slavemaker presence−1|population ID/colony ID/individual ID).

Table 5.2. The 31 hydrocarbons or co-eluting compounds in the cuticular profile of *Temnothorax longispinosus* workers, including their relative abundance and their effect on aggression. Effect sizes were calculated based on the GLMM estimates of the Bray-Curtis similarity between non-nestmates on the aggression intensity. The change in effect size represents the proportional increase in effect size upon inclusion of a substance in the chemical similarity calculation. Compounds in bold were identified as putative recognition cues because they increased the effect size in the analyses of both the absolute (Abs. aggr) and relative aggression intensity (Rel. aggr).

Substance	Rel. abundance mean ± s.e. (%)	Δ Effect size (%) Abs. aggr	Rel. aggr
n-C25	2.01 ± 0.21	-4.31	-1.90
n-C26	1.14 ± 0.10	-0.95	-2.32
n-C27	38.40 ± 2.15	-59.96	-36.24
5-MeC27	0.50 ± 0.07	**0.99**	**0.74**
3-MeC27	2.74 ± 0.20	**2.94**	**5.79**
n-C28	2.33 ± 0.14	-1.91	0.93
4-MeC28	0.61 ± 0.15	**4.81**	**0.54**
n-C29	16.35 ± 1.17	-68.09	-33.79
11/13/15/17-MeC29	1.27 ± 0.20	-1.02	-5.30
7-MeC29	0.87 ± 0.10	-2.67	-3.92
5-MeC29	1.19 ± 0.13	**5.11**	**12.10**
11,21-;15,19-;13,17-; 11,15-DiMeC29	0.71 ± 0.21	-7.52	-6.99
3-MeC29	7.23 ± 0.28	**20.19**	**26.45**
n-C30	0.74 ± 0.08	-5.88	-7.02
unknown	0.98 ± 0.12	-2.82	-1.28
4-MeC30	0.51 ± 0.12	0.21	-1.09
n-C31	2.42 ± 0.20	-8.56	-7.93
7-MeC31	0.58 ± 0.14	**0.96**	**1.04**
5-MeC31	0.07 ± 0.07	-2.75	-2.38
11,15-DiMeC31	5.43 ± 0.89	**18.39**	**17.83**
3-MeC31	3.23 ± 0.27	**1.68**	**0.62**
5,11-DiMeC31	1.07 ± 0.31	-1.01	2.02
unknown	1.39 ± 0.18	-10.89	-8.23
11;13;15;17-MeC33	1.21 ± 0.20	-1.65	-2.83
15,19-;13,17-; 11,15-DiMeC33	3.34 ± 0.57	-17.15	-18.38
5,17-DiMeC33	1.53 ± 0.32	**3.11**	**1.76**
11,15,19,23-TetraMeC33	0.19 ± 0.10	-5.28	-7.78
3,7,11-TriMeC33	0.72 ± 0.14	1.08	-5.72
11-;13-MeC35	0.33 ± 0.09	-9.36	-11.83
11,21-;11,23-; 13,23-DiMeC35	0.27 ± 0.20	-10.53	-5.51
5,11-;5,17-DiMeC35	0.60 ± 0.19	-5.25	1.32

For the second approach, we compared the chemical similarity in recognition profiles between parasitized and unparasitized populations at two levels of biological organization: within colonies and between colonies. We calculated the Bray-Curtis similarity between each pair of focal workers based on the putative recognition compounds. For each colony, this score was then averaged across comparisons within colonies and between colonies, representing 1) the average similarity between nestmates, and 2) the average similarity between non-nestmates. We used a LMM (Pinheiro *et al.* 2013) with the average similarity as dependent variable. Comparison type (within colony / between colony), parasite presence (present / absent) and their interaction served as fixed predictors. Colony ID, nested in population ID, was included as random factor to account for pseudo-replication. All analyses were performed in R version 3.1.1 (R Core Team 2014)

RESULTS

Identification of recognition compounds

Worker aggression was unrelated to the chemical similarity between non-nestmates when taking all 31 cuticular hydrocarbons into account (quasi-binomial GLMM: t_{373} = 0.461, p = 0.645). However, aggression did decrease with increasing chemical similarity based on a subset of these compounds (estimate ± s.e. = -2.989 ± 0.843, t_{373} = -3.546, p < 0.001). Specifically, our substance selection procedure identified nine chemical compounds involved in worker aggression, since the chemical similarity between non-nestmates based on these nine putative recognition compounds yielded the strongest effect size on the aggression intensity of *T. longispinosus* workers (table 5.2). In particular, the inclusion of 3-MeC29, 11,15-DiMeC31 and 5-MeC29 into the chemical similarity calculation increased the effect size on the relative aggression intensity by 26.4, 17.8 and 12.1%, respectively, whereas the effect of the remaining six substances was only 1.7% on average (table 5.2). None of the seven *n*-alkanes in the cuticular hydrocarbon profile of *T. longispinosus* workers appeared to serve as recognition cue, whereas seven out of 13 mono-methyl alkanes and two out of seven di-methyl alkanes were identified as putative recognition compounds. Similar to the aggressive responses of individual workers, we found that colony-level aggression decreased with increasing average chemical similarity between colonies, based on the nine putative recognition cues (quasi-binomial GLMM: estimate ± s.e. = 10.396 ± 2.825, t_7 = 3.680, p = 0.008), but not based on all 31 compounds (t_7 = 0.973, p = 0.363).

Recognition cue variability and slavemaker presence

Out of the nine putative recognition compounds, seven showed a stronger colony-specific signal in parasitized compared to unparasitized *T. longispinosus* populations (i.e. $\text{repeatability}_{\text{parasitized}} > \text{repeatability}_{\text{unparasitized}}$; figure 5.1). Comparing variance components across these nine compounds showed no difference between parasitized and unparasitized populations in the intra-colonial (Wilcoxon signed-rank test: individual ID var. comp: V = 8, n = 9, p = 0.098) or population-level variance component

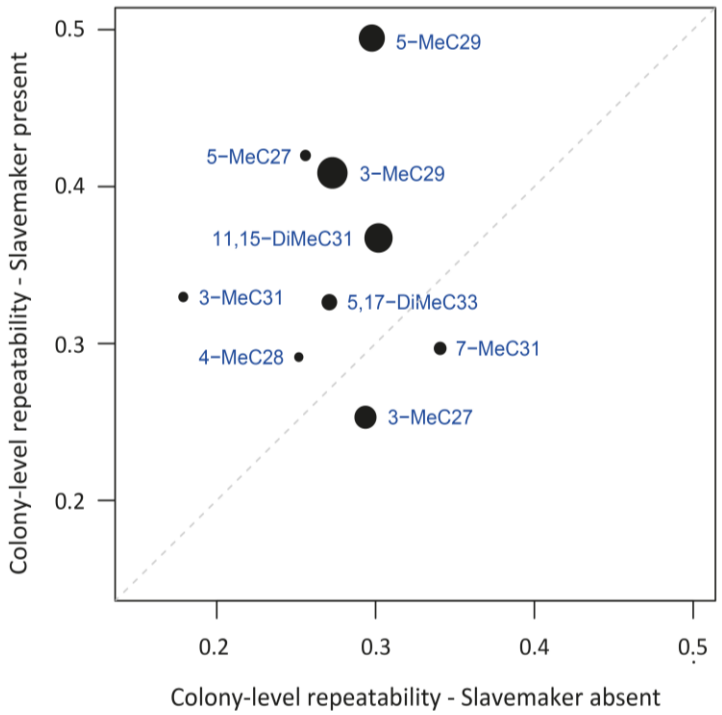

Figure 5.1. The colony-level repeatability in putative *Temnothorax longispinosus* recognition compounds in relation to slavemaker presence in the ant community of origin. Symbol sizes are proportional to the change in the effect size of chemical similarity on the relative aggression intensity (table 5.2; i.e. log(Δ effect size)+1).

(V = 16, n = 9, p = 0.496). However, we find a stronger colony identity in recognition compounds in the presence of slavemakers (colony ID var. comp: V = 2, n = 9, p = 0.012).

As predicted, the chemical similarity based on the nine putative recognition compounds between colonies was lower in the presence of slavemakers (figure 5.2; t_7 = -4.210, p = 0.004). Parasitized and unparasitized populations showed equal levels of within-colony chemical similarity (t_7 = -1.154, p = 0.286). Hence, we found a significant interaction effect of slavemaker presence and level of organization ($F_{1,108}$ = 18.544, p < 0.0001). Not surprisingly, the chemical similarity within colonies was higher than that between colonies ($F_{1,108}$ = 683.821, p < 0.0001), in both parasitized (t_{108} = 23.457, p < 0.0001) and unparasitized populations (t_{108} = 12.333, p < 0.0001).

