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UNIVERSITY OF SUSSEX

Submitted for the Degree of Doctor of Philosophy

CHRISTOPHER TRANTER

# Defending the Fortress

comparative studies of disease resistance in ant societies

## SUMMARY

Parasites represent a considerable and ubiquitous threat to organisms, and studies of host-parasite interactions can demonstrate important insights into key biological processes. Identification and quantification of host defences and their role in parasite resistance is an important part of understanding these effects. Additionally, life-history traits can have significant effects on host-parasite interactions. For example, living in groups has many benefits, but also may have associated costs in terms of increased parasite transmission. Thus group-living animals may be predicted to invest heavily in disease resistance strategies, though which may depend on each species' parasite pressure. Social insects, and ants in particular, are an ideal model with which to test these evolutionary and ecological hypotheses, as they possess an array of mechanisms to defend themselves against disease and have highly diverse life-histories. However, previous studies into disease resistance tend to have been performed on single species, often looking at just single measures of investment of defence. In this thesis I explore the comparative importance of disease resistance in different ant species. I show that ants possess a variety of defence mechanisms to protect themselves against the threat of parasites and demonstrate how investment into these important defences can vary between individuals and species, and may depend on context, type of parasite, and life-history of the host. Work such as this, demonstrating the costs of individual components of disease resistance in multiple species, is important in developing our understanding of how changes in parasite pressures can influence host biology and how organisms can survive in a world abundant with parasites.

# Defending the Fortress

comparative studies of disease resistance in ant societies

Christopher Tranter

Submitted for the Degree of Doctor of Philosophy  
University of Sussex  
Faculty of Life Science  
September 2015

# Declaration

I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications is included. The contributions of other authors are indicated for published chapters below. This thesis has not been and will not be submitted to another institution for the award of any other degree.

List of published chapters and contributions:

**Chapter 2.** Tranter, C., LeFevre, L., Evison, S.E.F. & Hughes, W.O.H. 2015. **Threat detection: contextual recognition and response to parasites by ants.** *Behavioural Ecology*. **26**: 396–405.

CT designed the experiments carried out the experimental work, analysed the data, and wrote the manuscript. LF conducted preliminary experimental trials. SEFE supervised the work of LF. WOHH supervised the work, assisted with the design of the experiments, the data analysis, and proofing the manuscript.

**Chapter 3.** Tranter, C., Graystock, P., Shaw, C., Lopes, J.F.S. & Hughes, W.O.H. 2014. **Sanitizing the fortress: protection of ant brood and nest material by worker antibiotics.** *Behavioural Ecology and Sociobiology* **68**: 499–507.

CT designed the experiments carried out the experimental work, analysed the data, and wrote the manuscript. PG aided with the experimental work in blocking the glands of ants. CS conducted preliminary experimental trials. JFSL supplied the ant colonies. WOHH supervised the work, assisted with the design of the experiments, the data analysis, and proofing the manuscript.

**Chapter 4.** Tranter, C. & Hughes, W.O.H. 2015. **Acid, silk and grooming: alternative strategies in social immunity in ants?** *Behavioural Ecology and Sociobiology*. **69**: 1687-1699

CT designed the experiments carried out the experimental work, analysed the data, and wrote the manuscript. WOHH aided in the manuscript proofing.



**Chapter 5.** Tranter, C., Fernandez-Marin, H., & Hughes, W.O.H. 2015. **Quality and quantity: transitions in antimicrobial gland use for parasite defence.** *Ecology and Evolution (in press)*

CT and WOHH designed the experiments, CT carried out the fieldwork and experimental work, analysed the data, and wrote the manuscript. WOHH aided in the manuscript writing and HFM aided with fieldwork.

**Chapter 6.** Tranter, C. & Hughes, W.O.H. 2015. **Simple societies and sanitary syndromes: species and individual differences in ant social immunity.** *Biology Letters (in review)*

CT and WOHH designed the experiments, CT and TM carried out the experimental work, CT analysed the data, and wrote the manuscript. WOHH aided in the manuscript writing.

**Chapter 7.** Tranter, C. & Hughes, W.O.H. 2015. **Sanitary slaves and slovenly slavemakers: disease resistance of social parasites and their hosts.** *Proceedings of the Royal Society of London: B (in review)*

CT and WOHH designed the experiments, CT carried out the experimental work, analysed the data, and wrote the manuscript. WOHH aided in the manuscript writing.

**Appendix** Tranter, C. & Hughes, W.O.H. 2015. **A preliminary study of nest structure and composition of the weaver ant *Polyrhachis* (Cyrtomyrma) *delecta* (Hymenoptera: Formicidae)** *Journal of Natural History. In press*

CT designed the experiments carried out the experimental work, analysed the data, and wrote the manuscript. WOHH aided in the manuscript proofing.

Signed \_\_\_\_\_

Date \_\_\_\_\_

# Acknowledgments

Thanks to Bill Hughes for the opportunity to undertake this PhD, for being ever supportive, no matter where in the world either of us might have been, and for giving me the chance to prove myself as a scientist through work that has been both exciting and rewarding.

To the members of the evolving Hughes Lab: thank you to those who welcomed me to Leeds and to PhD life, when I was first starting out, and provided me with the technical knowledge and friendship that has carried through my project and to the current members of the Hughes Lab who have helped feed ants, or with feedback on manuscripts, and have enabled me to produce the best work I can. I always manage to find someone to organise me (a skill in itself), and Jasmine Parkinson, this was you. We've shared 4 years together and I am immeasurably grateful for your help, patience and compassion. Thank you to Victoria Norman for being a tolerant fieldworker, an enthusiastic biker, and a great friend. I'd also like to mention all the other students, scientists and organisations, I've met and collaborated with, both at home and abroad, who helped with ant collection and identification, or lent equipment, or a hand, and who without which this PhD would have been considerably more challenging.

Thanks to my family who have always been supportive of whatever I've done and have helped get me where I am today. To my mother and father, thank you for the years of selfless commitment, the hours of your evenings spent driving me to and from activities, the places you took me, and chances you gave. I have certainly not said it enough, but that these things seemed routine, that you made these opportunities normal, demonstrates how truly lucky I have been. Finally thanks to Jen for unending support; I've taught you about ants, and you've taught me about myself. Thank you for your patience at the countless times we've been out and I've chased around after an ant, for the 5am starts in the dark for the perfect photo, for the mountains we've climbed, for the memories, for our future.

# Abstract

Parasites represent a considerable and ubiquitous threat to organisms, and studies of host-parasite interactions can demonstrate important insights into key biological processes. Identification and quantification of host defences and their role in parasite resistance is an important part of understanding these effects. Additionally, life-history traits can have significant effects on host-parasite interactions. For example, living in groups has many benefits, but also may have associated costs in terms of increased parasite transmission. Thus group-living animals may be predicted to invest heavily in disease resistance strategies, though which may depend on each species' parasite pressure. Social insects, and ants in particular, are an ideal model with which to test these evolutionary and ecological hypotheses, as they possess an array of mechanisms to defend themselves against disease and have highly diverse life-histories. However, previous studies into disease resistance tend to have been performed on single species, often looking at just single measures of investment of defence. In this thesis I explore the comparative importance of disease resistance in different ant species. I show that ants possess a variety of defence mechanisms to protect themselves against the threat of parasites and demonstrate how investment into these important defences can vary between individuals and species, and may depend on context, type of parasite, and life-history of the host. Work such as this, demonstrating the costs of individual components of disease resistance in multiple species, is important in developing our understanding of how changes in parasite pressures can influence host biology and how organisms can survive in a world abundant with parasites.

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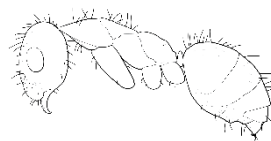


*Monomorium pharaonis*

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*Paratrechina longicornis*

# I. General introduction

## I.I Introduction

### 1.1.1 Parasites

The variety of life we see today represents the product of millions of years of evolution shaped by interactions between different organisms and their environments via natural selection (Anderson and May 1978; Poulin and Morand 2000). Where organisms have particularly close interactions with one another these selective forces may be especially strong and result in dramatic transitions in their biology, such as the evolution of eukaryote cells from their prokaryote ancestors, the formation of multicellular organisms or the transition to obligatory sociality (Margulis and Bermudes 1985; Szathmáry and Smith 1995; Grosberg and Strathmann 2007). Parasitic organisms have by definition detrimental fitness consequences for their hosts, which in return are under selection to counteract such effects. The resulting reciprocal co-evolutionary dynamics, can escalate into an ‘arms race’ where host and parasite are locked in a stepwise evolutionary dynamic and an evolutionary innovation on one side will results in a counter-adaptation on the other side (Gotwald 1996; Wojcik et al. 2007; Poulin 2007; Decaestecker et al. 2007) (Box 1). Such runaway processes have produced some truly remarkable phenotypes, ranging from deadly brain-eating amoeba, to three-metre-long tapeworms, and parasitic crustaceans which functionally replace the tongue of their host (Stevens et al. 1980; Brusca and Gilligan 1983; Hoberg 2002). Such extreme examples highlight the selective force that parasites exert across taxa and environments, shaping the evolutionary trajectory of both species

and communities (Hay et al. 2004; Boots et al. 2004; Poulin 2007; Schmid-Hempel 2011).

### Box 1. Parasites of social insects

Parasites are organisms which live in or on another organism (the ‘host’) and derive benefits at the host’s expense (Poulin and Morand 2000). Traditionally these can be divided taxonomically into macroparasites and ‘microparasites’ based on their scale (Schmid-Hempel 1995). Parasitism is one of the most successful modes of life and their infections are found almost ubiquitously across taxa, both in the distribution of parasites themselves and their host organisms (Poulin 2007). Social insects are no different, and are host to a wide range of parasites, the major groups of which are listed below (modified from Schmid-Hempel 1998; Boomsma et al. 2005).

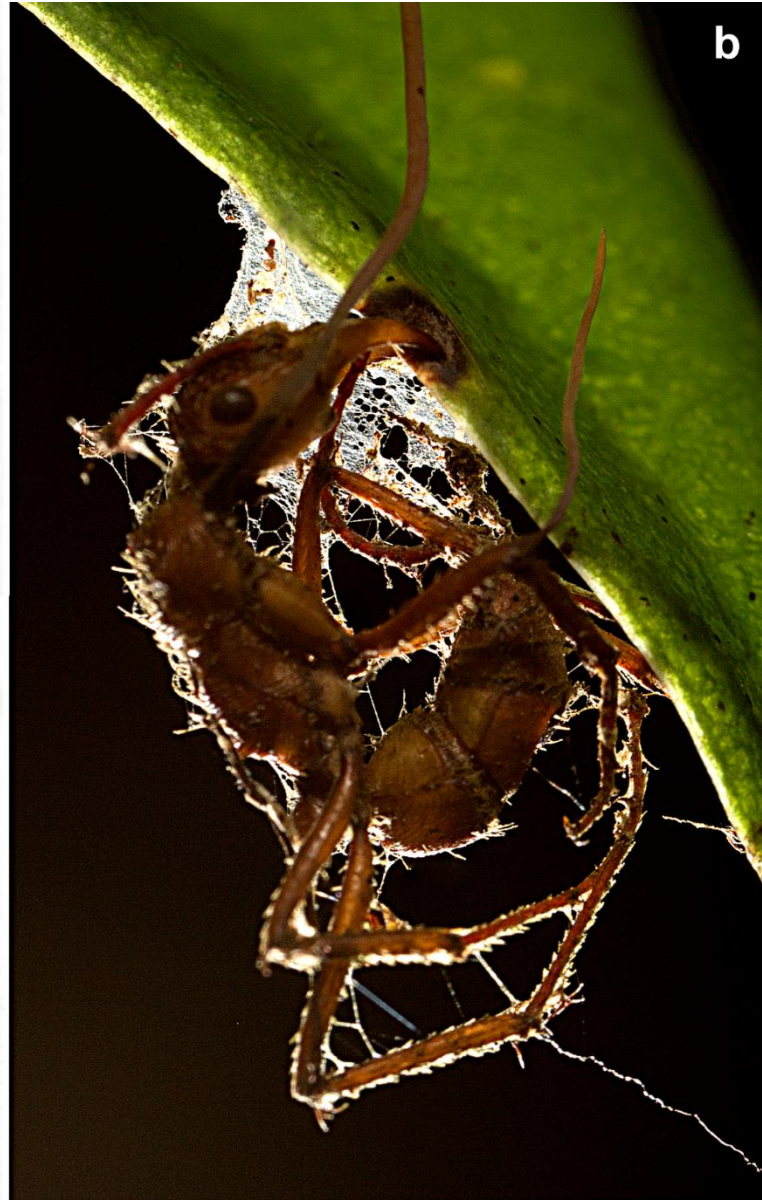
Parasite type	Relative size	Generation time	Primary infection route	Virulence to individual	Virulence to colony
<i>Macroparasites</i>					
Insect parasitoids	Medium-large	Very long	Active entry	Very high	Low
Mites	Small-medium	Very long	Active entry	Low to medium	Low to medium
Helminths	Small	Very long	Per os	Low	Low
Nematodes	Small-medium	Quite long	Per os / integument openings	Low to high	Moderate
<i>Microparasites</i>					
Protozoa	Small	Quite short	Per os	Low to medium	Low to medium
Fungi	Very small	Short	Through cuticle	Low to high	Low to high
Bacteria	Very small	Very short	Per os	Low to high	Low to high
Viruses	Extremely small	Very short	Per os	Low to high	Low to high

**Figure 1.1.** Examples of social insect species (*overleaf*). a) *Dinoponera quadriceps*, a primitive ant species, b) *Nasutitermes* termites, c) *Azteca* ants, d) Meliponine bees, e) Bee carrying pollen, f) *Bombus terrestris* bumblebees.











**Figure 1.2.** Examples of microparasitic infection in ants. a) A dead leaf-cutting *Acromyrmex* ant showing early signs of infection, b) *Ectatomma ruidum* killed by the parasitic *Ophiocordyceps* fungus which can be seen emerging from the cadaver, c) Leaf-cutting ant pupae sporulating with *Metarhizium*, d) A dying queen of the leaf-cutting ant *Atta*, amongst the remains of its fungal crop which has been destroyed by the parasitic fungus *Escovopsis*.