Discussion

When parasites break their host's nestmate recognition code, hosts are expected to shift their recognition cues away from that of the original host, targeted by mimetic parasites. Such recognition cue diversification through negative frequency-dependent selection favors rare host phenotypes and renders it impossible for parasites to match the recognition profile of all potential hosts. In line with this hypothesis, we demonstrate that *Temnothorax* hosts exposed to slavemakers in the field show a stronger colony

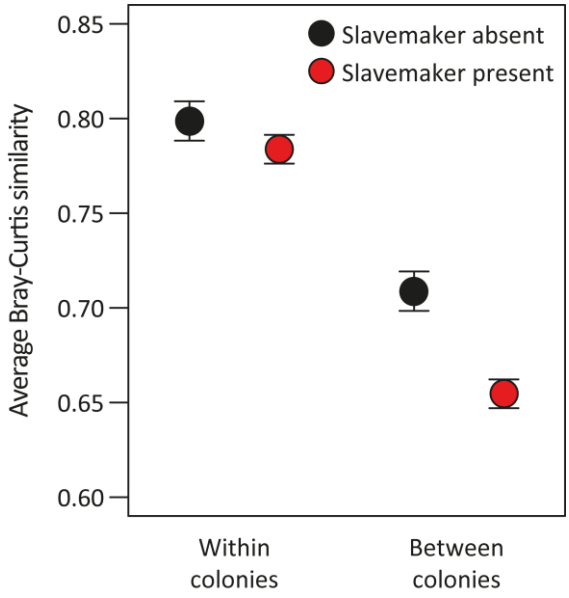

Figure 5.2. Chemical similarity between *Temnothorax longispinosus* nestmates (within colonies) and non-nestmates (between colonies) based on nine putative recognition compounds. Symbols represent the LMM estimates ± s.e. of the Bray-Curtis similarity, averaged per colony. Bray-Curtis similarity scores from colonies that co-occur with slavemakers are depicted in red, those from colonies originating from unparasitized populations in black.

identity in recognition compounds and a larger differentiation in recognition profiles between colonies, compared to host colonies from unparasitized populations. Importantly, the differences between parasitized and unparasitized populations in recognition cue diversity appear to be regulated by processes that specifically act upon the level of the colony, as neither the chemical variability within colonies nor that between populations was associated with slavemaker presence. This rules out potentially confounding effects on cuticular hydrocarbon profiles, such as consistent differences in abiotic conditions or standing genetic variation between parasitized and unparasitized populations. Instead, our findings indicate that slavemakers drive recognition cue diversity in their ant hosts, in much the same way that avian hosts appear to have diversified their egg appearance in response to brood parasite pressure (Øien *et al.* 1995; Spottiswoode and Stevens 2011).

Our findings corroborate with previous work on the role of temporary social parasites in the evolution and maintenance of recognition cue diversity in *Formica fusca* ants (Martin *et al.* 2011). By comparing two highly parasitized Finnish populations with two almost unparasitized UK populations, Martin *et al.* (2011) demonstrate higher diversity in chemical recognition cues when social parasites are common (i.e. in the Finnish populations). While *F. fusca* is targeted by a large variety of social parasitic wood ant species, none of our nine sampling populations harbored any other socially parasitic species than *P. americanus* slavemakers. Hence, our findings suggest that a single slavemaker species can promote the evolution and/or maintenance of recognition cue diversity in its host. The coevolutionary arms race between a single host and parasite

species provides interesting opportunities to further elucidate the chemical adaptations involved in recognition in the hosts of social parasites.

We can rule out that differences between parasitized and unparasitized populations in the recognition cue and profile variability are driven by intra-colonial variation. Indeed, we do not find lower intra-colonial variation in the putative recognition cues in parasitized populations, nor do colonies that co-occur with slavemakers show lower chemical similarity in recognition profiles. The maintenance of such strong colony identity in the presence of large recognition cue diversity between colonies allows for accurate nestmate recognition and is likely achieved through the continuous exchange of compounds within colonies (Stuart 1988; Soroker *et al.* 1995; Van Zweden and D'Ettorre 2010). Conveniently, the similarity in intra-colonial variability also negates the potentially confounding effect that colonies were more often polygynous in unparasitized populations, since polygyny would affect intra-colonial, not inter-colonial variation. At any rate, there is little empirical evidence that genetic variation correspond to changes in a colony's recognition cue variability (Martin *et al.* 2009; Helanterä *et al.* 2011).

Aggression bioassays have proven particularly useful to test for the role of hydrocarbons in nestmate recognition (Lahav *et al.* 1999; Wagner *et al.* 2000; Dani *et al.* 2001; Akino *et al.* 2004; Dani *et al.* 2005; Greene and Gordon 2007; Martin, Vitikainen, *et al.* 2008; Guerrieri *et al.* 2009), but, to our knowledge, have not been applied to identify recognition cues among the full complement of hydrocarbons present on the cuticle. Our study combined the biological relevance of aggression bioassays with multivariate statistical approaches to identify putative recognition cues. We did not alter the structural complexity of the cuticular hydrocarbon profile, but merely assessed which compounds present on the cuticle of workers were associated with aggression. Hence, our approach accounted for the notion that information on colony membership may not be encoded in isolated components, but rather in the mixture of structural classes found in the cuticular hydrocarbon profile (Greene and Gordon 2007). Despite the intuitive appeal of aggression bioassays, however, many different methods are currently applied. Although variation in methodology may be imposed by species-specific responses towards intruders, methods do greatly differ in their ability to detect aggression, depending in particular on sample size and context (Roulston *et al.* 2003). Therefore, our study combined the benefits of the large replication in our individual aggression bioassays with the more natural context of aggression by colonies as a whole. Both these methods showed a decrease in aggression with the chemical similarity between non-nestmates, based on the nine putative recognition cues, but not on the full hydrocarbon profile (see also Martin *et al.* 2012).

Previous studies on ants have suggested that, of the three main hydrocarbon classes, alkenes and methyl-branched alkanes are more often used in nestmate recognition than linear alkanes (Akino *et al.* 2004; Martin, Helanterä, *et al.* 2008). Our recognition cue identification support these earlier findings since none of the linear alkanes were implicated in aggression. Instead, we identified nine mono- and di-methyl alkanes as putative recognition compounds in *T. longispinosus* - a species that lacks alkenes in its

cuticular hydrocarbon profile. Among these putative recognition compounds, three in particular were involved in aggression, including 3-MeC29, 5-MeC29 and 11,15-DiMeC31. In the carpenter ant, *Camponotus aethiops*, 5-MeC29 was likewise implicated in nest mate recognition, as were several 11,15-dimethylalkanes (Van Zweden *et al.* 2009). Interestingly, the latter are the most commonly produced dimethylalkanes among ants and also the 3- and 5-monomethylalkanes are found in the majority of ant species studied to date (Martin & Drijfhout 2009). The ubiquity of those compounds we identified as putative recognition cues sets them apart as interesting candidates for enemy recognition in other species.

In conclusion, our study provides empirical support that slavemakers are a driving force in the evolution and maintenance of recognition cue diversity. Such diversity likely aids host colonies in distinguishing nest mates from slavemakers or conspecific slaves, as well as non-nestmates from free-living host colonies. Indeed, a previous study showed that colony aggression towards conspecifics increased with parasite pressure (Kleeberg *et al.* 2015; chapter 3), which is likely the result of the larger inter-colonial variation in host recognition cues reported here. The fact that parasite-free populations are less chemically diverse and less aggressive towards non-nestmates, suggests that competition among conspecific colonies is not as potent a driver of recognition cue diversity as slavemakers. Hence, our findings support theory predicting that external selection pressures such as parasites are needed for the evolution and maintenance of recognition cue diversity (Crozier 1986; Gardner and West 2007; Rousset and Roze 2007).

Both authors designed the study and contributed to the manuscript. EJ collected and analysed the data. Ant collection permits were 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.

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CHAPTER 6

Fitness Costs of Worker Specialisation for Ant Societies

Evelien Jongepier & Susanne Foitzik

Submitted

ABSTRACT

Division of labour is of fundamental importance for the success of societies, yet little is known about how individual specialisation affects the fitness of the group as a whole. While specialized workers may be more efficient in the tasks they perform than generalists, they may also lack the flexibility to respond to rapid shifts in task needs. Such rigidity could impose fitness costs when societies face dynamic and unpredictable events, such as an attack by socially parasitic slavemakers. Here, we experimentally assess the colony-level fitness consequences of behavioural specialisation in *Temnothorax longispinosus* ants that are attacked by the slavemaker ant *Protomognathus americanus*. We manipulated the social organization of 102 *T. longispinosus* colonies, based on the behavioural responses of all 3842 workers. We find that strict specialisation is disadvantageous for a colony's annual reproduction and growth during slave raids. These fitness costs may favour generalist strategies in dynamic environments, as we also demonstrate that societies exposed to slavemakers in the field show a lower degree of specialisation than those originating from slavemaker-free populations. Our findings provide an explanation for the ubiquity of generalists and highlight their importance for the flexibility and functional robustness of entire societies.