### 1.1.2 Parasite pressures in social insects societies

Living in groups has many benefits. Social species can have more efficient food acquisition, better defence against predators, and improved brood care, but these benefits are often traded-off against costs in terms of increase transmission of disease due to the high densities and close relationships between individuals. Such trade-offs have been documented in a diverse range of organisms including insects, mammals, birds, and lizards (Møller et al. 1993, 2001; Cote and Poulin 1995; Schmid-Hempel 1998, 2011; Altizer, Nunn, and Thrall 2003; Lourenço and Palmeirim 2007; Godfrey et al. 2009). In social insects, these costs are likely amplified, as their nests provide highly buffered habitats in terms of temperature and humidity, with a reliable population of hosts, which facilitate the evolution and transmission of parasites. Additionally many social insects exhibit low genetic diversity between individuals which can increase susceptibility to disease and combined with homeostatic nest conditions provides an environment ideal for parasites to thrive (Schmid-Hempel 1994; O'Donnell and Beshers 2004; Calleri et al. 2006). Consequently it may seem initially perplexing that although early simple models based on host density predict a net cost in terms of disease to group-living (Schmid-Hempel 1998), sociality is a widespread and highly successful strategy. More in depth considerations of group-living have since



produced models which suggest that while group-living may in some cases increase within-group transmission, this may be counter-balanced by reduced between-group transmission (Watve and Jog 1997; Wilson et al. 2003). Thus sociality itself may provide the opportunity to reduce the costs from the risk of parasites, which may partly explain how group-living was able to evolve and persist (Schmid-Hempel 2011). Social insects (Figure 1.1), in particular, have shown surprisingly few signs of any of the predicted costs of sociality for parasite transmission, in some cases showing the opposite effect (Rosengaus et al. 1998; Hughes et al. 2002; Ugelvig and Cremer 2007; Reber et al. 2011), and consequently are thought to have especially effective disease resistance strategies. These are expressed individually by ants in the form of innate immunity and physiological responses, and also collectively via hygienic behaviours which help individuals and the colony as a whole resist the effects of disease (Cremer et al. 2007; Cremer and Sixt 2009; Figure 1.2; 1.3).

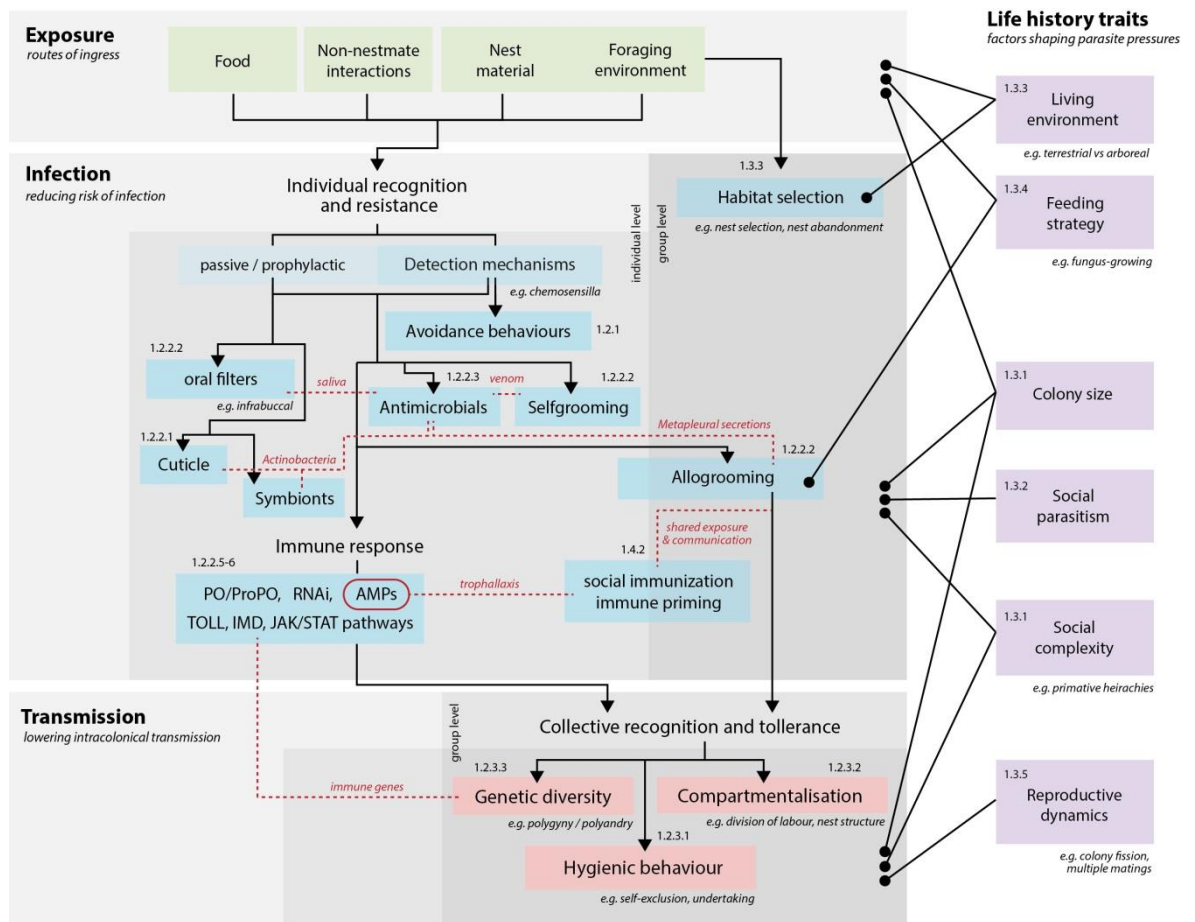
### **1.1.3 Comparative studies**

Social insect species exhibit a wide range of life-history strategies (Hölldobler and Wilson 1990; Boomsma et al. 2005). Consequently, social insect lineages provide many good examples for comparative studies. Social insects must defend against an array of parasites and have evolved a suite of responses. Many of these characters show considerable variation between species or in response to threats and can provide useful measurements of investment into disease resistance. Because the defence strategies of social insects are costly to maintain (Poulsen, Bot, Nielsen, et al. 2002), the relative investment of social insects into disease resistance through morphological, physiological, and behavioural adaptations can be used to infer the strength of parasite pressure, and through comparative studies, the effect of this on

the evolution of life history and sociality in insect societies can be inferred (Boomsma et al. 2005; Hughes, Pagliarini, et al. 2008). Current comparative studies have mainly focussed on just single components of disease resistance, or looked at multiple aspects of resistance but only in a single species (Fernández-Marín et al. 2006; Hughes, Pagliarini, et al. 2008; Adams et al. 2012; Fernández-Marín and Bruner 2013; Tragust, Mitteregger, et al. 2013). Whilst these have been very useful in answering specific questions and hinting at more general trends and significances, studies of a much larger range of species and resistance mechanisms are needed. In particular, studies which examine the investment of social insects into disease resistance at multiple levels, e.g. behavioural and physiological, will be important to identify the precise drivers of these defensive adaptations.

#### **1.1.4 Introduction aims**

This review will explore recent work on social insect disease resistance, beginning with an outline of the different levels of defence mechanisms, and following up with a summary of how the life-history of social insects can affect parasite pressures and hence investment into disease. Finally I discuss how these defences may be employed and traded-off at both individual and group levels, and outline the aims of this thesis.



**Figure 1.3.** Summary parasite dynamics schematic showing simplified parasite exposure, infection and transmission dynamics in social insects, modified from Boomsma et al. (2005). Dashed lines indicate relationships and examples of synergistic effects. Examples are given in italics. Life history traits of social insects which may influence various aspects of parasite pressure and host defence are listed on the right. Inset numbers reference chapter sections for further details

## 1.2 Defending the fortress: disease defence mechanisms in social insects

### 1.2.1 Exposure: detection and avoidance

Social insect individuals can become infected when they encounter a parasite in the environment. This can be in the course of foraging, from food or external surfaces, or from interactions with non-nestmates or even from nest material itself. The first line of defence for any organism is to reduce the risk of exposure, and social insects have consequently evolved to detect and avoid parasites to minimise costs to individuals, and ultimately the colony as a whole.

#### *1.2.1.1 Individual avoidance*

It is possible to classify responses to parasitic threats as either proactive defences which prevent infection occurring in the first place, or reactive defences which are initiated after exposure (Hart 1990; Cremer et al. 2007). The ability of an organism to detect a threat is fundamental to them mounting a successful reactive defence against it. This is particularly key for resisting parasites, whose co-evolutionary arms race with their host can select for better defended and more vigilant hosts, but also for more exploitative and harder to detect parasites (Ebert and Hamilton 1996; Decaestecker et al. 2007). In social insects, microparasites such as entomopathogenic fungi, are often highly lethal to the host, and proactive avoidance of exposure is invariably a better strategy than relying on resistance

post-exposure (Shah and Pell 2003). Early and accurate detection allows for avoidance of the threat altogether, or at least the initiation of early defence mechanisms (Hart 1990, 1994; Schmid-Hempel and Ebert 2003; Wisenden et al. 2009). Consequently social insects are able to detect a variety of parasites in different environments and contexts. Ants (Feener 1981; Diehl-Fleig et al. 1992; Tragust, Mitteregger, et al. 2013; Parmentier et al. 2015), termites (Kramm and West 1982; Zoebisch and Stimac 1990), bees (Arathi and Spivak 2001; Goode et al. 2006), and wasps (Glare et al. 1996; Harris et al. 2000) are all able to detect both macro- and microparasites, and initiate disease resistance mechanisms to them. For example, bumblebees are able to detect and avoid parasite-contaminated flowers (Durrer and Schmid-Hempel 1994; Fouks and Lattorff 2011), and ants, bees and termites groom more frequently in fungal infected environments in addition to cleaning infected brood and foodstuffs (Ugelvig and Cremer 2007; Rosengaus et al. 2011; Tragust, Mitteregger, et al. 2013; Tranter et al. 2014, 2015).

This detection is likely mediated through chemical cues, and can utilise similar highly sophisticated chemoreception systems to those that social insects use for recognition of nestmates and communication (Fernando et al. 2005; Drilling and Dettner 2009; Meunier et al. 2011; Mburu et al. 2011; Baracchi, Fadda, et al. 2012; Diez, Moquet, et al. 2013). Termites have proven to be especially good models to test this hypothesis as many have limited vision. Studies have found strong evidence of the sensitivity of termite antennae to the odours of entomopathogenic fungi, and suggest an ability to discriminate parasites based on their virulence, and then implement defensive grooming (Myles 2002; Yanagawa et al. 2008, 2009; Mburu et al. 2009, 2011; Yanagawa and Fujiwara-

Tsujii 2011). Bees are also able to similarly detect the volatile chemicals produced by chalkbrood infected larvae, and initiate hygienic behaviour to mitigate the cost (Swanson et al. 2009). Interestingly colonies that have been selectively bred for hygienic behaviour can detect larvae infected with chalkbrood at much lower stimulus levels, which suggests a strong heritable component to disease detection. (Spivak 1996; Spivak and Reuter 1998). Additionally, overall differences in colony fitness through the effectiveness of hygienic behaviour can result from the efficiency with which individual bees are able to detect and remove diseased larvae. (Arathi and Spivak 2001; Arathi et al. 2006), which demonstrates how vital detection is to individual and colony fitness.

#### ***1.2.1.2 Group level avoidance***

As well as individual-level detection it is possible that parasite threat avoidance operates at group or population levels. It may make sense for ant colonies to avoid environments or situations which may be more hazardous, for example social insect species nest and forage in a multitude of ecological conditions with a wide range of associated parasite risks (Curtis and Sloan 2002; Van Borm et al. 2002; Keller et al. 2003; Hughes et al. 2004; Reber and Chapuisat 2011). Additionally through geographical spacing of foraging ranges and territorial marking, social insects may be able to minimize horizontal transmission from other colonies (Boomsma et al. 2005). However, in some cases social insects do not show avoidance strategies, which suggests that the benefits from reduced exposure are not as simple as it may at first appear. This expectation is reversed in Pharaoh ant colonies and individual founding queens of *Formica selysi*, which

demonstrate an active preference for fungal contaminated environments during colony relocation and nest foundation (Brütsch et al. 2014; Pontieri et al. 2014). This surprising finding may be due to manipulation by the parasites, or more likely that fungal-presence is signal for otherwise good quality nesting sites or is beneficial through ‘immune priming’ (see 1.4.2).

### **1.2.2 Disease resistance: internal and external individual defence**

After detection of a threat, the first line of insect defence against parasites is for individuals to minimise or remove the risk through cleanliness strategies. These external defences consist of both group and individual behaviours, and antimicrobial compounds, all of which are employed after detection of the threat but before the parasite has penetrated internally into the host. These defences are common in both vertebrate and invertebrate animals and range widely in complexity (Basibuyuk and Quicke 1999; Siva-Jothy et al. 2005). In the social insects both behavioural and antimicrobial components of disease resistance have been well described (Cremer et al. 2007; Otti et al. 2014), and include actions such as removal of dead or diseased brood, removal of dead individuals (‘undertaking’), general clearing of waste material from the nest (‘nest hygiene’), and grooming (Rothenbuhler 1964; Hart and Ratnieks 1998; Boomsma et al. 2005; Cremer et al. 2007). In general, these defences describe an action or set of antiseptic behaviours which promote colony and individual hygiene, with the collective defences being termed ‘social immunity’ (Cremer et al. 2007; Wilson-Rich et al. 2009). This initial definition has since been refined and expanded to include broader defence definitions, including within it for example social immunization, and has been applied to analogues beyond social insects (Wilson-

Rich et al. 2009; Cotter and Kilner 2010). When referred to here, social immunity broadly describes the combined effects of individual-level and group-level parasite resistance mechanisms, which act together to resist or reduce the impact of parasite infections on the colony. For example, one group-level external social-immune defence is the thermoregulatory behaviour of honeybees. Several bee parasites are sensitive to temperature, and honeybees may elevate the nest temperature prior to the establishment of infection which has been suggested to be an adaptive response to kill parasites (Starks et al. 2000; Thomas and Blanford 2003; Campbell et al. 2010). This warmer temperature may however benefit the parasite (Fialho and Schall 1995; Campbell et al. 2010), and so provides an important example of the need to disentangle the effects of host manipulation and disease defence.

#### ***1.2.2.1 The cuticle***

The cuticle of insects covers the entirety of their external surface and acts as an important physical barrier to infection from parasites (Neville 1974; Andersen 1979; Wilson et al. 2001; Siva-Jothy et al. 2005; Moret and Moreau 2012). Its role in parasite defence is recognised from arthropods that undergo moults, where the newly-formed, not-yet-hardened cuticle is substantially more vulnerable to penetration by parasites (Moret and Moreau 2012). In social insects this means that the brood stages may be especially vulnerable due to the reduction of this physical defence. In many ant species, the pupae are enclosed in a cocoon which can act as a proxy physical barrier until the cuticle is fully hardened, through melanisation (Tragust, Ugelvig, et al. 2013; see also the phenoloxidase cascade:



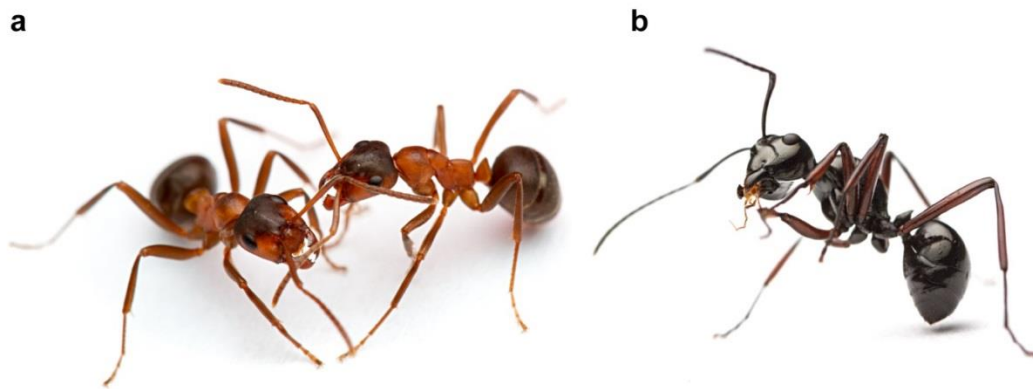
1.2.2.7). Similarly, the cuticle is formed of different layers and its structural characteristics range from tough and brittle to highly flexible, and in a range of thicknesses (Hopkins and Kramer 1992; Siva-Jothy et al. 2005). It is these thin flexible points, for example around joints, which are often most vulnerable. Although bacteria may penetrate the cuticle, fungi present the greatest risk and possess both physical and chemical methods to penetrate the cuticle of their prospective hosts (Khan and Aldrich 1973; Hänel 1982; St Leger et al. 1991). The primary defence of the cuticle itself to these infections is likely through physical thickness and the degree of sclerotisation (Wilson et al. 2001; Andersen 2010; Moret and Moreau 2012), but there are important synergistic effects with antimicrobial compounds which are spread actively or passively over the surface of the cuticle (Hopkins and Kramer 1992; Andersen 2010; Otti et al. 2014). Insects may increase the melanisation of their cuticle in response to increasing populations densities, perhaps in a prophylactic attempt to counteract the additional threat of parasite transmission (Barnes and Siva-jothy 2000; Gillespie et al. 2000; Wilson et al. 2001). However, bees infected with mites showed little change in exoskeleton investment (Daly et al. 2015). Although cuticular defense may be simplistic compared with internal physiological defences, its role in parasite resistance is not well studied in social insects, which can demonstrate considerable variations in cuticle morphology (Neville 1974; Moret and Moreau 2012). This is an area in need of detailed work in order to fully determine the role the cuticle plays in disease resistance in social insects.

### ***1.2.2.2 Grooming***

Grooming is a complex, multipurpose behaviour, but which serves a major role in the cleaning of the external surface of an insect (Figure 1.4). Ants and bees possess tibial spurs which they may use as an antennal cleaner, or may simply rub together legs or body parts to physically remove particles (Basibuyuk and Quicke 1999). Honeybees use their mouthparts to remove parasitic mites from the body of nestmates but do not actively ingest them (Peng et al. 1987). Ants may ingest debris groomed from the body of themselves or their nestmates, which they can filter out using a specialised infrabuccal filter to produce a pellet of waste that is accumulated in an infrabuccal pocket and then regurgitated (Quinlan, R. J. Cherrett 1978; Little et al. 2006). In termites, spores groomed from the body are completely ingested, but may then be inactivated by gut microorganisms (Yanagawa et al. 2008). The smallest caste of leaf-cutting ants ‘hitchhike’ on leaf-fragments being brought into the colony by returning foragers, defending foragers against macroparasites such as phorid flies, and also cleaning the leaves of microparasitic fungi through grooming (Feener and Moss 1990; Orr 1992; Griffiths and Hughes 2010). Additionally ants and bees returning to the nest will stop and increase grooming prior to returning, and contaminated individuals may even be disallowed entry, through nest guarding behaviours (Drum and Rothenbuhler 1985; Morelos-Juárez et al. 2010; see also 2.3.2 nest compartmentalisation)

These actions in social insects can be highly effective in the mechanical removal of parasites including mites, nematodes, fungi and bacteria, from exposed individuals (Kermarrec 1975; Oi and Pereira 1993; Spivak 1996; Rosengaus et

al. 1998, 2000; Hughes et al. 2002; Traniello et al. 2002), and many studies document upregulation of grooming in response to parasites (Rosengaus et al. 1998; Currie and Stuart 2001; Walker and Hughes 2009; Ugelvig et al. 2010; Reber et al. 2011; Tragust, Mitteregger, et al. 2013).



**Figure 1.4.** Grooming defensive behaviours in ants. a) Allogrooming involves the grooming of one ant by another as seen in these *Formica rufa* workers, b) selfgrooming is performed by an individual social insect, such as this *Polyrhachis delecta* worker, and can be highly effective at removing parasites from the cuticle.

Grooming may remove ectoparasitic mites which are known vector of viral diseases, but there is no evidence that the behaviour directly lowers the risk from viruses (Evans and Spivak 2010). Experimental suppression of grooming behaviour can increase mortality after parasite exposure, demonstrating the direct fitness benefits of grooming (Kramm and West 1982; Shimizu and Yamaji 2003; Yanagawa et al. 2008). In particular, some pesticides such as Imidacloprid suppress grooming responses and in so doing increase the susceptibility of ants to infection (Galvanho et al. 2013). Rapid removal of threats from the external surface of the insect is vital to stop penetration of the host and successful infection.

Allogrooming involves the grooming of one individual by another and is important in ant and termite social immunity for group-level removal of fungal conidia (Rosengaus et al. 1998; Yanagawa et al. 2008; Chouvenc et al. 2010; Graystock and Hughes 2011; Okuno et al. 2011). Allogrooming is especially useful as it allows for cleaning of areas otherwise inaccessible to selfgrooming. Both ants and termites allogroom nestmates as part of the maintenance of general nest hygiene, which may be influenced by group size, and parasite risk (Okuno et al. 2011), or as proactive prevention of disease (Schmid-Hempel 1998). For example, leaf-cutting ants routinely groom returning foragers that are more likely to be contaminated with parasites (Morelos-Juárez et al. 2010). Ants (Liersch and Schmid-Hempel 1998; Walker and Hughes 2009), bees (Arathi et al. 2000; Wilson-Rich et al. 2009), wasps (Sumana and Starks 2004; Turillazzi 2012), and termites (Rosengaus et al. 1998; Traniello et al. 2002) also show directed allogrooming in response to pathogen exposure. The behaviour thus seems to be a readily observable and quantifiable trait which correlates with parasite risk.

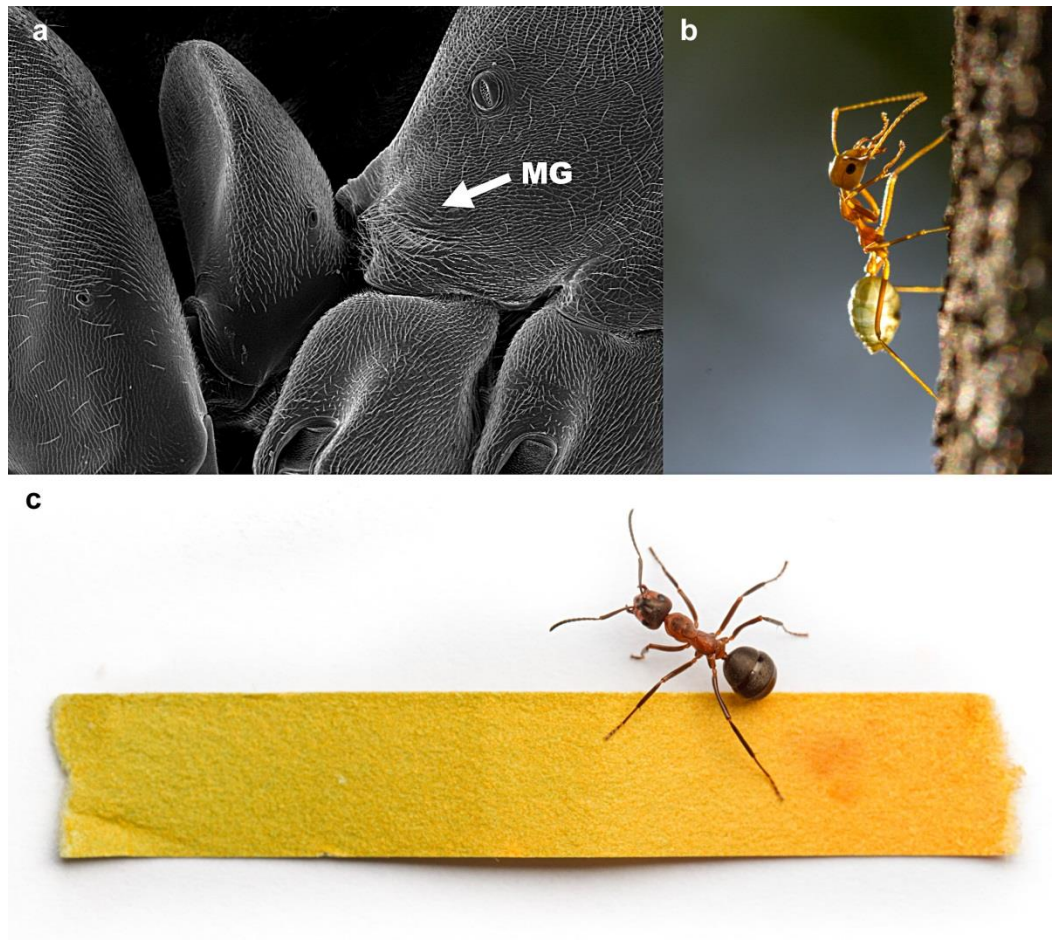
Because ants may spend a considerable proportion of their time grooming (Cole 1986), the behaviour is likely to be traded-off with other activities. Allogrooming may also present conflicting costs and benefits at different levels within the colony, as although there may be a group-level benefit in terms of overall disease reduction, grooming may carry an individual-level cost in terms of exposure to the parasite. (Rosengaus et al. 1998; Schmid-Hempel 1998). However, current experimental data from leaf-cutting ants suggests that the cost to the grooming individual in terms of increased transmission may be minimal (Hughes et al. 2002). It seems that individual ants exposed to fungi may up-regulate self-grooming but decrease allogrooming of nestmates, which models

predict will thereby provide disease resistance to the individual whilst minimising disease spread (Theis et al. 2015).

### ***1.2.2.3 Antimicrobials***

Social insects possess a variety of secretory glands (Wilson 1965; Billen 2009, 2011; Rosengaus et al. 2011; Adams et al. 2012; Figure 1.5). There is significant interspecific variation in the secretions of a particular gland between species, with closely related species producing a largely unique combination of chemical components that are important not just in communication, but also recognition, trail finding, and disease resistance (Attygalle and Morgan 1984; Adams et al. 2012). In some cases these gland secretions may be used as an external antimicrobial defence in social insects, in order to maintain hygiene at both individual and group levels (Attygalle and Morgan 1984; Tragust, Mitteregger, et al. 2013; Otti et al. 2014; Tranter et al. 2014; Tranter and Hughes 2015).

In leaf-cutting ants, mandibular secretions contain substances which inhibit fungal (North et al. 1997; Marsaro Júnior et al. 2001; Rodrigues et al. 2008) and bacterial growth (Mendonça et al. 2009), and sterilize the infrabuccal pellet that is produced during grooming (Little et al. 2006). Similarly termites produce proteins in their saliva which are incorporated into their nest environment and are active against gram-negative bacteria (Bulmer et al. 2009; Rosengaus et al. 2011).



**Figure 1.5.** Antimicrobial glands and secretions. a) The metapleural gland reservoir or 'bulla' is located at the end of the propodeum and can be seen externally as in this Electron Micrograph image of a *Formica rufa* worker, b) *Oecophylla* workers like many social insect species possess antimicrobial venom which they can expel from a venom gland in their gaster. c) This venom is highly acidic as shown by the colour change of this pH indicator paper placed with a *Formica rufa* worker.

Termites also secrete terpenoids and other chemicals from their cephalic and sternal glands which have antimicrobial activity (Rosengaus et al. 2011). In many species of ants the role of the venom, or poison, gland, which secretes high concentrations of formic acid for use in chemical defence in formicine species, aids

in disease resistance through its antimicrobial properties (Blum 1992; Graystock and Hughes 2011; Tragust, Mitteregger, et al. 2013; Otti et al. 2014; Tranter et al. 2014; Tranter and Hughes 2015). Antimicrobial venom has also been demonstrated in bees (Baracchi and Turillazzi 2010; Baracchi et al. 2011), and wasps (Turillazzi et al. 2006; Baracchi, Mazza, et al. 2012).

The metapleural glands (MG) are a pair of exocrine glands found only in the ants, which has been extensively studied for its production of antimicrobial compounds in many species (Brown 1968; Beattie et al. 1986; Hölldobler and Wilson 1990; Angus et al. 1993; Ward 2007; Yek and Mueller 2011; Adams et al. 2012). Antimicrobial activity of the metapleural gland secretion has been documented against both bacteria (Iizuka et al. 1979; Veal et al. 1992), and fungi (Beattie et al. 1985, 1986; Bot et al. 2002; Poulsen, Bot, Nielsen, et al. 2002; Fernández-Marín et al. 2006, 2009). Importantly, experimental closure of the metapleural gland results in significantly reduced resistance of ants to the pathogenic fungus *Metarhizium anisopliae*, proving the importance of the gland *in vivo* for disease resistance (Poulsen, Bot, Nielsen, et al. 2002; Tranter et al. 2014). Both venom and the metapleural gland secretions in ants may be actively spread onto the cuticle via specialized grooming (Brown 1968; Basibuyuk and Quicke 1999; Currie and Stuart 2001; Fernández-Marín et al. 2006). This behaviour allows antimicrobials to be actively dispersed and also allows for regulation of dispersal through variation in the extent of grooming.

Recently a number of studies documenting the antimicrobial properties of substances used by honeybees has emerged (McCleskey and Melampy 1939; Bilikova et al. 2001; Fontana et al. 2004; Melliou and Chinou 2005; Alreshoodi

and Sultanbawa 2015; Bílikova et al. 2015; Brudzynski and Sjaarda 2015). This work has identified multiple antimicrobial peptides from royal jelly (a honey bee secretion that is used in the nutrition of larvae) that show broad activity against bacteria, including important bee parasites such as foulbrood, and may work synergistically with other antimicrobials (Romanelli et al. 2011; Alreshoodi and Sultanbawa 2015). Honey itself has long been known to possess antimicrobial properties, and molecular and chemical analyses have begun to isolate specific proteins and chemicals responsible (Viuda-Martos et al. 2008; Brudzynski and Sjaarda 2015). Propolis (a resinous substance that bees collect from the exudates of plants) also demonstrates *in vitro* antimicrobial effects, and colonies bred for increased propolis production showed increased brood viability and lifespans (Banskota et al. 2001; Viuda-Martos et al. 2008; Boonsai 2014; Campos et al. 2014; Marques et al. 2014; Nicodemo et al. 2014; Lopez and Lourenço 2015). Colonies of bees exposed to the chalkbrood parasite may up-regulate resin collection as a form of ‘self-medication’ (Simone-Finstrom and Spivak 2012). There is also evidence that exposure to this propolis resin can affect immune gene expression in larvae, in addition to lowering bacterial loads (Simone et al. 2009).