Keywords: Behavioural specialisation; Division of labour; Dynamic conditions; Colony fitness; Social insects; Slavemaker ants

INTRODUCTION

Division of labour into specialized units is a highly successful strategy, found at all levels of biological organization. It is associated with the major evolutionary transitions, such as those from prokaryotes to eukaryotes, single cell to multi-cellular organisms and solitary to social life (Szathmaty and Maynard Smith 1995). The early economist Adam Smith proposed that societies in which each worker specializes on a subset of the tasks capitalize on increased individual efficiency and avoid the costs of task switching (Smith 1776). These benefits however fail to explain the enormous variation in division of labour observed in nature. Ant colonies typically face between 20 and 40 different tasks, yet the maximum number of morphologically differentiated worker castes is only three, and 80% of the ant genera lack such physical worker castes altogether (Oster and Wilson 1978). Even among these monomorphic species, the number of tasks greatly exceeds the number of distinct behavioural castes, which is rarely more than six (Oster and Wilson 1978). Despite this variation, little is known about the constraints on division of labour and how it affects the fitness of societies as a whole. Indeed, over 35 years ago, Oster and Wilson highlighted that the relationship between division of labour and fitness awaits empirical testing (Oster and Wilson 1978), and little progress has been made since (Duarte *et al.* 2011; Jeanson and Weidenmüller 2014).

Empirical evidence shows that the presumed advantage of behavioural specialisation for individual efficiency is not universal, even in ants, the exemplars of division of labour (Dornhaus 2008). Moreover, the adaptive significance of division of labour has been repeatedly questioned on theoretical grounds, since specialization is thought to constrain a worker's flexibility in task performance, and hence the ability of societies to cope with change (Tofts and Franks 1992; Bourke and Franks 1995). Indeed, the major factors underlying worker specialisation, such as morphology, genes or age (Oster and Wilson 1978; Hölldobler and Wilson 1990; Page and Robinson 1991), only enable societies to respond to environmental change over relatively long time-spans, sometimes generations. This is hard to reconcile with the need to respond to short-term fluctuations in task needs. Theoretical models indeed predict that selection for flexibility and functional robustness under dynamic conditions counter-acts the evolution of division of labour (Wakano *et al.* 1998; Waibel *et al.* 2006; Rueffler *et al.* 2012). Yet, an ecological perspective on the social organization of insect colonies is largely missing from the empirical literature (Gordon 2014).

Here, we experimentally assess the colony-level fitness consequences of behavioural specialisation in *Temnothorax longispinosus* ants during an attack by the slavemaker ant *Protomognathus americanus*. To exploit the work force of its *Temnothorax* hosts, this social parasite conducts slave raids on neighbouring host colonies, capturing brood and frequently killing adult workers and the queen (Wesson 1939; Alloway 1979; Foitzik *et al.* 2001). Slave raids thus impose severe costs in terms of the host's annual reproductive success and the potential for colony survival and growth; the three main fitness components of insect societies. Importantly, slave raids are representative of dynamic conditions frequently faced by societies, causing dramatic changes in task needs

(supplementary material; video S1). Since a failure to respond likely results in severe fitness losses, flexibility in task performance is expected to confer the largest fitness benefits to *T. longispinosus* colonies under slavemaker attack. We therefore test experimentally whether colonies composed of specialised workers suffer higher fitness losses during slave raids than colonies composed of less specialized workers. In addition, we use a population-comparative approach to investigate whether natural levels of worker specialisation are related to slavemaker presence in the local ant community.

METHODS

Experimental study design

We manipulated the social organization of 102 *T. longispinosus* colonies, based on the behavioural responses of all 3842 workers. Mechanistically, division of labour is often explained by response threshold models, which assume that workers differ in their intrinsic responsiveness towards stimuli, determining the likelihood that they perform the associated tasks (Bonabeau *et al.* 1996; Beshers and Fewell 2001). To assess worker specialization, we therefore tested their responsiveness towards two types of stimuli: an opponent and a brood stimulus (figure 6.1a). These stimuli were chosen because they are associated with the two main, fitness-determining tasks during slave raids: nest defence and brood evacuation (Foitzik *et al.* 2001). Workers were grouped into four categories, based on whether they were more responsive than the average response of their colony towards the brood stimulus (*brood-responsive*), the opponent stimulus (*opponent-responsive*), neither of the stimuli (*unresponsive*), or both stimuli (*both-responsive*; figure 6.1b). We established generalist colonies by removing workers responding to only one stimulus type and specialist colonies by removing workers responding to neither or both stimuli (figure 6.1c). One day after the manipulation of colony composition, each experimental colony was exposed to a slavemaker colony (figure 6.1d). A major

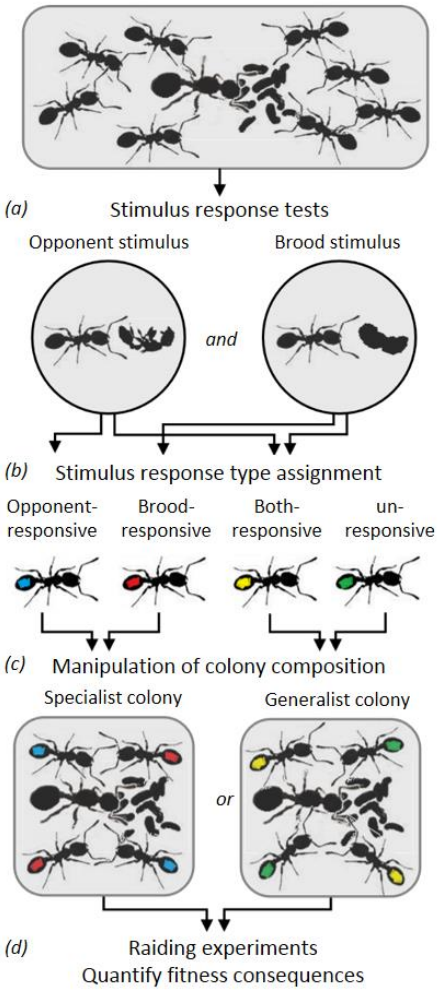

Figure 6.1. Experimental procedure. (*a*) Shortly before the emergence of a new, annual worker generation, each *Temnothorax longispinosus* worker was subjected to an opponent and a brood stimulus test, (*b*) based on which individuals were assigned to one of four response groups. Arrows indicate that workers were more responsive than their colony's average response. (*c*) Specialist colonies were established by the removal of *both-responsive* and *unresponsive* group members, generalist colonies by the removal of *opponent-* and *brood-responsive* group members (see methods section for further details). (*d*) The fitness consequences of colony composition were assessed during raiding experiments.

experimental benefit of slave raids is that fitness losses become immediately apparent and can therefore be quantified before workers change their behavioural profile following experimental manipulation of the composition of their colony.

Colony collection and maintenance

In June 2013, we collected 159 *Temnothorax longispinosus* ant colonies containing 30 to 60 workers and 100 *Protomognathus americanus* colonies with three to ten slavemaker workers at the E. N. Huyck preserve, Rensselearville New York, USA (42°19'10", -074°5'15"). Colonies were transported to the laboratory, where they were counted, transferred to artificial nest sites and housed in plastered boxes to prevent desiccation. Prior to the experiments, *T. longispinosus* colonies were kept at a constant 14° C and a 12h:12h light / dark cycle to delay brood development. Five days before the stimulus response tests, they were gradually acclimatized to the experimental temperature regime of 27° C. *Protomognathus americanus* slavemaker colonies were kept at a constant 25° C and a 12h:12h light / dark cycle until the start of the raiding experiments, when temperatures were increased to 27° C to promote raid willingness. Colonies kept at temperatures below 20° C were fed weekly, those kept at higher temperatures twice a week, with honey and crickets. For our experiments, we selected 102 *Temnothorax longispinosus* colonies with 37.6 ± 7.6 (mean ± s.d.) workers.

Stimulus response tests

One day prior to the raiding trials, each *T. longispinosus* worker was isolated and subjected to a standardized opponent and brood stimulus test (figure 6.1a) (Modlmeier *et al.* 2012). For the opponent stimulus test, we introduced a freshly frozen, non-nestmate worker into an 8 mm ø circular arena with the focal worker. We recorded the occurrence of aggression (i.e. biting, holding, dragging and stinging) every 10 seconds for 3 min 20. We chose a dead opponent to exclude effects of variation in opponent aggression on the responsiveness of the focal ant. An earlier study confirmed that responses towards live opponents were correlated with those towards freshly frozen, dead opponents (Modlmeier and Foitzik 2011). Opponents originated from the same population but a different collection site as the focal ant to ensure that focal ants were not tested against a colony member from a polydomous nest part. Each opponent was used only once.

For the brood stimulus test, we introduced a focal ant into the arena with a worker pupa from its own colony, and recorded the occurrence of grooming or pupa-carrying every 10 seconds for 3 min 20. Brood in the pupal stage undergoes restructuring during which it cannot actively signal its needs or be fed. This precludes that variation in begging intensity of the brood affected the focal ant's responsiveness to the brood stimulus. If there were fewer worker pupae than workers in a colony, we re-used pupa in the brood stimulus tests. The average responsiveness to the brood stimulus did not differ between colonies where pupae were re-used or not ($F_{1,100} = 1.96$, $p = 0.165$). Stimuli were provided in the same order as they are perceived by workers during a raid (i.e. the opponent stimulus test preceded the brood stimulus tests with a 30 min. interval).

In total, we recorded 20 observations per worker per stimulus, based on which we grouped each worker into one of four response groups (figure 6.1b). For each experimental colony, the *opponent-responsive* group consisted of workers that were more responsive to the opponent stimulus, but less responsive to the brood stimulus, than the average colony responsiveness. Visa versa, the *brood-responsive* group consisted of workers that were above averagely responsive to the brood stimulus but below averagely responsive to the opponent stimulus. Workers with a lower or higher than average responsiveness to both stimuli were assigned to the *unresponsive*- or *both-responsive* group, respectively. Workers were marked on the gaster with edding® 750 paint marker in accordance with their response group. Colours were randomized between colonies to allow for our blind experimental design.

Manipulation of colony composition

Prior to the stimulus response tests, colonies were randomly assigned to either specialist (n = 50) or generalist (n = 52) colony type treatment. Colony composition was manipulated by excluding *opponent*- and *brood-responsive* group members from generalist colonies, or *unresponsive*- or *both-responsive* group members from specialist colonies (figure 6.1c). The reason we also removed the *unresponsive*-group from specialist colonies was not because these workers were considered to be generalists, but because a pilot study showed that this approach equalized the average responsiveness towards the two stimuli in the two colony treatment groups (see supplementary material for pre- and post-manipulation colony characteristics). The number of excluded workers (mean ± s.d. = 25.8 ± 5.2%) equalled twice the number of workers of the smallest response group. If this resulted in only the partial removal of a response group, we excluded those workers that showed the most extreme responsiveness in the stimuli tests. For instance, if the smallest response group of the original colony contained 5 workers, then we established a generalist colony by excluding from the *opponent-responsive* group the 5 workers that were most responsive to the opponent stimulus and from the *brood-responsive* group the 5 workers that were most responsive to the brood stimulus. Alternatively, to establish a specialist colony, we excluded from the *both-responsive* group the 5 workers with the strongest bivariate response and from the *unresponsive* group, the 5 workers with the weakest bivariate response. This way, we avoided that natural variation in the relative abundance of the different response groups caused consistent biases in experimental colony sizes between generalist and specialist colonies.

For each colony, we calculated a $D_{y|x}$ index (Gorelick *et al.* 2004) before and after manipulation. Although the $D_{y|x}$ index was developed to quantify division of labour among members of a social group, we apply it to reflect worker specialisation in their responses towards the two stimuli. Hence, an index value of 1 represents maximal specialisation, where each worker in the colony is responsive to only a single stimulus type, whereas a value of 0 means that each worker is equally responsive to both stimuli. This index is invariant to the number of workers in a colony and thus allowed for direct comparisons despite variation in colony size (Gorelick *et al.* 2004).

Manipulation of colony composition resulted in a 39.8 ± 2.8% lower $D_{y|x}$ index for generalist colonies compared to specialist colonies (t-test: t_{100} = 14.36, p < 0.0001; supplementary figure S6.1). Manipulation did not affect the level of behavioural variation within colonies, nor was intra-colonial behavioural variation related to the fitness outcomes of the raiding experiments described below (See supplementary material).

Raiding trials

One to three days after manipulation, a *T. longispinosus* and a slavemaker colony were positioned in opposite corners of a 30 cm equilateral triangular arena. Each *T. longispinosus* colony was only allowed to be raided once. In total, we staged 165 trials resulting in 77 raids: 55 raids on the first day, 13 on the second and 9 on the third. Including trial day as random factor did not explain significant variation in colony fitness (all p > 0.9). At the onset of a raid, we provided an escape nest at a distance of 15 cm from the *T. longispinosus* nest. Since the confines of the arena prevented colonies from relocating outside the raiding radius of the slavemaker, raiders were physically restrained from attacking the escape nest by the observer. Successful trials were terminated one hour after the last brood item was removed from the *T. longispinosus* nest site, at which point the slavemaker colony and any slavemakers inside the arena were removed. Trials that did not result in a raid were terminated at 18:00 h by returning the *T. longispinosus* and slavemaker colony, as well as any of their workers in the arena, to their own, separate nest boxes.

Verification that stimulus responsiveness reflects task performance

To confirm that stimulus responsiveness reflected task performance in the colony context during the experiments, we scored the number of workers per stimulus response group that showed aggression towards slaves and slavemakers in the arena. This scan sampling was repeated every 30 minutes up to the start of the raiding attack, yielding on average (± s.d.) 9.20 ± 9.00 scans for each of the 71 observed *T. longispinosus* colonies. We confirmed that workers that were above averagely responsive towards the opponent stimulus were also more likely to attack slaves and slavemakers when their colony was exposed to a slavemaker colony (supplementary figure S6.2). Hence, stimulus responsiveness of individual workers outside the colony context, reflected task performance in the colony context.

Fitness consequences of colony composition

One day after a raiding trial we counted the number of sexual and worker brood items that were saved by the *T. longispinosus* colony or lost to the slavemakers; the number of *T. longispinosus* worker and queen fatalities/survivors; and the number of fatalities among slavemaker colony members. This one day interval was chosen because many workers were still engaged in fights with slaves when a raiding trial was terminated, which often resulted in the death of combatants. We further recorded the time it took workers from *T. longispinosus* colonies to carry their first five brood items into the escape nest, once discovered, based on which we calculated the brood saving speed. Because not

all raids were continuously observed when multiple raids occurred simultaneously, brood-saving speed was documented for a random subset of the raids (n = 28).

We used Generalized Linear Models (GLM) with colony type as fixed predictor for the analyses of the relative number of saved brood items and worker fatalities (quasi-binomial GLMs with a logit-link function), as well as the likelihood that a queen died (binary GLM – logit-link). The √-transformed number of fatalities among slavemaker colony members and the log-transformed brood saving speed were analysed with t-tests. Residual distributions did not deviate significantly from normality (Kolmogorov-Smirnov tests). Where appropriate, we used Jack-knife methods to assess the effect of influential data points, which did not yield qualitatively different results. We used R v3.1.1 (R Core Team 2014).

Worker specialisation and slavemaker sympatry

We further assessed whether the predicted colony-level fitness costs of worker specialisation during slave raids favour generalist strategies in the field. From each of six *T. longispinosus* populations (figure 6.3a) we selected 10 (MA and NH) to 15 (ME, NY, QC and VT) colonies. This was a subset of the minimally 100 colonies collected per community in June 2012 (Jongepier *et al.* 2014; chapter 2), based on which we determined slavemaker prevalence. Since there was little variation in prevalence in parasitized populations (figure 6.3a), we used slavemaker presence / absence in the analyses. To control for potential effects of prior experience with slavemakers in the field, colonies were collected shortly before the annual raiding season in summer and maintained under common garden conditions for three months. Because the sole reason of *P. americanus* workers to leave their nest is to perform a raid, *T. longispinosus* colonies only encounter slavemakers in their natural habitat during the two month raiding season. Hence, the older worker generation could not have encountered slavemakers for over a year, whereas the young worker generation (which emerged after collection) did not have prior contact with slavemakers at all. Any differences in the organization of work between populations is therefore likely driven by local adaptation rather than plasticity.

From each colony we selected 10 focal workers, which were subjected to an opponent and a brood stimulus test as described above. To capture maximum behavioural variation, focal individuals were selected sequentially in the order in which they left the nest site upon opening it (e.g. for an average ± s.d. colony of 20.0 ± 3.8 workers, every second worker served as focal individual). Based on the two stimulus response tests, we calculated the $D_{y|x}$ index per colony (Gorelick *et al.* 2004), which increases with the level of worker specialisation in stimulus responsiveness.

Colony size did not differ between colonies originating from communities where slavemakers were present or absent (Poisson GLMM with population ID as random factor: $\chi^2_1 = 0.51$, $p = 0.474$). Out of the 80 experimental colonies, 87.5% had a queen(s). Populations that occurred in the absence or presence of slavemakers did not differ in queen presence ($\chi^2_1 < 0.01$, $p > 0.999$), although colonies were more often polygynous in slavemakers-free populations (binomial GLMM with population ID as random factor: estimate ± s.e.Δ = -2.10 ± 0.57, z = -3.70, $p < 0.001$; see also (Foitzik *et al.* 2009)).