As well as propolis in bees, ants can also use externally derived antimicrobials for immune defence. The wood ant *Formica paralugubris* uses antimicrobial resin in nest construction, and this resin can reduce microorganism loads (Christe et al. 2003). The resin increases survival of individual ants when they are exposed to fungal and bacterial parasites, and could lead to a relaxation in their internal immune function (Christe et al. 2002; Chapuisat et al. 2007; Castella, Chapuisat, and Christe 2008; Castella, Chapuisat, Moret, et al. 2008; Brüttsch and Chapuisat 2014). Similarly, there is active use of antimicrobials in



the facultatively social earwig, which uses faeces to line the soil of their burrows (Diehl et al. 2015). Some ants may actively ingest ‘reactive oxygen species’ compounds which, although harmful to the individual, can provide a net benefit through disease defence; healthy ants will avoid these substance, but infected ants will actively consume them to self-medicate, and in sodoing improve their survival (Bos et al. 2015).

#### ***1.2.2.4 Symbiont defense***

Fungus-growing attine ants possess a vertically transmitted bacterial ectosymbiont which forms white blooms on their cuticle (Little et al. 2006). This symbiotic bacteria produces antimicrobial compounds which are highly active and specialized against the *Escovopsis* fungus which parasitizes the ant’s fungal food crop (Currie, Scott, et al. 1999; Currie 2001). Attine ants possess specialised structures for culturing the filamentous bacteria and show a highly developed relationship with these mutualists which they employ as a parasite defense mechanism (Zhang et al. 2007). Similarly there is recent evidence that *Allomerus* ants, which also cultivate beneficial fungi, possess similar bacteria on their cuticle which can produce antimicrobial compounds, though the extent of their use and mutualistic relationship is currently less clear (Seipke et al. 2012; Gao et al. 2014). These symbiotic actinobacteria are also found in a solitary bee species and many other insect species, but are utilised in social insects for group level-defense (Kaltenpoth 2009; Souza et al. 2012).

Although not well studied compared to in vertebrates, the gut biota of social insects may also have an important immunological role (Dillon and Dillon 2004) . Studies mainly from bees (Koch and Schmid-Hempel 2011; Vásquez et al. 2012), and termites (Chouvenc et al. 2010; Chouvenc and Su 2010, 2012; Sen et al. 2015), have shown a diverse gut microflora that includes many bacteria important in defence from microbial threats. These bacterial communities are different in social and solitary species, and their infections are established by exposure to faeces from nestmates after pupal eclosion (Koch and Schmid-Hempel 2011; Korb et al. 2015).

It has been demonstrated that *Wolbachia*, a common symbiont in many insects lineages, can provide its host with increased resistance against a range of viral infections and thus constitutes an additional level of host antiviral defence (Martinez et al. 2014). However this work has focussed on the model organisms *Drosophila melanogaster* and *Aedes aegypti*, and the study of symbionts for defence against parasites, in particular viral infections, and their consequences for host fitness has been little studied in social insects.

#### ***1.2.2.5 Internal immunity and physiological defences***

Classically, internal, physiological insect defences can be split into cellular and humoral immunity (Siva-Jothy et al. 2005; Viljakainen 2015). The Toll and Imd, and multipurpose JAK/STAT, signalling pathways work synergistically in insect humoral immunity and are triggered in response to the recognition of microbes, particularly bacteria and fungi (Kingsolver and Hardy 2012; Johnson 2015) or

cell damage (Dostert et al. 2005). This results in multiphase signal transduction and the secretion of antimicrobial peptides, lysozymes and general antimicrobial substances. The cellular component of the insect immune system consists of haemocytes which may aid in phagocytosis or encapsulation of foreign bodies through the activation of phenoloxidase. Viruses also represent important threats and have been confirmed in all major social insect groups including bees (Chen and Siede 2007; Oldroyd and Fewell 2007; McMahon et al. 2015), wasps (Rose et al. 1999), ants (Avery et al. 1977; Valles et al. 2004, 2007) and termites (Gibbs et al. 1970), with often detrimental outcomes for their hosts species. Comparatively little is known about the defence mechanisms social insects utilize against viral infections. In social insects the innate response to viral infection seems independent of the antimicrobial and antifungal responses associated with the Toll and Imd pathway (Azzami et al. 2012), which may not be the case for insects in general (Vodovar and Saleh 2012). RNA interference may also plays an important role in the specific defense against viruses (Nazzi et al. 2012). The RNAi response is triggered by the presence of double-stranded RNA (dsRNA), which is a virus specific, and ultimately results in the direct degradation of the viral genome utilizing the RNAi machinery (Hannon 2002) to limit virus replication, and consequently damage to the host.

#### ***1.2.2.6 Antimicrobial peptides***

Antimicrobial compounds are important components of the insect innate immune response (Bulet et al. 1999). Insect antimicrobial peptides can be grouped into three main categories based on their chemical composition. The first are the cysteine-containing peptides that include defensins and drosomycins. Insect

defensins are most active against Gram-positive bacteria, with limited activity reported against Gram-negative bacteria and fungus, and are one of the few compounds well defined in ants (Bulet et al. 1999). Drosomycin, however, has strong antifungal properties but has so far only been found in *Drosophila* and as analogous sequences in some coleopterans (Zhang and Zhu 2009). Both compounds are quick-acting and kill microorganisms within a few minutes of contact (Bulet et al. 1999). The other two categories of insect antimicrobial peptides are the proline-rich peptides and glycine-rich molecules, such as gloverins and attacins. These antimicrobial peptides are much slower acting (Bulet et al. 1999). Proline-rich peptides isolated from Hymenoptera haemolymph include apidaecins and abaecins (Casteels et al. 1990, 1993; Schlüns and Crozier 2009), as well two formaecins detected in the metapleural gland secretion of the bulldog ant *M. gulosa* (Mackintosh et al. 1998). Combinations of these AMPS have important defensive interactions with Gram-negative bacteria (Viljakainen 2015; Rahnamaeian et al. 2015). Another important antimicrobial protein is the enzyme lysozyme that breaks down bacterial cell walls by hydrolysing linkages in peptidoglycan (Gillespie et al. 1997). In insects, its presence in the haemolymph usually increases after infection (Hultmark 1996), and is thought to be one of the primary components in insect disease response (although its role in *Drosophila* may be more focussed on digestion; Daffre et al. 1994). Like other antimicrobial compounds lysozyme is mostly produced in the fat body, and to a certain extent from circulating haemocytes, and released into the haemolymph (Hultmark 1996; Vizioli et al. 2001). Termite nymphs and workers exposed to the entomopathogenic fungus *Metarhizium anisopliae* up-regulate the levels of existing, and induce production of novel, protective antimicrobial proteins (Rosengaus et al. 2007).

### ***1.2.2.7 Haemocytic defence and the phenoloxidase cascade***

Social insects possess three main types of general insect haemocyte, prohaemocytes, plasmocytes and granulocytes (Lavine and Strand 2002; Wilson-Rich et al. 2009). Although a number of studies have counted humoral haemocyte numbers (Ashida and Brey 1995; Baer et al. 2006; Wilson-Rich et al. 2008, 2012), often inferring levels of immunity from these, there has been little descriptive or experimental work on their role in social insects. These three cell types are known to work together and constitute the cellular response in a paper wasp and the honeybee (Manfredini et al. 2008; Negri et al. 2015), and correlate with infection time in a termite (Avulova and Rosengaus 2011), but much more work is needed. From other insects, plasmocytes are known to aggregate together to form nodules on foreign bodies, marking them for removal. Similarly plasmocytes may differentiate into flat lamellocytes which encapsulate a foreign body (Siva-Jothy et al. 2005; Wilson-Rich et al. 2009).

Cuticular melanin is produced through enzymatic oxidation of tyrosine derivatives to quinones, and their resulting polymerisation, in a process known as the prophenoloxidase (proPO) cascade (Ashida and Brey 1995; Wilson et al. 2001). This process is not only a vital structural component of the cuticle but is important in broad-action insect innate responses including cellular and humoral encapsulation and nodule formation (Ashida and Brey 1995; Siva-Jothy et al. 2005). PO also produces reactive quinone intermediates and oxygen species which are toxic to invading microorganisms (Zhao et al. 2007). Prophenoloxidase is abundant in the insect haemolymph and cuticle (Ashida and Brey 1995). It is activated to phenoloxidase (PO) by serine proteases through immune triggers

such as penetration of the cuticle or the presence of foreign cellular components. Insects bred for reduced disease susceptibility exhibit higher PO activities and increased PO levels following exposure to a parasite (Paskewitz et al. 1989; Nigam et al. 1997; Reeson et al. 1998). Artificially reducing proPO levels in non-social insect species has resulted in reduced melanisation and a lessened ability to resist bacterial infection (Shiao et al. 2001; Liu et al. 2007), while termites show variation in their encapsulation response to infection by the fungal parasite *Metarhizium* and also variation in the physiological costs this imposes (Chouvenc et al. 2010; Chouvenc, Su, et al. 2011). Thus cuticular melanisation, encapsulation response and PO levels may be a useful indicator of investment into disease resistance, particularly in social insects which experience high parasite pressures (Barnes and Siva-jothy 2000; Wilson et al. 2001; Chouvenc, Su, et al. 2011).

**Figure 1.6.** Brood care in ants (*overleaf*). Brood are particularly vulnerable to the threat of disease as they lack behavioural and developed physiological immunity and so rely heavily on adults for their care a) *Dinoponera quadriceps* worker with pupae, b) *Polyrhachis* brood, adults of c) *Sericomyrmex amabilis*, d) *Formica fusca* and e) *Acromyrmex echinator* tending to brood.



**a**



**b**



**c**



**d**



**e**



### **1.2.3 Intracolony group defences: tolerating disease and reducing transmission**

#### ***1.2.3.1 Hygienic behaviour***

The greatest threat of transmission will occur within the densely populated, homogenous environment, within the nest, and consequently social insects attempt to minimise the initial entry of a parasite. However, once inside the colony social insects are able to minimise the threat of disease through removing the threat, or rendering it less harmful. Initially behaviours are employed which inform other members of the presence of a parasite. In bees, individuals which have detected a threat may perform a ‘grooming dance’ that elicits allogrooming from nestmates (Rath 1999; Peng et al. 2002), and termites exhibit vibrational displays that act as a parasite alarm (Rosengaus et al. 1999). Once detected by the collective, the threat can then be reduced or removed. For example honey bees infected with deformed-wing virus are detected and removed from the colony (Baracchi, Fadda, et al. 2012; Schöning et al. 2012). The removal of cadavers, ‘necrophoric’ or ‘undertaking behaviour’ (Sun and Zhou 2013), is displayed in ants (Haskins and Haskins 1974; Howard and Tschinkel 1976; Julian and Cahan 1999; Choe et al. 2009; Renucci et al. 2010; Diez et al. 2012; Diez, Le Borgne, et al. 2013), bees (Visscher 1983; Trumbo and Robinson 1997; Spivak and Gilliam 1998), and termites (Chouvenc, Robert, et al. 2011; Chouvenc and Su 2012; Neoh et al. 2012), along with burial and cannibalism behaviours (Marikovsky 1962; Zoberi 1995; Myles 2002; Su 2005; Renucci et al. 2010). In ants, cadavers are carried away more rapidly than other waste in the nest and are



disposed of at specific sites (Wilson et al. 1958; Gordon 1983), and ant cadavers infected with fungi will be moved even further away than those uninfected (Diez et al. 2015). Similarly bees will remove old corpses more rapidly than freshly killed workers (Visscher 1983). In termites, cannibalism is performed more often on individuals with higher spore concentrations of the entomopathogenic fungus, *Metarhizium anisopliae* (Rosengaus and Traniello 2001). Additionally termites may display density-dependent, or time-dependent, switching between cannibalism at low levels of mortality and burial at higher levels (Chouvenc and Su 2012; Sun et al. 2013), and ants show differing necrophoric responses to different brood and workers (Figure 1.6). In addition termites may bury cadavers in soil mixed with saliva, secretions or faecal material with antimicrobial properties (Chouvenc and Su 2010; Chouvenc, Robert, et al. 2011). The removal of corpses is important for breaking the transmission cycle (Diez et al. 2014, 2015). Bees will also proactively remove larvae infected by parasitic fungi, bacteria or mites (Spivak and Gilliam 1998; Spivak and Reuter 1998). Interestingly this hygienic behaviour in bees is highly heritable, with selective breeding producing lines of ‘hygienic colonies’ that are more successful in disease resistance (Spivak and Gilliam 1998; Spivak and Reuter 1998; Arathi and Spivak 2001; Arathi et al. 2006).

Although many hygienic behaviours are inherently risky to the individual carrying them out whilst decreasing the group-level threat, one of the most extreme examples of hygienic behaviour is ‘self exclusion’ (Shorter and Rueppell 2012). This is where sick ants (Heinze and Walter 2010; Chapuisat 2010), or bees (Rueppell et al. 2010), will leave the colony in a form of altruistic self-sacrifice to minimise the transmission of disease to their nestmates (Trail 1980).

Bees infected with parasitic mites show a reduced ability to navigate back to the hive which has been hypothesized to be an adaptive behaviour to stop introduction of the threat into the colony (Kralj and Fuchs 2006, 2009; Higes and Martín-Hernández 2008). However, it is very possible that being infected has negative effect on the bee's navigation ability in general, both physically and neurobiologically, and the effect is not necessarily an adaptive behaviour. Some parasites are however skilled manipulators of host behaviour (Libersat et al. 2009), for example a liver fluke (*Dicrocoelium dendriticum*) can hijack the navigational system of its host ant, *Formica fusca*, causing it to climbing to the tip of a blade of grass, thereby adaptively promoting its transmission via consumption by the vertebrate final host (Moore 1995; Poulin 1995). This highlights how difficult it can be to disentangle host adaptation from coincidental side-effects or parasite adaptations, especially where fitness effects are hard to measure.