We used a linear mixed model (Pinheiro *et al.* 2013) including the $D_{y|x}$ index per colony as dependent variable, slavemaker presence as binary predictor, population ID as random factor and poly- vs monogyny as a binary covariate. We further fitted latitude (LR = 9.43, p = 0.002) and longitude (LR = 0.26, p = 0.605) of the collection sites as covariates in the initial model, but only the former was retained because the $D_{y|x}$ index decreased with latitude (t_3 = -3.74, p = 0.033; i.e. northern populations showed a lower level of worker specialisation).

RESULTS

Fitness consequences of colony composition

We found no difference between generalist and specialist colonies in the likelihood that they were raided (71 and 80%, respectively; χ^2-test: χ^2 = 0.65, p = 0.419). However, generalist colonies that were attacked by slavemakers saved a larger fraction of their sexual (figure 6.2a; GLM: t_{51} = 2.75, p = 0.008) and worker brood (figure 6.2b; t_{75} = 3.44, p = 0.001) than specialist colonies. Moreover, generalist colonies were, on average, 2.6 times faster in carrying their brood to safety (figure 6.2c; t-test: t_{26} = 3.25, p = 0.003),

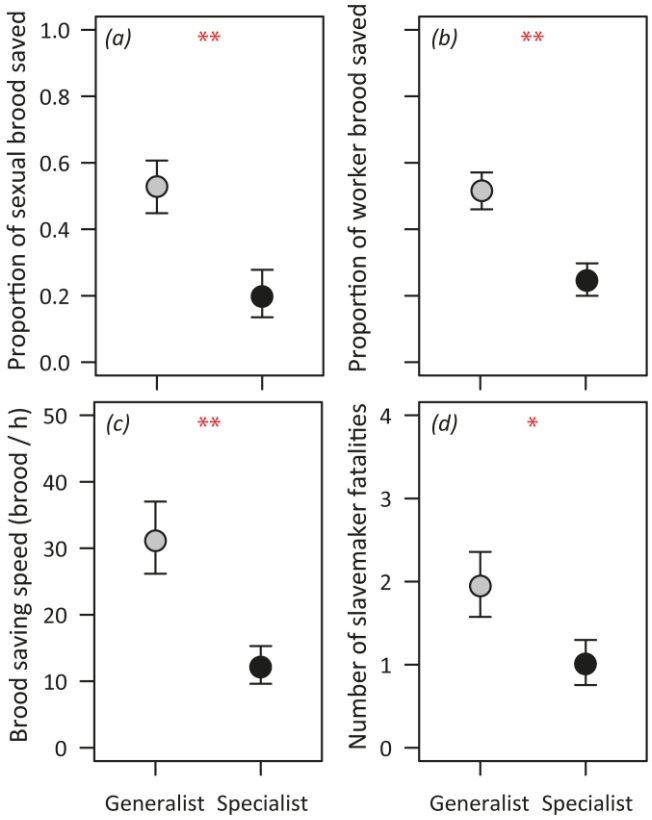

Figure 6.2. Fitness consequences of colony composition. Differences between specialist and generalist *Temnothorax longispinosus* colonies in (*a*) the proportion of sexual and (*b*) worker brood saved during slave raids. (*c*) Difference in the speed with which workers carried their brood to safety in an escape nest. (*d*) The number of fatalities among slavemaker colony members raiding specialist or generalist colonies. Symbols represent estimates ± s.e.; * p < 0.05, ** p < 0.01.

which contributed to the higher fraction of brood saved (i.e. the faster colonies saved their brood, the more brood they managed to save in total; GLM: estimate ± s.e. = 0.028 ± 0.011, t_{26} = 2.50, p = 0.019). Generalist colonies did not trade off brood rescue for adult survival, as generalist and specialist colonies did not differ in the relative number of worker fatalities (GLM: t_{75} = 1.22, p = 0.228) or the likelihood that a queen died (GLM: z_{68} = -0.42, p = 0.673). However, slavemaker colonies that raided generalist *T. longispinosus* colonies suffered higher fatalities than those raiding specialists (figure 6.2d; t-test: t_{75} = -2.01, p = 0.048).

Worker specialisation and slavemaker sympatry

As predicted, *T. longispinosus* colonies from communities where the slavemaker was present showed a lower $D_{y|x}$ index than those where the slavemaker was absent (figure 6.3; LMM: t_3 = -3.90, p = 0.030). This difference was not driven by variation in the level of polygyny, and hence intra-colonial genetic variation, since the $D_{y|x}$ index was unrelated to whether colonies were headed by a single or multiple queens (LR = 0.22, p = 0.636).

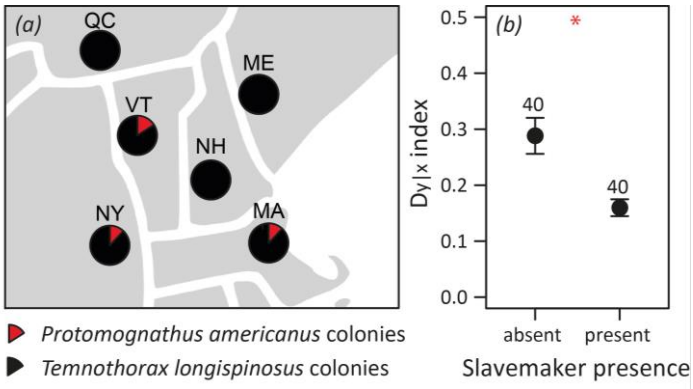

Figure 6.3. Natural variation in the level of worker specialisation in relation to slavemaker presence, (*a*) based on six *Temnothorax longispinosus* populations from Massachusetts (MA), Maine (ME), New Hampshire (NH), New York (NY), Quebec (QC) and Vermont (VT). Pie charts represent slavemaker prevalence (i.e. *T. longispinosus* colony to slavemaker colony ratio). (*b*) *Temnothorax longispinosus* colonies originating from communities where the slavemaker was present showed less division of labour. Symbols represent the estimates ± s.e.; numbers represent the $n_{colonies}$; * $p < 0.05$.

DISCUSSION

Contrary to popular belief, our findings demonstrate that specialisation can have detrimental consequences for the annual reproductive success of a colony and it's potential for growth. Ant societies composed of specialists lost almost 80% of their worker and sexual brood during a raid, whereas generalist colonies managed to save more than half of their brood. Such heavy losses of sexual brood represent severe fitness costs to a species that only reproduces once per year. Moreover, the loss of a substantial part of the colony's new workforce likely compromises future colony survival and productivity. Further support for the ecological significance of these fitness costs of strict

specialisation is provided by our population comparison: workers from colonies that co-occur with slavemakers adopt a more generalist strategy compared to colonies from slavemaker-free sites. Hence, colonies exposed to the dynamic conditions posed by slavemaker attack appear to generate a robust, risk-tolerant organization of work by relying more on generalist workers.

Specialisation is, in essence, a behavioural trait, and variation in task performance among workers frequently occurs independently of their morphology, genes or age (Robinson 1992). In the absence of unifying organizational principles, it is the fitness consequences of behavioural specialisation *per se* that beg for experimental support (Jeanson and Weidenmüller 2014). Conveniently, *T. longispinosus* lacks physical worker castes, which rules out differences in task efficiency due to morphological adaptation. Moreover, we have shown that genetic variation is of minor importance for the division of labour in *T. longispinosus*, a species in which queens mate with a single male (Foitzik and Herbers 2001). Thirdly, because our study population only produces a single worker generation per year, the assessment of worker specialization shortly before brood emergence ensures that all workers are at least one year old. This greatly limits the potential for age-dependent task performance, especially since there is little evidence for such age-polyethism in *Temnothorax* ants (Sendova-Franks and Franks 1995). Hence, differences in worker behaviour alone are most likely responsible for the higher success of colonies composed of generalist workers compared to specialists during slave raids.

Although it is generally assumed that specialists are more efficient at their respective tasks than generalists, this "Jack-of-all trades, master of none" hypothesis has rarely been tested. A noticeable exception is a study by Dornhaus, who demonstrated that specialization in *Temnothorax albipennis* ants does not predict individual efficiency (Dornhaus 2008). In our study, we investigated how worker specialisation affects the efficiency of entire societies, which is the main unit of selection in social insects. We find that colonies composed of generalists inflicted more fatalities among slavemaker colony members than specialist colonies, without suffering higher losses among their own workers. This suggests that *T. longispinosus* colonies composed of specialists were actually less, not more efficient in their aggressive defences, which is at odds with the "Jack-of-all trades, master of none" hypothesis implicit in many discussion on division of labour.

Proximately, higher fitness losses suffered by societies composed of specialists are probably due to a smaller subset of workers responding to a particular stimulus. The costs of not performing a necessary task likely outweigh any advantages of specialization in terms of efficiency gain, especially since there is no evidence that specialization increases individual efficiency in *Temnothorax* ants (Dornhaus 2008). Moreover, potential benefits of specialization for the avoidance of costs associated with switching between different tasks (Goldsby *et al.* 2012) are likely negated during dynamic conditions such as slave raids, due to the disruption of the colony's spatial organization (supplementary material; video S1). Indeed, spatial fidelity is a key regulator of social organization in ants because different sets of tasks are usually segregated within a colony (Sendova-Franks and Franks 1995; Mersch *et al.* 2013). In its absence, workers that

perform any task they encounter likely contribute more to colony fitness than workers that reject tasks unless they fall within their narrow behavioural repertoire. This contribution of generalists to flexibility and functional robustness provides a key benefit of decentralized control of division of labour when groups face sudden shifts in group demand.

Fitness costs of division of labour likely apply especially to small societies, which are particularly prone to lose vital functions with the death of few highly specialized workers. We however do not believe this marginalizes the implications of our findings, since many taxa that exhibit division of labour are characterized by small group sizes. Indeed, the majority of social insect colonies do not resemble the large societies of honey bees or leafcutter ants (Wilson 1971; Hölldobler and Wilson 1990; Dornhaus *et al.* 2012), and social groups of cooperative birds and mammals rarely exceed several dozen of individuals. Moreover, most species that form large societies will cross a phase in their ontogeny where they are composed of only few individuals, during which the division of labour into highly specialized modules may jeopardize the survival of the group as a whole. Hence, small or young societies are expected to rely more on generalists in their work force to absorb fluctuations in the environment, and there is indeed ample empirical evidence supporting the positive association between group size and division of labour (Holbrook *et al.* 2011; Dornhaus *et al.* 2012; Ferguson-gow *et al.* 2014). From an evolutionary perspective, the fitness pay-offs of division of labour in small societies is of particular interest, as social life likely evolved starting with small groups rather than large, complex societies. Hence, our findings provide insights into the evolutionary drivers of social life and the ultimate causes of variation in social organisation.

Our findings underline the importance of an ecological perspective on the organization of work and the behavioural rules of individuals that give rise to beneficial outcomes for societies as a whole. Although we focused on the enigmatic but idiosyncratic conditions caused by slave raids, social groups frequently face dynamic conditions, be it small day-to-day perturbations or sudden catastrophic shifts in task needs. Such conditions likely favour flexibility, not only in ant societies but across all levels of organization. For instance, metabolic networks that computationally evolve in fluctuating environments increase robustness through the acquisition of generalist enzymes than those evolved in stable environments (Nam *et al.* 2012), and the recent financial crisis the human economy has demonstrated how the reliance on few specialized financial institutions can lead to the collapse of the global economy (Arinaminpathy *et al.* 2012). Hence, societal costs of strict division of labour likely apply whenever conditions demand flexible task performance by group members.

Both authors designed the study and contributed to the manuscript. EJ collected and analysed the data. Ant collection permits were 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.

ACKNOWLEDGEMENTS

[removed for privacy purposes]

SUPPLEMENTARY MATERIAL

Pre- and Post-Manipulation Colony Characteristics

Colony types did not differ in colony size before (*t*-test: t_{100} = 0.08, p = 0.936) or after manipulation (t_{100} = -0.12, p = 0.903). Colony sizes after manipulation (mean ± s.d. = 27.9 ± 5.8 workers) lay well within the natural range (Foitzik and Herbers 2001). Ninety percent of the colonies had a queen(s). Queen presence did not differ between colony types (χ^2-test: χ^2_1 = 0.07, p = 0.789). Excluding queen-less colonies did not qualitatively change the fitness results (not shown).

Colony types did not differ in the average brood stimulus responsiveness before (t_{100} = -1.05, p = 0.294) or after manipulation (t_{100} = 0.33, p = 0.742). The average responsiveness towards the opponent stimulus did not differ *a priori* (t_{100} = -0.66, p = 0.510) between colony types, although the removal of workers resulted in a higher average responsiveness towards the opponent stimulus in generalist colonies (t_{100} = -2.75, p = 0.007). To control for this, we included the average colony responsiveness towards the opponent as a covariate in the analyses, which showed that it was unrelated to our fitness measures (all p > 0.7).

Although experiments were conducted before the emergence of a new worker generation, 40.2% of the experimental colonies already contained newly emerged sexuals, which we excluded from the manipulated colonies. Darkly coloured pupae, likely to emerge before or during the raiding experiments were also removed, resulting in the exclusion of 2.4% [0, 9.7] (median [IQR]) of the brood. Specialist and generalist colonies did not differ with respect to the proportion of excluded brood (quasi-binomial GLM: t_{100} = 0.78, p = 0.436) or newly emerged sexuals (t_{81} = 0.65, p = 0.519).

Manipulation of colony composition did not affect intra-colonial variation in behaviour

Because previous studies have shown that societies benefit from increased behavioural variation among group members (Pruitt and Riechert 2011; Modlmeier et al. 2012), we manipulated the level of specialisation while maintaining similar levels of behavioural variation within colonies. That is, generalist and specialist colonies did not differ in intra-colonial variation in opponent stimulus responsiveness (MCMCglmm posterior variance [95% CI] = 17.48 [15.08, 19.87], 16.73 [14.05, 19.20], respectively) or brood stimulus responsiveness (1.23 [1.08, 1.39], 1.23 [1.06, 1.38], respectively), as shown by the substantially overlapping 95% confidence intervals. These results were obtained by decomposing the variance in individual behavioural responses. We fitted separate Generalized Linear Mixed Models (MCMCglmm package) (Hadfield 2010) for the opponent and brood stimulus responsiveness of workers from generalist and specialist colonies. Models included colony ID and individual ID as "random" factors, in addition to the intercept. Opponent stimulus models were run for 1.5 x 10^7 iterations, with 10^5 initial iterations removed as a burn-in and a sampling interval of 5000. For the brood stimulus models we chose 10^6 iterations, a burn-in of 10^5 and a sampling interval of 500. We used inverse-gamma priors for the random effects (V = 1, nu = 0.002) and fixed the residual

variance at a value of 1. To determine whether colony types differed in intra-colonial behavioural variation, we assessed whether the 95% confidence intervals of the posterior distribution of individual ID overlapped between specialist and generalist colonies.

No fitness consequences of intra-colonial variation in behaviour

Potential fitness consequences of intra-colonial behavioural variation were assessed using quasi-binomial GLMs with logit-link function. Colony type and the colony's median absolute deviation in either opponent stimulus or brood stimulus responsiveness were fitted as fixed predictors. This showed that the proportion of brood saved by *T. longispinosus* colonies was unrelated to intra-colonial variation in opponent (GLM: t_{74} = 1.63, p = 0.107) or brood stimulus responsiveness (t_{74} = 0.45, p = 0.652).

Video S6.1. [will be made available upon request] Slave raids by *Protomognathus americanus* on *Temnothorax longispinosus* colonies. The recording shows three colonies attacked by a different slavemaker colony in a separate arena, demonstrating high variability in raiding and defence strategies characteristic of slave raids. Left: pre-raid phase where only the queen and brood remain inside the nest while most workers are engaged in fights with slaves and slavemakers in the arena. Centre: ongoing raid with slavemakers entering the nest, followed by brood evacuation and fights between host workers and slaves / slavemakers. Right: final stages where slavemakers reside inside the nest, eliciting little response from the few remaining host workers.

SUPPLEMENTARY FIGURES

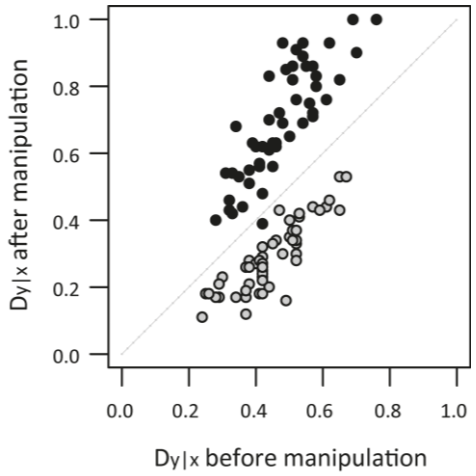

Figure S6.1. The effect of manipulation on the level of specialisation in generalist and specialist colonies. Each point depicts a *Temnothorax longispinosus* colony. The level of specialisation is represented by the $D_{y|x}$ index. Black symbols: specialist colonies; grey symbols: generalist colonies. $D_{y|x}$ of specialist and generalist colonies differed after (*t*-test: $t_{100} = 14.36$, $p < 0.0001$), but not before manipulation ($t_{100} = 1.27$, $p = 0.207$).