### ***1.2.3.2 Compartmentalisation and division of labour***

The structured organisation of a colony is a quintessential trait in most social insects; involving complex physical and social division within the colony (Hölldobler and Wilson 1990; Bourke and Franks 1995; Ross and Keller 1995). The highly developed waste management strategies employed by ants involves the synergistic partitioning of both behaviours and nest compartmentalisation to isolate potentially hazardous waste through group level organisational immunity (Hart and Ratnieks 1998, 2001; Bot, Currie, et al. 2001). The strict division of labour can minimise the risk of within-colony transmission by separating tasks

that pose a high risk of infection (waste tenders, foragers), from those that interact with the most vulnerable areas of the colony (brood and queen-tenders) (Wang and Mofller 1970; Hart and Ratnieks 2001; Naug and Camazine 2002; Hart et al. 2002; Baer and Schmid-Hempel 2003; Cremer et al. 2007; Naug 2008; Baracchi and Cini 2014). This may result in spatial separation of different caste-types within the nest (Appendix 1). In leaf-cutting ants this separation can be enforced by aggressive behaviours directed towards waste-heap workers (Hart and Ratnieks 2001; Ballari et al. 2007; Waddington and Hughes 2010; Abramowski et al. 2011). Nest architecture and environment itself may also influence parasite pressures within a colony (Schmid-Hempel 1998). Models suggest that more complicated nest structures result in both temporal and spatial heterogeneity which reduces the opportunity for disease transmission (Pie et al. 2004). This effect can also work in conjunction with plastic avoidance behaviours where sections, or even whole nests, may be abandoned if environmental parasite loads become too great (Marikovsky 1962; Epsky and Capinera 1988; Drees et al. 1992; Oi and Pereira 1993). Compartmentalising the nest can also reduce the number and virulence of strains within the nest by minimizing the selective effect of between-strain competition (Ulrich and Schmid-Hempel 2015).

### ***1.2.3.3 Genetic diversity***

Haplodiploidy in hymenopterans results in high coefficients of relatedness between female nestmates, and because all the offspring in most insect societies are borne from a single, singly mated queen, there is limited genetic variation and

consequently the possibility of increased susceptibility to disease. It is thought that heterozygosity in disease resistance loci improves immune response to parasites, but because haploid males cannot be heterozygous, males may be intrinsically more vulnerable to disease (known as the ‘haploid susceptibility hypothesis’). However, males may alternatively have undergone relaxation in selection for disease defences because their lives within the colony involve minimal risks of parasite exposure and, unlike the queens they mate with, there is no fitness advantage to surviving after they have finished mating. Indeed male leaf-cutting ants have significantly lower innate immune responses than workers (Baer et al. 2005).

Within the worker population, the impact a parasite has on a colony can be reduced if individuals within the group are composed of multiple genotypes, which vary genotypically in their resistance to disease (Poulin 2007). Genetic diversity may hinder transmission as parasites adapted to infect one genotype may be less successful at infecting others, and result in an overall ‘herd immunity’ where the proportion of susceptible individuals is low enough that the disease epidemic cannot be maintained (Anderson and May 1978). Similarly, the presence of multiple genotypes will in general hinder the evolution of parasites. Additionally high genetic diversity improves the probability that resistant individuals will occur, and these resistant individuals may then be able to offer protection to susceptible individuals through social immunity (Hamilton 1987; Harpur et al. 2014). Genetic variation in disease resistance has been demonstrated in bumblebees, honeybees and ants (Baer and Schmid-Hempel 2003; Palmer and Oldroyd 2003; Hughes and Boomsma 2004b; Hughes et al. 2010). Experimentally produced polyandry has been studied in artificially inseminated

bumblebees, where more genetically diverse colonies were less affected by disease (Baer et al. 1999; Baer and Schmid-Hempel 2003). Additionally genetic diversity in bumblebees can reduce the load of parasitic mites (Whitehorn et al. 2014). In honeybees, results relating genetic diversity to disease resistance have been more conflicting. Studies have demonstrated both a reduction in the variation of disease prevalence (Tarpy 2003; Palmer and Oldroyd 2003; Tarpy and Seeley 2006; Invernizzi et al. 2009; Evison et al. 2013; Desai and Currie 2015), and in some cases no effect (Page et al. 1995; Neumann and Moritz 2000; Wilson-Rich et al. 2012; Lee et al. 2012), in response to variations in genetic diversity. Reduced heterozygosity and allelic diversity due to inbreeding has a negative effect on social immune response and survival in termites (Calleri et al. 2006). In the ants there has been evidence for genetic variation in disease resistance from experiments on leaf-cutting ants. Worker nestmates from different patriline of the polyandrous species *Acromyrmex echinator* vary in their resistance to the parasite *Metarhizium anisopliae* (Hughes and Boomsma 2004b), and in the size of their antimicrobial-producing metapleural glands (Hughes et al. 2010). As these individuals shared a common environment and differed only in their paternal genotype, the patriline differences are strongly indicative of being due to the different paternal genotypes. Genetics has also been shown by a cross-fostering experiment in leaf-cutting ants to influence both individual immunity and the size of the metapleural gland (Armitage et al. 2011). Additionally when leaf-cutting ants were placed into high and low genetic diversity groups, resistance to a fungal parasite was highest in the genetically diverse group (Hughes and Boomsma 2004b). Lower levels of genetic diversity also reduce the effectiveness of behavioural responses to disease in *Cardiocondyla obscurior*, with inbred colonies

having a compromised ability to detect and remove diseased brood (Ugelvig et al. 2010)

#### ***1.2.3.4 Social insect genomics***

In insects, the genetic and molecular basis for insect immune responses has been best studied in *Drosophila melanogaster* (Sackton et al. 2007), but in general the genetic mechanisms for innate responses are thought to be similar across insect taxa (Gillespie et al. 1997). The first studies on social insect genomics in the honeybee *Apis mellifera* suggested that it has a reduced set of immune genes compared with *Drosophila*, and it was hypothesised that was because colony-level defences allowed reduced investment in individual-level defences (Evans et al. 2006). However, it is also possible that insects as a whole possess only a limited suite of immune-related genes and that *Drosophila* represents an anomaly (Fischman et al. 2011). Recently, published genome sequences from bumblebees (Sadd et al. 2015), solitary *Nasonia* wasps (Werren et al. 2010), the solitary pea aphid *Acyrtosiphon pisum* (Consortium and others 2010), and several ant species (Bonasio et al. 2010; Smith, Smith, et al. 2011; Smith, Zimin, et al. 2011; Wurm et al. 2011; Nygaard et al. 2011; Gupta et al. 2015) have also shown a small number of immune genes, and thus sociality now does not appear to be the predictor of the number of immune genes that was originally suggested (Fischman et al. 2011). Recent genomic studies in termites similarly found no evidence for enrichment of immune defense genes, but did find the existence of termite-specific AMPs and evidence of positive selection of many of these genes in the soil-foraging species (Lamberty et al. 2001; Bulmer and Crozier 2006;

Bulmer et al. 2010; Korb et al. 2015). The number of AMP genes in bumblebees is reduced, a state that appears to be basal in bees, and predates the evolution of sociality (Sadd and Schmid-Hempel 2006; Barribeau et al. 2015).

Social insects do, however, seem to show examples of fast rates of molecular evolution and evidence that sociality has driven selection on immune genes. Selection on genes can be inferred from comparisons with the substitution rates in related non-immunity genes or from the overall ratios of nonsynonymous to synonymous substitutions (Graur and Li 2000; Viljakainen et al. 2009). Because advantageous mutations reach fixation more quickly than neutral changes, and nonsynonymous substitutions change the function of a coded protein, the rate of nonsynonymous substitutions should exceed that of synonymous substitutions if positive selection for an advantageous mutation is occurring (Graur and Li 2000). Molecular evolution of immune genes in social insects may be likely to show evidence of positive selection if parasite pressures are great. In other species selection on immune genes may be relaxed if they experience a reduced environmental parasite threat. If nucleotide substitution rates are related to the stringency with which a structural protein coding region is maintained, then there may be observable increases in the rate of nucleotide substitutions in genes with a relaxation in their functional use (Graur and Li 2000). Concordantly, rates of evolution may vary between lineages depending on the relative pressures that different species experience to maintain and express disease resistance traits. Termites show strong positive selection in the duplicated antifungal peptide termicin (Bulmer and Crozier 2006). Bee (Xu and James 2012; Harpur and Zayed 2013; Zayed and Kent 2015; Barribeau et al. 2015; Sadd et al. 2015), and ant defense genes show evidence of positive selection

based on dN/dS values and population genetics studies (Viljakainen and Pamilo 2005, 2008; Viljakainen et al. 2009; Roux et al. 2014; Gupta et al. 2015). In honeybees, however, some changes in immune genes seem to be best explained by relaxed selection (Wallberg et al. 2014; Zayed and Kent 2015). It is possible that social insects could experience more rapid rates of evolution due to their small effective population sizes which leads to faster fixation of mutations due to drift (Bromham and Leys 2005). However this rate should be uniform across the genome and current studies indicate faster rates of substitution in immune genes (Viljakainen and Pamilo 2008; Viljakainen et al. 2009), which lends weight to the inference of positive selection on immune genes. Some of these immune genes may also be differentially expressed, with AMP genes up-regulated more strongly in larvae compared with adults in immune-challenged *Camponotus floridanus* ants (Gupta et al. 2015). The differential expression of immune traits between individuals and life-stages, along with the increasing number of published genomes, makes social insects good models for studying the epigenetics of immunity in the future (Yan et al. 2014).

### 1.3 Factors driving parasite defence

The life-histories of social insects are both shaped by parasite pressures, and themselves, help shape the external threat of disease they face.

**Figure 1.7.** Colony sizes and modes of transmission (*overleaf*). a-b) Army ants such as *Eciton*, pictured here, can reach colony sizes of many millions and reproduce through colony fission, unlike most ants c) which reproduce through the nuptial flight of winged sexuals, such as this *Tapinoma* queen. d) Leaf-cutting ants have large colonies that produce long foraging trails across the forest floor.







### 1.3.1 Colony size

Ant colonies range from just a few tens of individuals in some ponerine species, to populations of many millions in *Dorylus* army ants and *Atta* leaf-cutting ants (Hölldobler and Wilson 1990; Figure 1.7). Larger colonies may be generally better at maintaining a stable thermal optimum, and are typically warmer than smaller colonies in similar ecological conditions (Schmid-Hempel 1998). These conditions may be favourable for raising brood, but they also generally favour parasite survival. Thus although suboptimal external environments may reduce between-colony transmission, internal conditions could benefit maintenance of parasites within the nest, especially in larger colonies (Schmid-Hempel 1998).

More importantly though, larger colonies may also be exposed to greater risk through the presence of a larger number of foragers, which can introduce external pathogens to the nest when they return, and through increases in density-dependent within-colony transmission. These more populous colonies will effectively sample a greater proportion of the external environment and may thus be more likely to be exposed to parasites to which they are susceptible that are rare or scattered in the environment. Larger colonies tend also to be longer-lived, with the potential for more within-colony transmission. Models suggest that colony growth can rapidly increase parasite transmission and that this pathogen risk can substantially reduce the benefits from increasing colony size (Schmid-Hempel 1998). In species with strict division of labour and task partitioning, the increased disease risk associated with larger colonies could be mitigated through separation of, for example, foragers and nest workers. However even in societies with more complex organisation, models and meta-analyses still predict that

larger group sizes will correlate with increased parasite pressures (Alexander 1974; Cote and Poulin 1995; Rifkin et al. 2012; Patterson and Ruckstuhl 2013). Empirical tests from social insects, however, have been inconclusive. Studies in bees and wasps have shown both weak reductions and slight increases in parasite prevalence in larger colonies (Macfarlane and Pengelly 1974; Schmid-Hempel 1998), but comparative studies in ants tend to suggest that there may be a positive relationship between colony size and investment in immune defence (Hughes, Pagliarini, et al. 2008; Fernández-Marín et al. 2009, 2013). Whether this is due to species with larger colonies being able to invest more due to increased energy budgets, or needing to invest more because of greater parasite pressures, is an open question.

### **1.3.2 Social parasitism**

Some species of social insect have evolved to exploit other social insect colonies in a relationship termed ‘social parasitism’ (Buschinger 1986; Hölldobler and Wilson 1990). Because social parasites rely on their host species for many activities including food acquisition and brood care (Hölldobler and Wilson 1990), it seems possible that the parasitic species may also take advantage of their host for defence against other parasites. If socially parasitic species can gain sufficient disease resistance from host secretions, grooming and general hygienic behaviour, then they may be able to invest less of their own resources into disease resistance. The most obligate, ‘inquiline’ social parasites seem to show a number of characteristic ‘symptoms’ as they evolve towards a more specialized degree of host dependence, in what has been termed ‘inquiline syndrome’ (Wilson 1971;

Hölldobler and Wilson 1990). For example, in many social parasites, the energetically costly metapleural gland has been reduced or lost altogether (Sumner et al. 2003; Yek and Mueller 2011). In a sample of 44 socially parasitic ant species from three subfamilies, 67% of inquiline species, but only 9% of temporary parasitic species, showed a loss of the metapleural gland. This is logical, as the temporary parasites would only benefit from host defences for initial colony foundation, and must be sufficiently able to defend against other parasites on their own once the original host workers have died. Thus, investment in disease resistance cannot be relaxed as much in facultatively social parasites as in obligate social parasites which have a closer and longer-term relationship with their hosts. This idea broadly predicts a correlation between the level of host dependency of social parasites and the degree of reduction in their individual disease resistance. This hypothesis will be investigated further in Chapter 7.

### **1.3.3 Living environment**

Ant species nest and forage in a multitude of ecological conditions with a wide range of associated parasite risks. Within ecosystems, bacterial and fungal loads are commonly highest in the soil and much lower on surrounding vegetation (Curtis and Sloan 2002; Keller et al. 2003). Ground-dwelling or subterranean ant species are therefore predicted to suffer greater exposure to environmental parasites than arboreal species. Because parasite pressures may therefore be lower for ants that nest and forage away from the soil, arboreal ants may have evolved to reduce their investment in resistance (Johnson et al. 2003; Schmid-Hempel and Ebert 2003; Baer et al. 2005). However, a study of seven ant

species, exhibiting a spectrum of arboreal nesting and foraging behaviours, did not support this hypothesis (Walker and Hughes 2011). Instead, those species which spent time both in terrestrial and arboreal habitats were most resistant to infection, suggesting that it may be the diversity, rather than quantity, of parasites which is the most important factor in influencing the evolution of disease resistance. A second aspect which may affect disease exposure is nest material. The weaver ant species belonging to *Polyrhachis*, *Camponotus* and *Oecophylla* form colonies in nests weaved from larval silk instead of using substrates obtained from the environment. Although this may seem likely to provide the ants with a more aseptic living environment, in experiments on the weaver species *Polyrhachis dives*, nest material did not seem to confer any increased resistance against a fungal parasite or inhibit *in vitro* fungal growth (Graystock and Hughes 2011). Furthermore, the silk building material harbours large quantities of the opportunist fungal pathogen *Aspergillus flavus*, which infected ants much more commonly when they were kept with the silk nest material than without it (Fountain and Hughes 2011). However, because the weaving behaviour of *Polyrhachis* is relatively primitive, comparisons with other species with more advanced weaving behaviour may produce different results and will be useful in elucidating any relationship between nest weaving and disease resistance (see Chapter 4).