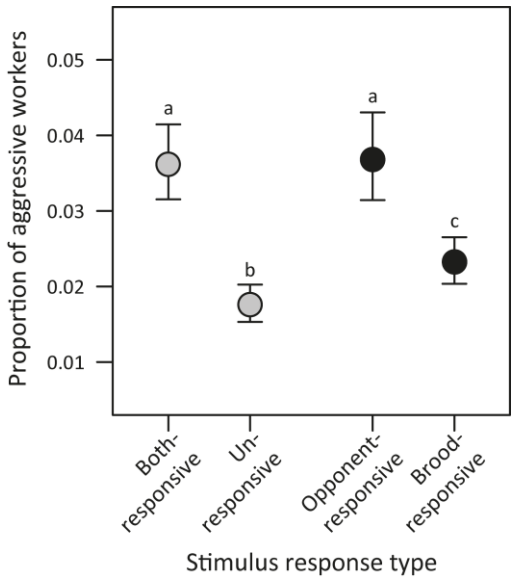

Figure S6.2. Differences between response groups in their aggressive task performance in the colony context. The proportion of *Temnothorax longispinosus* workers showing aggression towards slaves and slavemakers (binomial GLMM with colony ID as random factor: $\chi^2_3 = 52.97$, $p < 0.0001$). Symbols represent estimates ± s.e. and different letters indicate significant differences between response groups ($\alpha = 0.05$). Based on 71 colonies and 653 scans performed prior to the onset of a raid.

CHAPTER 7

Perspectives

Evelien Jongepier

Many hosts lack particular defences that have proven highly efficient against parasites in others (Britton *et al.* 2007; Kilner and Langmore 2011); and in some systems, parasites even appear to select against anti-parasite defences (Zamora-Munoz *et al.* 2003; Krüger 2011). Such counter-intuitive findings are plentiful in the literature as well as in this thesis. For instance, we show that collective aggression and brood evacuation can eliminate slavemaker threat (chapter 2), yet, slavemakers seem to select against workers that are specialized in either task (chapter 6); Aggressive anti-slavemaker defences decrease with slavemaker pressure (chapter 2), despite the obvious advantages of aggression during host-slavemaker encounters (Foitzik *et al.* 2001; Pamminger *et al.* 2012; Kleeberg *et al.* 2014); And hosts that most effectively attacked and killed an intruding slavemaker (chapter 2) were found to have the lowest, not the highest aggressive potential (chapter 3). These findings are difficult to interpret as long as each defence strategy is viewed in isolation, and necessitates a more comprehensive approach to study host defences.

Despite the different objectives of the chapters in this thesis, the question how variation in host defences relates to slavemaker pressure takes central stage in each. This common denominator and the fact that many experiments were performed on the same *Temnothorax* ant hosts, now allows us to speculate on the outcomes of the coevolutionary interactions between slavemaker and their hosts. The purpose of this perspective is not to repeat or summarize the diverse and detailed scientific discussions presented in the previous chapters, such as the discussion on our experimental demonstration of fitness costs of worker specialisation (chapter 6), or the discussion on slavemakers driving host recognition cue diversity (chapter 5). Rather, I aim to provide some perspective on how the collective findings presented in this thesis contribute to our understanding of the coevolutionary outcomes of host-parasite interactions. Any claims about the evolutionary mechanisms, trajectories and outcomes in species with long generation times such as ants are, by necessity, speculative. Especially where complex, multidimensional and interrelated host defence portfolios are concerned. This perspective therefore serves to inform future studies rather than confirm current coevolutionary theory.

The evolution of the first line of defence

Behavioural adaptations that that help to avoid conspecifics, parasites or vectors are the first line of defence against parasites. Such frontline defences can entirely prevent infection and are therefore thought to convey the highest fitness benefits to the host (Feeney *et al.* 2012; Curtis 2014). Examples include spiny lobsters that refuse to share a den with lethally infected conspecifics (Behringer *et al.* 2006); Killifish that preferentially shoal with unparasitized conspecifics (Krause and Godin 1994); or humans that avert, socially exclude or quarantine individuals which may pose infection risks (reviewed by Curtis 2014). Hosts of brood or social parasites likewise exhibit defences aimed at avoidance of parasitism, such as the collective mobbing behaviour of reed warblers towards cuckoos (Welbergen and Davies 2009), or the collective aggression of *Temnothorax* workers towards slavemakers (Wesson 1939; Jongepier *et al.* 2014, chapter 2). In *Temnothorax* ants, this first line of defence likely yields the highest fitness benefits because killing a scouting slavemaker entirely eliminates the threat of an upcoming raid. In line with this we found that *Temnothorax* hosts that respond aggressively towards slavemakers are less likely to let the slavemaker escape and suffer lower parasite prevalence in the field (Jongepier *et al.* 2014, chapter 2).

The fact that frontline defences can reduce or entirely eliminate parasitic exploitation probably drives counter-adaptations in the parasite. Such counter-adaptations include

mimicry or stealth, aimed to avoid detection by the host; superb fighting ability to counter the host's aggressive defences; or manipulation of host behaviour to disable its frontline defences (reviewed by Feeney et al. 2012). This latter offensive strategy, and the evolutionary response by the host was assessed in chapter 4, where we found that inter- and intra-specific variation in host aggression could be explained by difference in the host resistance to manipulation by the slavemaker. Such manipulation impaired colony defences and increased the likelihood that an intruding slavemakers escaped unharmed, able to recruit nestmates and initiate a slave raid. Hence, inter- and intraspecific variation in host resistance to manipulation can have important implications for the evolutionary outcome of host-parasite interactions, as detailed below.

Successful avoidance of parasitic exploitation

Efficient frontline defences may be decisive for the outcome of coevolutionary interactions, to the point that parasitic exploitation ceases entirely. Examples include small pox vaccination in humans, which has effectively eradicated the virus. Similarly, *Formica rufibarbis* colonies are rarely parasitized by the slavemaking ant *Polyergus rufescens*, which has been attributed to efficient and relentless aggressive attacks on invading parasites (Mori *et al.* 1995). We likewise show that colonies that are susceptible to manipulation by the slavemaker are more likely to be parasitically exploited than resistant colonies. Most noticeably, the preferred host *T. longispinosus* is more susceptible to manipulation than the less preferred host *T. curvispinosus*, which explains why *T. curvispinosus* responded with more aggression towards an intruding slavemaker, why they were less likely to let slavemakers escape and why they were less frequently parasitized by the slavemaker than *T. longispinosus*.

Although behavioural data for long-lives species such as ants is not available over evolutionary time-scales, local records of slavemaker prevalence exist for as far back as 1927 (Sturtevant 1927). Together with our own data it suggest that slavemaker prevalence is locally subject to change, although the magnitude and direction of this change differs between different locales. For instance, slavemaker prevalence has gradually increased over the last four decades in the intensely studied *T. longispinosus* population in New York, suggesting that the slavemaker is leading the coevolutionary arms race with its host. Interestingly, we found that this host population was highly susceptible to manipulation by the slavemaker, with over 70% of the colonies showing slavemaker induced nestmate attack. It also showed little aggression towards an intruding slavemaker, most of which managed to escape unharmed. Hence, the near lack of frontline defences in this *T. longispinosus* population may explain why slavemakers are on the winning hand in New York. Contrastingly, Sturtevant (1927) found an average slavemaker prevalence of 6% in a *T. curvispinosus* population in New Jersey, which contrasts with the fact that we did not find a single slavemaker colony at a nearby locale 85 years later. Interestingly, the New Jersey *T. curvispinosus* population was the only one where none of the colonies were manipulated into attacking their own nest mate, and these hosts showed both the most intense and effective collective aggressive defences against an intruding slavemaker. This implies that *T. curvispinosus* from New Jersey has won the coevolutionary arms race with the slavemaker, causing host-parasite interactions to cease entirely.

Host shifts, alternations and cycles

When hosts successfully resist parasitic exploitation, parasites either go locally extinct or are forced to switch to another host species. Coevolutionary interactions often

involve multiple species, which facilitates complex evolutionary outcomes such as host switching, alternations or coevolutionary cycles. Host switching can occur when the preferred host either evolves efficient defences or becomes too rare in the local community to support the parasite. Host switching may escalate into coevolutionary alternation if parasite preference changes repeatedly between potential hosts according to the constantly evolving levels of host defences (Davies & Brooke 1989b; Nuismer & Thompson 2006). Alternatively, host-parasite systems may become locked in perpetual coevolutionary cycles when natural selection favours parasites that target the most common host species or genotype (Dybdahl & Lively 1998; Nuismer *et al.* 2005).