#### **1.3.4 Feeding strategy**

One of the primary routes of ingress for parasites into a host organism is via ingestion (Figure 1.8). This is particularly applicable in insects where the majority of the body is clad in protective cuticle. The midgut is the only area







### **1.3.5 Sexual dynamics and colony reproduction**

The life cycles and composition of ant colonies may share a basic plan but many lineages can express key differences. Where no nest material or other provisions are transported to the new nest, only parasites of adults are able to be vertically transmitted. In most ant colonies, which do not reproduce via fission, vertical transmission is thought to be minimal or only occur in particularly avirulent parasites, as any cost during colony founding is likely to be enough to cause failure. However, in colonies that reproduce via fission there may be additional scope for vertical transmission because colony establishment is much less risky and not only the queen, but also a large number of workers (often 50% of the existing colony (Kronauer et al. 2004)) transfer to the new colony. From the perspective of the parasite this effectively increases the lifespan of the colony, which could lead to an increased accumulation of parasites from the environment and transmission between individuals within a colony (Boomsma et al. 2005). However from an epidemiological perspective, models predict that the force of infection should be inversely proportional to colony age (Schmid-Hempel 1998). Thus long-lived colonies with minimal horizontal transmission, may show a reduced level of infection, not due to efficient disease defence per se, but due to the co-evolutionary dynamics of the infection (Schmid-Hempel 1998).

In most horizontally transmitted parasites, transmission rates are heavily influenced by host density. Therefore in group-living species, especially social insects, which live in very high-population nests with often little genetic diversity, the threat from parasites is often considered very high. However the dynamics of vertically transmitted parasites within social insects are especially complex. The

primary mode of transmission of many endosymbionts, such as *Wolbachia* or *Spiroplasma*, is vertically through generations and through the founding of new colonies by queens (Riesa and Amazineb 2001; Baldo et al. 2008). As the effectiveness of their transmission may rely on the fecundity of the host, the fitness of both parties is especially closely linked (Bull et al., 1991). As a result these endosymbionts, known as reproductive parasites, may alter the biology of their hosts in order to increase their own transmission. For example, *Wolbachia* may feminise hosts or kill males, as it is only transmitted through females (Duron et al. 2008; Werren et al. 2008). Because these parasites are mainly vertically transmitted we may expect them to be particularly prevalent in ants that reproduce via colony fission. However, evidence from army ants, which principally reproduce via fission, suggests if anything that army ants harbour fewer intracellular parasites, particularly *Wolbachia*, than average (Mitteldorf and Wilson 2000; Funaro et al. 2011; Kautz et al. 2013). Honeybees may reproduce at a colony level through the process of swarming, which is the same as fission in army ants. This behaviour is thought to be the main source of vertical transmission within honeybee populations, although some studies have reported decreased parasite loads in swarming, compared with, non-swarming colonies (Fries et al. 2003). Thus the diversity of reproductive strategies in social insects can have complex consequences for parasite risk. Through comparisons of both host and parasite biology it may be possible to infer modes of parasite transmission (Chapter 8).

The traditional view of the colony with a single mated queen, may be complicated by polygyny (colonies have multiple queens) and polyandry (queens mate with multiple males) (Boomsma and Ratnieks 1996; Crozier and Pamilo



1996; Hughes, Ratnieks, et al. 2008). As these reproductive strategies both have considerable costs (Hughes, Ratnieks, et al. 2008), their evolution has been studied in order to ascertain how they have arisen and been maintained and what benefit they afford. Polygyny may arise due to nest site limitation during colony founding, where the advantages gained from allowing help from other queens outweighs fitness loss through shared reproductive output. However, this cannot explain the occurrences of polyandry. Instead it is likely that the increased colony genetic diversity, which results from the queen mating with multiple males, reduces the cost of incompatible matings, improves task specialization and produces a colony more resistant to disease (Boomsma and Ratnieks 1996; Crozier and Fjerdingstad 2001; Oldroyd and Fewell 2007; Hughes, Ratnieks, et al. 2008; Reber et al. 2008). Given the evidence for this from a range of social insects it is likely that life-history traits such as polygyny and polyandry may affect disease resistance through increases in genetic diversity. Consequently, parasite pressures may play an important role in the shaping of reproductive strategies of ant societies, which in turn may change the threat from parasites.

## 1.4 A hierarchy of insects and defences: unifying multilevel defences

**Figure 1.9.** Synergistic defences (overleaf). a) Trophallaxis is a behaviour where substances are exchanged orally between two social insects. This can result in not only the transfer of nutrition but also compounds important in disease defence. B) Hitch-hiking small workers of these leaf-cutting ants are responsible for cleaning leaf-fragments prior to their entry into the nest, which ensures the colony is kept parasite-free.

a



b



The multi-level picture of disease defence mechanisms set-out here is undeniably a simplistic one. There is a great degree of overlap to many defence mechanisms, for example, antimicrobial compounds are actively spread over the cuticle by grooming and AMPs may be spread via trophallaxis (Figure 1.9). Information from the complete spectrum of immune responses: behavioural, physiological chemical and genetic, will help build a more complete picture of the relative importance of each component. Similarly just as it is vital to understand the interplay between the different aspects of defence mechanisms, so too is it important to consider the various levels of selection which operate within social insect communities, in order to build an accurate picture of how traits benefit individuals and the colony (Schmid-Hempel 1998; Keller 1999; Michod 2006; Cremer and Sixt 2009). For example, it may be that the behavioural group-level defences in social insects reduce the selection pressure on individual-level defences. Thus individual defence mechanisms which are important in other, non-social, insect taxa may be less important in social insects.

#### **1.4.1 Synergistic external defences**

Selfgrooming and allogrooming may be used in conjunction with the application of antimicrobial compounds. This is well documented in ants in conjunction with antimicrobial venom or metapleural gland secretions (Veal et al. 1992; Mackintosh et al. 1995; Ortius-Lechner et al. 2000; Bot, Obermayer, et al. 2001; Fernández-Marín et al. 2006; Poulsen et al. 2006; Tragust, Mitteregger, et al. 2013), but also gram-negative binding proteins in termites (Bulmer and Crozier 2006). Social insects are also able to enhance disease resistance through the

transfer of immune factors, such as up-regulated immune effector molecules, to other nest mates (Hamilton et al. 2011). Where this transfer occurs from an exposed individual to a naïve individual, it effectively acts as ‘social immunization’ (Traniello et al. 2002; Fefferman et al. 2007). This is believed to occur during trophallaxis in ants (Figure 1.9): where, during feeding, immune effector molecules as well as liquid food, are shared between two individuals via the mouth (Hölldobler and Wilson 1990; Hamilton et al. 2011). *Camponotus pennsylvanicus* increased its trophallactic behaviour when immune-challenged and also produced regurgitated droplets with increased antimicrobial activity (Hamilton et al. 2011). In this way, an individual-level physiological response can be externalized and distributed to the rest of the colony via social behaviours, ultimately facilitating colony-level disease resistance. These external defences work together to defend not only the individual social insect but also to promote the wider group hygiene.

#### **1.4.2 Immune and behavioural priming**

There is some recent evidence, that much like in the vertebrate immune system, some exposure to parasites can be beneficial for disease defence through ‘immune priming’ (Little and Kraaijeveld 2004). A repeated parasite exposure can affect both the physiological immune system and behavioural social immune responses (Siva-Jothy et al. 2005; Wilson-Rich et al. 2009). Ants are known to up-regulate the expression of social immunity behaviours such as allogrooming after previous contact with fungal parasites (Walker and Hughes 2009; Westhus et al. 2014; but see Reber et al. 2011, where ants upregulated selfgrooming but not

allogrooming), although honeybee undertakers were not found to improve their cadaver removal with experience (Trumbo and Robinson 1997).

Although there is currently no known system in invertebrates that can serve as an immunological memory in the way that leucocyte clonal expansion provides in vertebrates, priming has been shown both in individuals, between individuals and across generations. Trans-generational immune priming, that is increased immunity conferred from parent to offspring, has been demonstrated in bumblebees challenged with bacterial parasites (Sadd et al. 2005). This inherited immunity may also be r in males, which once dispersed will not return to the nest, compared to workers (Sadd et al. 2005; Haine et al. 2008). Both ants (Rosengaus et al. 1999; Traniello et al. 2002; Konrad et al. 2012), and bees (Sadd et al. 2005; Sadd and Schmid-Hempel 2006), show lower susceptibility to reinfection, which may be parasite-specific (Sadd and Schmid-Hempel 2006). This priming can occur during social contact with other nestmates and lead to upregulation of immune defences and increased survival in termites and ants (Rosengaus et al. 1999; Traniello et al. 2002; Konrad et al. 2012). This priming may be up-regulated through the act of mating in the ant *Lasius niger*, where virgin queens showed no beneficial response to priming (Gálvez and Chapuisat 2014). In the ant *C. pennsylvanicus*, this social prophylactic priming seems to be mediated through trophallactic transfer of immune related proteins, which are up-regulated after parasite exposure (Hamilton et al. 2011). Additionally the beneficial gut microbiota of termites are transmitted to young through exposure to the faeces of adult workers (Koch and Schmid-Hempel 2011). This demonstrates how exposure to low level, non-fatal doses, of a parasite can provide a benefit in social insects. This may explain why hygienic behaviour and allogrooming of

contaminated nestmates, which at first seems to have significant costs to the performer, may in fact be adaptive not just for improving group-level, but also for individual-level, defence (Konrad et al. 2012). It also demonstrates how behavioural defences such as grooming and trophallaxis can work synergistically to improve defences at both the individual physiological levels, but also more broadly at the group level through social immunization.

### **1.4.3 Alternative defence strategies**

Because defence mechanisms are costly to express and maintain, in terms of time and energy expenditure, and even in terms of maintaining neural tissue responsible for behaviours, they may be traded-off with other traits (Lochmiller and Deerenberg 2000; Poulsen, Bot, Nielsen, et al. 2002; Sulger et al. 2014). In other organisms this may represent a trade-off with life-span or fecundity (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Siva-Jothy et al. 2005; Gwynn et al. 2005). However in social insects, where the majority of the workforce is sterile, this is more complicated. Trade-offs may be made at the individual level, where for example individual ants may possess a more robust antimicrobial defence but have fewer AMP genes, or at the group level, where for example ants may spend longer allogrooming or cleaning brood or the nest rather than investing in individual defences.

In the fungus-growing ants there is also evidence for trade-offs between the use of antimicrobial symbiotic bacteria, grooming and weeding behaviours, and the use of the metapleural glands (Poulsen, Bot, Currie, et al. 2002; Poulsen



et al. 2006; Fernández-Marín et al. 2009, 2013). Some traits, such as grooming, may be more plastic and can be lost or gained more quickly under selection than physiological defence components. Flexibility in general may be a good strategy to possess. At its simplest, this idea can be seen in social insects which up regulate their defence in response to a threat, instead of maintaining constantly high levels (Rosengaus et al. 2007; Bocher et al. 2007; Tranter et al. 2015). Given accurate detection abilities, these defences can then be employed effectively in various contexts to efficiently combat the threat. Additionally this flexibility allows social insects to respond differently to fungal parasites presented in different contexts, perhaps because they represent differing levels of threats (Tranter et al. 2015).

#### **1.4.4 Summary and thesis aims**

Social insects possess an array of mechanisms to defend themselves against disease. These may be employed at the individual physiological and behavioural levels, but also at the group-level in social insects. However, previous studies into disease resistance tend to have been performed on single species, often looking at just single measures of investment of defence. In this thesis I aim to use ants as a model system in which to explore the comparative importance of disease resistance in different species and at multiple levels. I aim thereby to improve our understanding of how these defences are employed and traded-off, both within individuals and colonies, and between species. Additionally, I hope that by using physiological and behavioural responses as indicators of investment, it may be possible to build a picture of how the life-history of social insect species can both affect, and in turn be affected by, parasite pressures. In Chapter 2 I test the

ability of different ants to detect and recognise parasitic threats in various contexts, and demonstrate how these responses are important in promoting individual survival, and that they may vary between species. Then in Chapter 3 I investigate how ant species may use antimicrobial secretions from the metapleural and venom glands to help keep, not only themselves, but also their brood and nest material hygienic. I extend this in Chapter 4 to focus on comparing how ants may use different strategies, including grooming and antimicrobial secretions, to combat similar threats and highlight the importance of acidic venom as an antifungal agent in social immunity in weaver ants. In Chapter 5 I study how shifts in the quality and quantity of a different defence, the metapleural gland, in a range of Attine ants map to transitions in life-history, specifically colony size and social complexity, within the group. Then I investigate this further in Chapter 6 by comparing the degree of social immunity between ant species with simple and complex societies, and identifying the presence of ‘hygienic personalities’ which can improve individual ant survival. Chapter 7 also looks at how variations in life history can affect investment into disease resistance, but instead of colony size or social complexity, I investigate how parasite pressures and investment into disease resistance can change in socially-parasitic ant species, which rely on their hosts for many aspects of their life history. Then Chapter 8 I present comparative infection data from 83 species of tropical Panamanian ant with different life-histories screened for the presence of the reproductive parasite *Wolbachia*, using molecular typing of strains to elucidate transmission dynamics between host and parasite in this system. Finally in Chapter 9 I discuss the findings of this thesis and their wider implications.



## 2. Threat detection: contextual recognition and response to parasites by ants

Tranter C, LeFevre L, Evison SEF, Hughes WOH (2015) Threat detection: contextual recognition and response to parasites by ants. *Behav. Ecol.* 26:396–405

### 2.1 Abstract

The ability of an organism to detect threats is fundamental to mounting a successful defence and this is particularly important when resisting parasites.

Early detection of parasites allows for initiation of defence mechanisms which are vital in mitigating the cost of infection and are likely to be especially important in social species, particularly those whose life-history makes parasite pressure more significant. However, understanding of the relative strength of behavioural responses in different species and situations is still limited. Here we test the response of individual ants to fungal parasites in three different contexts, for four ant species with differing life-histories. We found that ants from all four species were able to detect fungi on their food, environment and nestmates, and initiate avoidance or up-regulate grooming behaviours accordingly to minimise the threat to themselves and the colony. Individuals avoided fungal-contaminated surfaces and increased grooming levels in response to fungal-contaminated nestmates.

Ants from all species responded qualitatively in a similar way, although the species differed quantitatively in some respects that may relate to life-history differences. The results show that ants of multiple species are capable of recognising fungal threats in various contexts. The recognition of parasite threats may play an important role in enabling ant colonies to deal with the ever-present threat from disease.

## 2.2 Introduction

Organisms possess an array of defences to help combat potential threats from predators and parasites. Organisms can increase their fitness by monitoring predator abundances, parasite levels and habitat stability, and acting accordingly (Hart 1990; Blaustein et al. 2004; de Roode and Lefèvre 2012). In each case detection of the threat is a fundamental prerequisite for the launch of any targeted response or decision. This is particularly key for resisting parasites, whose co-evolutionary arms race with their host can select for better defended and more vigilant hosts, but also for more exploitative and harder to detect parasites (Ebert and Hamilton 1996; Decaestecker et al. 2007). In insects, microparasites such as entomopathogenic fungi are often lethal to the host, and proactive avoidance of exposure is invariably a better strategy than relying on resistance post-exposure (Shah and Pell 2003). Early and accurate detection potentially allows for avoidance of the threat altogether, or at least the initiation of early defence mechanisms, which may be vital in mitigating the cost of the infection (Hart 1990; Schmid-Hempel and Ebert 2003; Wisenden et al. 2009). Although there has been substantial work on the detection and triggering of physiological immune responses (Hoffmann et al. 1996; Medzhitov and Janeway 2000; Siva-Jothy et al. 2005), our understanding of the ability of insects to detect the threat of parasites prior to infection is less well developed, despite its probable importance (Hart 1990).

Eusocial insects are thought to be particularly at risk from the threat of disease due to living in dense groups, with homeostatic nest environments, and high levels of relatedness within a colony (Schmid-Hempel 1998). However, evidence

of this increased parasite pressure is often lacking and this is thought to be because social insects have reduced the cost of group living through the development of effective group-level defences, termed ‘social immunity’ (Cremer et al. 2007). These include behavioural adaptations such as undertaking, waste management, and grooming behaviour, which are effective at removing parasites from individuals and the colony as a whole (Boomsma et al. 2005; Wilson-Rich et al. 2009). As many of these behaviours rely on the ability to target a contaminated item or individual, parasite detection is likely to be particularly important in eusocial insects. Food sources, such as flowers or leaves (Durrer and Schmid-Hempel 1994; Griffiths and Hughes 2010; Parker et al. 2010; Fouks and Lattorff 2011), can become a dangerous hub for the horizontal transmission of parasites to visiting individuals, and consequently insects can detect and avoid contaminated food (de Roode and Lefèvre 2012). Similarly, termites, crickets, and ladybirds will preferentially avoid environments heavily contaminated with entomopathogenic fungi (Staples and Milner 2000; Thompson and Brandenburg 2007; Ormond et al. 2011), and ants and termites will increase self-grooming or allogrooming in the presence of fungal conidia (Rosengaus et al. 1998; Yanagawa and Shimizu 2006; Yanagawa et al. 2008; Walker and Hughes 2009; Morelos-Juárez et al. 2010; Reber et al. 2011). Responses have been shown to scale with the severity of the threat in termites, whose antennae play an important role in the detection of fungal conidia. (Rosengaus et al. 1999; Myles 2002; Yanagawa et al. 2009) Additionally, the ability to detect an external parasite threat may vary between species with different evolutionary parasite pressures and life histories, or between different parasites. Host species which are exposed to greater parasite pressure, for example because they have vulnerable food stores, feed on more contaminated food or in more contaminated environments, may be

more adept at detecting and recognising threats (Boomsma et al. 2005; Walker and Hughes 2011). Similarly, hosts will be selected to be better at detecting more dangerous threats, such as from virulent entomopathogens (Dieckmann 2002; Poulin 2007; Mburu et al. 2011). Both host life-history and parasite virulence have been shown to affect the evolution of host disease resistance (Currie 2001; Sumner et al. 2003; Fernández-Marín et al. 2006, 2009; Hughes, Pagliarini, et al. 2008), but their effects on disease avoidance are less clear.

Although there has therefore been much progress made in understanding the resistance to parasites of insects in general, and of social insects in particular, our understanding of the behavioural recognition phase of resistance is still limited. In particular, it is not clear how the recognition and response of social insects to parasites may vary depending on the context of exposure, for example whether the parasite is encountered in food, the general environment, or on a nestmate. It is also not clear the extent to which the ability to behaviourally recognise and respond to parasites is present across taxa, or indeed if the ability may differ between social insect species. The behavioural response of social insects to parasites can sometimes be counter-intuitive (Brütsch et al. 2014), so knowledge of this stage is important for a full understanding of the complete process that takes place from encountering a parasite to resistance or infection.

Here we test the ability of individual ants to detect and respond to the presence of two fungal pathogens. To determine how the response was affected by context, we presented the fungal pathogens under controlled laboratory conditions, without the confounding effects of other environmental cues, and mimicked exposure via the three key routes of ingress for a parasite into a colony: through

contaminated food, environment, and nestmates (Schmid-Hempel 1998; Boomsma et al. 2005). Studies typically focus on a single species, but interspecific variation in parasite resistance is likely, so we here tested the response of ants from four species with similar colony sizes but different life-histories: 1) *Polyrhachis dives*, an omnivorous Southeast Asian weaver ant which may frequently encounter contaminated food but which only has to protect its own nestmates from disease (Hung 1967); 2) *Messor barbarus*, a granivorous, European seed-harvesting ant which has, in addition to its nestmates, to protect its food store of seeds from microbial contaminants (Plowes et al. 2013); 3) *Acromyrmex echinator*, a mycophagous Panamanian leaf-cutting ant which also has to protect a food store, but in which this is in the form of a fungal crop that is highly vulnerable to foreign microbes (Currie, Mueller, et al. 1999); 4) *Formica rufa*, a European wood ant that build large nests out of conifer needles and plant debris, and feeds on a mix of insect honeydew and scavenged carcasses. Harvester ants, leaf-cutting ants and wood ants possess antimicrobial producing metapleural and venom glands, but weaver ants lack the metapleural gland, and the venom of wood ants and weaver ants may have particularly strong antimicrobial properties because it consists largely of formic acid (Attygalle and Morgan 1984; Hölldobler and Wilson 1990; Billen 2009; Yek and Mueller 2011; Tragust, Ugelvig, et al. 2013). As leaf-cutting ants and harvester ants have vulnerable food stores to protect, we would predict that they may be more vigilant at preventing the ingress of pathogens into the colony, but we would also predict that all ant species will show effective behaviours to minimise their risk from parasites in the environment and on themselves.

## 2.3 Methods

The experiments were conducted using randomly selected individual foragers. We tested individuals from four colonies each of leaf-cutting ants (Ae396, Ae398, Ae399, Ae088), harvester ants (Mb0801, Mb1201, Mb1301, Mb1302) and wood ants (Fr1301, Fr1302, Fr1303, Fr1304) and two colonies of weaver ants (Pd0701, Pd0704). The colonies had been kept at 27°C, 80% RH, 12h:12h photoperiod, on species-specific diets provided twice a week (*Tenebrio* larvae and 20% sucrose solution for weaver ants and wood ants, the same supplemented by grass seeds for harvester ants, and privet leaves for leaf-cutting ants). All leaf-cutting ant and weaver ant colonies and harvester ant colonies Mb0801 and Mb1201, had been kept as above for at least 12 months prior to the experiments, whilst all the wood ant colonies and the harvester ant colonies Mb1301 and Mb1301 had been kept for one month, and all appeared in good health (no signs of parasite infections or excessive mortality). The colonies were given *ad libitum* water and 20% sucrose solution throughout the experiment, but were starved of solid food during, and for three days prior to, Experiment 1: looking at the response of ants to contaminated food. Four treatments were tested: 1) conidia of the specialist entomopathogenic fungus *Metarhizium anisopliae* [strain ARSEF 144467, isolated from the soil of a Canadian maize field], 2) conidia of the facultative entomopathogenic fungus *Aspergillus flavus* [GU172440.1, isolated from bees in an experimental apiary West Yorkshire, UK; Foley et al. 2012], 3) talcum powder control (to control for the presence of a physical particulate; talcum particles were  $5.2 \pm 6.6\mu\text{m} \times 5.3 \pm 7.7\mu\text{m}$ , compared to  $5.5 \pm 0.2\mu\text{m} \times 3.3 \pm 0.2\mu\text{m}$  for the *M. anisopliae* conidia and  $3.6 \pm 0.1\mu\text{m} \times 3.6 \pm 0.2\mu\text{m}$  for the *A. flavus* conidia), and 4) blank control (to control for the Triton-X used as a

surfactant in delivery of fungal conidia). Both *Metarhizium anisopliae* and *Aspergillus flavus* are very common in the soil environment of ants at many locations and have been reported as natural parasites of ants on numerous occasions (Jouvenaz et al. 1972; Allen and Buren 1974; Alves and Sosa-Gómez 1983; Lofgren and Vander Meer 1986; Gilliam et al. 1990; Diehl-Fleig et al. 1992; Humber 1992; Sanchez-Pena and Thorvilson 1992; Quiroz et al. 1996; Schmid-Hempel 1998; Hughes et al. 2004; Poulsen et al. 2006; de Zarzuela et al. 2007, 2012; Rodrigues et al. 2010; Lacerda et al. 2010; Castilho et al. 2010; Ribeiro et al. 2012). Multiple species of *Aspergillus* have also been reported growing on the fungal garden of leaf-cutting ants or nest material of weaver ants, and will, given the opportunity, quickly overgrow them (Fountain and Hughes 2011; Tranter et al. 2014). Additionally many opportunistic fungal species are found on the seeds harvested and stored within the colonies of the granivorous harvester ants, and *Aspergillus* can be a common and important threat to seed stores (Klich et al. 1984; Crist and Friesse 1993; Satish et al. 2007). Although the generalist nature of both parasites makes coevolution with ant hosts unlikely, we used exotic strains of both parasites to avoid any potential for the parasites to have evolved to avoid recognition by any of the ant species used here.

*Metarhizium anisopliae* is a more virulent entomopathogen than *A. flavus* (Zimmermann 1993; Glare et al. 1996; Frazzon et al. 2000; Hughes et al. 2004; Scully and Bidochka 2005), and thus would be expected to stimulate a more extreme behavioural response from ants. In Experiments 1 and 2 below, the conidia and talcum particles were made up as suspensions of  $1.5 \times 10^8$  conidia or particles per ml in 0.05% Triton-X surfactant using a blank haemocytometer, with the control being pure 0.05% Triton-X solution. In Experiment 3, looking at the response of ants to contaminated nestmates, the conidia and talcum powder

were applied dry to avoid grooming being stimulated by the presence of a liquid on the cuticle. Fungal conidia were harvested from freshly sporulating media plates, and viability was confirmed to be  $> 90\%$  throughout the experiments by plating the conidia solutions onto Sabouraud dextrose agar, incubating for 24 h and quantifying successful conidia germination, defined as the production of a germ tube longer than the conidia diameter (Siegel 2012).

### **2.3.1 Exp. 1: Response to contaminated food**

In order to test the ability of the individual ants to detect and avoid contaminated food, ants from each species were presented with food treated with either *Metarhizium* or *Aspergillus* conidia, talcum powder control or Triton-X control solution. Weaver ant and wood ant workers were provided with a section of *Tenebrio molitor* larvae (length: 8 mm, diameter: 2.5 mm), harvester ant workers with two grains of rice (length: 7 mm, diameter: 1.8 mm), and leaf-cutting ant workers with a section of fresh privet leaf (*Lingustrum* sp. length: 8 mm, width: 8 mm), with the surface area of the food ( $\sim 64 \text{ mm}^2$ ) being the same in each case. Shortly prior to the experiment, an even coating of 8  $\mu\text{l}$  of the treatment solution was pipetted over the surface of the food and allowed to dry, resulting in a treatment density of approximately 1875 conidia/ $\text{mm}^2$  (the number of conidia adhering to the different food sources was very similar, see Table S2.1 p.274). A Fluon-lined 90 mm Petri dish was placed in the foraging arena of each colony on a Fluon-coated tripod so that ants could only enter via a removable bridge (Figure S2.1). The ants were allowed to acclimatise to the general apparatus over several days. For each trial, a piece of filter paper was placed in the dish and a single



foraging worker was allowed to enter the dish, with the ant then confined within an inverted transparent pot (25 mm diameter) for 5 min to allow it to acclimatise and ensure a consistent starting position within the dish to avoid biasing. The food was then placed in the centre of the Petri dish, the pot removed, and interactions between the ant and the food recorded by eye for 15 min. The behaviours recorded were: i) whether the ants appeared to attempted to harvest the food, i.e. transported the food from the centre of the Petri dish to the edge closest to their nest where the bridge was previously located, ii) the length of time spent interacting with the food (i.e. direct antennation, cutting or feeding, picking up without moving), and iii) the length of time spent self-grooming. For leaf-cutting ants and wood ants this was repeated with  $n = 64$  ants from each species for each of the four treatments (16 ants for each of the four colonies per species, per treatment). For harvester ants this was repeated with  $n = 48$  ants per treatment (16 ants from two colonies, 8 ants from two colonies), and with  $n = 32$  ants per treatment for weaver ants (16 ants from two colonies). The filter paper was replaced after each trial to remove any cues potentially left on the paper from the previous trial.

### **2.3.2 Exp. 2: Response to contaminated environment**

A 90 mm diameter filter paper of 2  $\mu\text{m}$  porosity, sufficient to prevent the passage of fungal conidia (see dimensions above), was divided in two, with one half infused evenly with 0.4 ml of one of the four treatments, resulting in an approximate treatment density of 1875 conidia/ $\text{mm}^2$  for the fungal treatments or 1875 particles/ $\text{mm}^2$  for the talcum powder treatment, and the other half infused

with 0.4 ml of 0.05% Triton-X control solution. Once dry, the two halves were placed in a Petri dish to provide a simple choice set-up, with one half of the Petri dish treated and the other untreated. The Petri dish arena was placed in an enclosure formed of blank white surrounding walls with diffuse lighting in order to remove visual orientation cues. For each trial, an ant was placed in the Petri dish and confined within a transparent pot (25 mm diameter) at the centre of the dish for 5 min to allow the ants to calm down after their initial alarm and ensure a consistent starting position. The pot was then removed and the ant video recorded (Logitech B910 HD) from a fixed and consistent position above the dishes for 15 min. Lighting was provided by strip lighting on the ceiling 170 cm above dish and trials were performed at a room temperature of 21 °C. For leaf-cutting ants and wood ants this was repeated with  $n = 64$  ants from each species for each of the four treatments (16 ants for each of the four colonies per species, per treatment). For harvester ants this was repeated with  $n = 48$  ants per treatment (16 ants from two colonies, 8 ants from two colonies), and with  $n = 32$  ants per treatment for weaver ants (16 ants from two colonies). The filter paper was replaced between each trial, and the Petri dish replaced and reoriented by 180° every fourth trial, to remove any chemical cues and control for any visual cues that may have influenced the results. The videos were analysed to obtain the length of time spent, speed travelled, distance covered, and time spent stopped, by the ant on either half of the Petri dish. Results from the video analysis were outputted from AntTrak (Table S2.2 p.274), a path analysis program designed for this task, as data for analysis and as images of the tracks for visual inspection.

### **2.3.3 Exp. 3: Response to contaminated nestmates**

The ability of individual ants to recognise contaminated nestmates was tested by applying dry conidia or talcum powder evenly to the gaster of a treatment ant with a cotton bud, before placing the treated ant with an untreated nestmate in a Petri dish. Application was performed so as to provide a constant treatment layer between trials, whilst accounting for species and body size. A clean blank cotton bud was brushed onto the ant in the same manner as above for the control treatment. Instances of contact (defined as any non-grooming interaction, e.g. antennation) between the two ants, self-grooming by the treatment ant, and allogrooming were tallied by eye during 10 min. For leaf-cutting ants and wood ants this was repeated with  $n = 64$  ants from each species for each of the four treatments (16 ants for each of the four colonies per species, per treatment). For harvester ants this was repeated with  $n = 48$  ants per treatment (16 ants from two colonies, 8 ants from two colonies), and with  $n = 32$  ants per treatment for weaver ants (16 ants from two colonies). None of the ants were used more than once and none were returned to the colony after use to avoid influencing the independence of other workers.