Although we lack historical records on degree of mobilisation, the relative abundance and the level of parasitic exploitation for the two host species, there is some indirect evidence that *P. americanus* has switched from one to the other *Temnothorax* host. Previous studies have demonstrated that *P. americanus* prefers *T. longispinosus* over *T. curvispinosus* hosts, and *T. longispinosus* is indeed more heavily exploited by the slavemaker in the field (Brandt and Foitzik 2004). Interestingly, *T. longispinosus* also showed less efficient first line defences than *T. curvispinosus*, as the former was more readily manipulated into attacking own nestmates and showed less intense and efficient collective aggression towards the slavemaker. Thus, *T. curvispinosus* appears to have evolved efficient anti-slavemaker defences, even in populations that are currently unparasitized. Such high level of mobilisation in the coevolutionary arms race with the slavemaker is likely reminiscent of a parasitized past. Indeed, there are several host communities that harbour both host species, providing the opportunity for slavemakers to switch to the less defended host. For instance, both *T. longispinosus* and *T. curvispinosus* occur at high densities in West Virginia, yet, *P. americanus* exclusively parasitizes the less defended *T. longispinosus* host population.

In addition, we found the footprint of coevolutionary cycles in our study system, albeit between different host phenotypes within populations of *T. longispinosus* host colonies. In chapter 5, we show that recognition cue diversity between *T. longispinosus* colonies is higher in the presence of slavemakers, rendering it impossible for parasites to match the recognition profile of all potential hosts. The evolution and maintenance of recognition cue diversity under slavemaker pressure can only be explained by balancing selection. Hence, the selective advantage of hosts with rare recognition cues in the presence of slavemakers appears to be a major driver of host diversity through coevolutionary cycles.

Escalating arms races and the evolution of defence portfolios

Once the first line of defence becomes permeable to parasitism, selection favours hosts that mount further lines of defence. Hence, if host cannot avoid parasitism they may use a defence-in-depth strategy against parasites (Welbergen and Davies 2009; Kilner and Langmore 2011). For instance, vertebrates possess an intricate immune system involving many different cell types (Roitt and Delves 2001; Holmes 1983); and plants frequently synthesise many toxic chemicals as a defence against herbivores (Emlen 1984).

In this thesis, we have shown that *Temnothorax* colonies do not rely only on efficient aggressive defences, but instead possess multiple lines of defence expressed at various stages of the host-parasite interaction. For instance, hosts that are targeted by a slavemaker scout can still salvage their brood and save the queen through colony evacuation. This second line of defence is likely to be more costly since evacuating hosts

have to compete for a new nest site, which is a limiting resource in *Temnothorax* ants (Herbers 1986). Nonetheless, colonies that evacuate their nest sites before a scout returns with a raiding party do evade the fitness loss of being raided, which likely favours flight strategies if fighting fails. Even if colonies are unable to avoid a raid, they still poses strategies that allow them to mitigate its costs. For instance, they can improve their ability to recognize and attack conspecific workers, such as the slaves that join slavemakers on a raid and are responsible for many host casualties and brood loss (Wesson 1939; Foitzik et al. 2001). Hence, the ability to recognize conspecific workers as non-nestmates, and the aggressive defences that follow enemy recognition, likely represent an important line of defence to hosts that are unable to resist the slavemaker itself. In addition, host colonies that co-occur with slavemakers adjust the social organisation of the colony, such that they are better able to cope with the unpredictable conditions posed by slave raids.

The depth of adaptive portfolios depends on how each successive line in the defence hierarchy affects the strength of selection on the next (Britton et al. 2007; Kilner and Langmore 2011). Thus, exposure to parasites drives behavioural adaptations in the host, which in turn affect the selection regime for other potential strategies. In this thesis, we indeed show that the level of mobilization in each successive line of defence is not independent, which allows us to speculate on the two mechanisms implicated in how evolution shapes the complexity and depth of host defence portfolios.

i) Strategy blocking

As long as the first lines of host defences are highly efficient against parasites, selection for further defences is diminished. Such strategy blocking has been invoked to explain the lack of obvious defences in some hosts species of avian brood parasites (Britton et al. 2007), but has not been demonstrated between populations of the same host species. For instance, the field fare does not show any aggression towards cuckoo models and has very low egg rejection rates. However, it does builds steep-sided nests that inhibits pre-egg laying ejection of host eggs or chicks by the cuckoo. The high efficiency of this first line of defence has been hypothesised to block the evolution of aggressive defences or egg rejection (Feeney et al. 2012). Likewise, cape bulbuls do not reject or abandon cuckoo eggs, despite their aberrant appearance. Instead bulbuls respond highly aggressively towards an adult cuckoo in the nest vicinity, which reduces the likelihood that that the cuckoo successfully deposits its egg in the host's nest (Krüger 2011). Presumably, fierce nest defence prior to parasitation relaxes selection for resistance strategies employed at later stages of the breeding cycle.

Along the same line, we show that colonies that are resistance to manipulation of their aggressive defences by the slavemaker rely less on further lines of defence. For instance, the more intense the hosts' collective aggressive defences, the less likely they were to evacuate their nest site upon slavemaker encounter. Presumable, the high efficiency of killing an intruding slavemaker and thus averting a slave raid diminishes the fitness returns of nest site evacuation to the point that the costs associated with giving up a limited resource are not offset by its marginal benefits. Likewise, *T. longispinosus* populations that were most aggressive towards an intruding slavemaker were less aggressive towards workers from conspecific colonies in their population of origin, which are the potential source of the slaves they would encounter during a slave raid. Hence, host populations with efficient first line defences appear to invest less in strategies that may aid them in later stages of host-parasite interactions.

ii) Strategy facilitation

The corollary of strategy blocking is strategy facilitation, where one line of defence increases rather than decreases selection for further defensive strategies. While it has been proposed that strategy facilitation can drive the evolution of elaborative defence portfolios, empirical support remains scant (Kilner and Langmore 2011). One possible example of strategy facilitation may be found in the superb fairy wren. In this species, only host populations that occur in sympatry with the Horsfield's bronze-cuckoo can recognize adult cuckoos and respond with aggression. Fairy wrens desert nests with a single cuckoo chick, but only if it has detected an adult cuckoo in the area. Hence, recognition of adult cuckoo may have facilitated the evolution of chick desertion, since the combined strategy is likely to reduce fitness losses due to recognition errors (i.e. the erroneous abandonment of the host's own chick).

This thesis provides two examples where the elaboration of host defence portfolios may be explained by strategy facilitation. Firstly, we show that host resistance to manipulation by the slavemaker enables hosts to collectively attack and kill the slavemaker. Secondly, *T. longispinosus* colonies that are more chemically distinct from each other are also more aggressive towards conspecifics. The more similar colonies are in their recognition profile the more likely it is that they make recognition errors, which is costly if those errors cause aggression towards nestmates. Hence, increased variability in the recognition profiles between host colonies allows for more accurate recognition of nestmates or enemies, facilitating the evolution of enemy discrimination through aggression. The balancing selection for rare recognition profiles exerted by slavemakers thus likely exerts positive selection on aggression towards conspecific workers, including slaves.

Outlook

This thesis demonstrates that ant colonies use a plethora of defence strategies to cope with social parasites. The level of expression of these strategies varies across populations and species, depending not only on parasite prevalence but also on other strategies in the host's defence portfolios. Hosts with efficient frontline defences suffered lower parasite prevalence in the field. Nonetheless, hosts whose frontline defences were breached by the parasite were found to mount further lines of defence. Hence, host defence portfolios can reach remarkable depths.

I conclude with highlighting four areas for future research.

1. Most of the evidence presented in this thesis is of a correlative nature, which begs for experimental confirmation. One approach is to manipulate colony defences by the removal of those workers that act as the primary defenders, not unlike how we manipulated the division of labour of colonies by removing generalist of specialist workers in chapter 6. Alternatively, one could exploit the genomic resources that are becoming rapidly available for our study system, as well as many others. Identification of the genes underlying host defence and slavemaker offence strategies opens up the possibility to experimentally assess host defences, when for instance the parasite's manipulative arsenal is knocked down.
2. The relative expression patterns of the different strategies in host defence portfolios is surprising consistent. However, little is known about the mechanistic underpinning of consistent variation in host defences. Whether the various strategies are under the control of the same or different regulatory pathways remains unknown, although such information could provide valuable insights into the proximate causes of convergent strategies across species and populations.

3. While long lived species, such as ants, are not accessible to experimental evolutionary approaches, the foot print of selection can be found in their genomes. Combining population genomic approaches with behavioural essays on host defences can not only elucidate the relationship between molecular and phenotypic variation, but also provides information on the mode of selection. Such an approach may for instance provide further support for negative, frequency-dependent selection acting on host recognition cues in the presence of slavemakers.
4. Coevolutionary dynamics involve, by definition, two or more interacting parties; yet, this thesis has focused exclusively on the defence portfolios of the hosts. Future studies into the different strategies and their level of expression in the parasite's offence portfolio may shed further light on the geographic variation in host defences and the distribution and abundance of hosts and parasites.

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