### **2.3.4 Statistical analysis**

Data from all experiments were non-normal so generalised linear mixed models were used throughout with model distribution determined based on AIC scores and the structure of the non-normal data. No overdispersion was observed based on model deviance/df values. Non-significant interaction terms within the models

were removed based on likelihood-ratio tests to achieve the minimum adequate models. The effect of the colony from which the individual ants tested were obtained was included in all GLMM analyses as a random factor, but was non-significant ( $P > 0.05$ ) in all cases except when comparing contact rates in Experiment 3. For all experiments, overall tests were run on total data with species and treatment as factors, but the effect of treatment was additionally analysed for each species individually. In Experiment 1, the effects of treatment and species on the length of time individual ants spent engaged in each of the activities was analysed using a GLMM with gamma distribution and log link function. The proportion of trials in which ants harvested food was analysed using a GLMM with binomial distribution and probit link function. For Experiment 2, a GLMM with gamma distribution and log link function was used to analyse the effect of treatment and species on the relative proportions of length of time, speed and distance travelled (see Figure S2.3 for distance travelled). A GLMM with negative-binomial distribution and log link function was used to analyse the effect of time spent inactive on the treated side. For Experiment 3, the number of contacts, allogrooming and self-grooming was tested between the four different treatments and four different species using a GLMM with gamma distribution and log link function. Post-hoc comparisons between individual treatment groups were conducted using a pairwise sequential Bonferroni comparisons for all three experiments. All analyses were conducted using SPSS 20.

## 2.4 Results

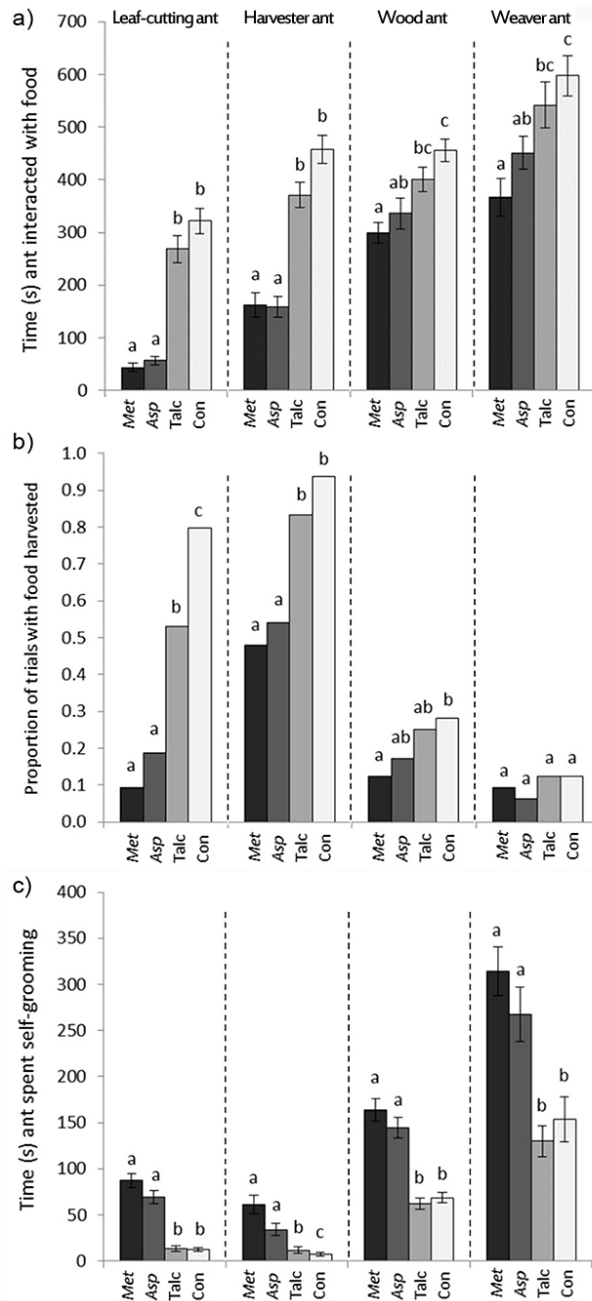
### 2.4.1 Exp. 1: Response to contaminated food

There was a significant overall interaction between ant species and treatment, indicating that ants from different species were responding differently to the various treatments, on the length of time ants spent interacting with food ( $F_{9,816} = 17.04, P < 0.001$ ), the proportion of trials in which ants harvested food ( $F_{9,816} = 3.45, P = 0.002$ ) and the length of time ants spent self-grooming ( $F_{9,816} = 4.49, P < 0.001$ ; Table S2.3 p.275). When analysed individually, all species showed a significant effect of treatment on the length of time spent interacting with, and in the time spent grooming after exposure to, contaminated food (Table S2.4 p.275). Both leaf-cutting and harvester ants also showed a significant difference in their propensity to harvest food (i.e. transporting the food towards the position where the bridge leading back to the nest had been located) depending on whether it was contaminated or uncontaminated, but the wood ants and weaver ants did not. Weaver ants interacted with both contaminated and uncontaminated food for the longest time, and leaf-cutting ants the least (Figure 2.1a; Figure S2.2). In all species there was no significant difference in interaction time with food between the two fungal treatments or between the two control treatments. Leaf-cutting ants and harvester ants showed a much greater difference in the time spent interacting with fungal-treated opposed to control-treated food, when compared to wood ants and weaver ants. In both wood ants and weaver ants there was no significant difference in food-interaction time between the *Aspergillus* and talcum powder treatments. Harvester ants harvested

the food in most trials, whereas weaver ants very rarely harvested the food offered (Figure 2.1b). Less food was harvested by leaf-cutting ants and harvester ants when it was contaminated with *Metarhizium* or *Aspergillus* conidia than when it had been treated with the blank control or talcum powder. Wood ants only showed a difference in the proportion of trials where food was harvested between the *Metarhizium* and blank control treatments, and weaver ants showed no significant difference between any of the treatments. Leaf-cutting ants also were significantly more likely to harvest food treated with the blank control compared with food treated with talcum powder. Harvester ants groomed in the fewest trials, but at a similar level to leaf-cutting ants, whilst wood ants and weaver ants self-groomed for much longer (Figure 2.1c). All species of ants groomed significantly more after interacting with *Metarhizium* and *Aspergillus*-treated food compared to the two control treatments, and harvester ants groomed less after interacting with on blank control-treated food compared to food treated with a talcum powder control.

#### **2.4.2 Exp. 2: Detection of contaminated environment**

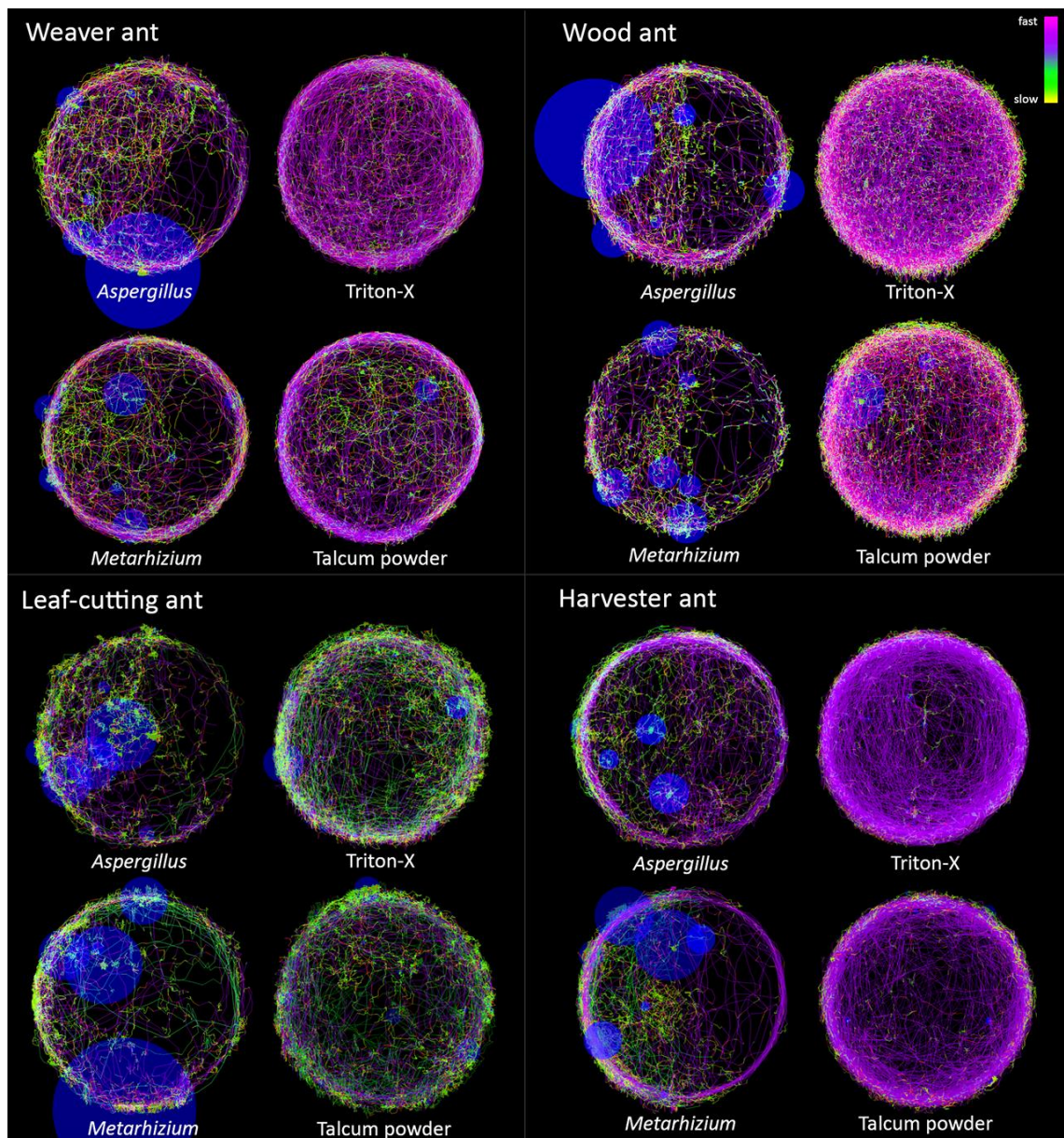
Data from the analysis of ant tracks (Figure 2.2) showed there was overall a significant interaction between the effect of species and treatment on the proportion of time spent ( $F_{3,816} = 5.98$ ,  $p = 0.001$ ), speed travelled at ( $F_{3,816} = 4.24$ ,  $p = 0.006$ ) and length of time spent inactive ( $F_{3,816} = 16.65$ ,  $p < 0.001$ ) by ants on the untreated side of the enclosure (Table S2.5 p.277). All four species, when analysed individually, showed a significant effect of treatment on the total time spent, and also the time spent inactive, on the treatment side.



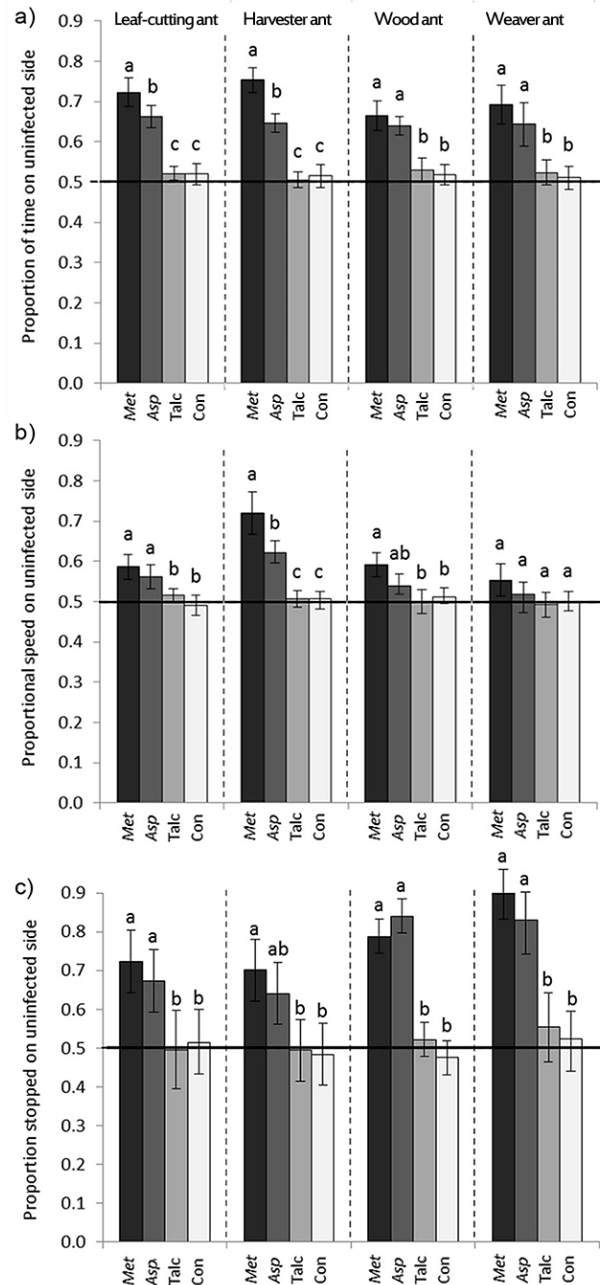
**Figure 2.1** Durations of feeding and grooming behaviours. The (a) mean  $\pm$  s.e. time spent interacting with food, (b) proportion of food harvested (transported the food to the position of the bridge leading back to the colony) and (c) mean  $\pm$  s.e. time spent self-grooming by leaf-cutting ants ( $n=64$ ), harvester ants ( $n=48$ ), wood ants ( $n=64$ ), and weaver ants ( $n=32$ ) in Experiment 1. Food was treated with either the *Metarhizium* (Met.) or *Aspergillus* (Asp.) fungal pathogens, talcum powder control (Talc.) or control solution (Ctrl.). Within each graph, treatments within each species group with different letters differed significantly from one another at  $P < 0.05$

Harvester ants and wood ants, also moved at significantly different speeds on the treated half depending on the treatment applied (Table S2.6 p.278). All species spent significantly longer on the uncontaminated half when the other side had a fungal treatment applied, compared to the blank and talcum powder control treatments where ants showed no preference for either side (Figure 2.3a; Figure S2.3). Additionally, leaf-cutting ants and harvester ants spent longer on the uncontaminated side when the other half had been treated with *Metarhizium*, compared to when it had been treated with *Aspergillus*. Leaf-cutting ants, harvester ants and wood ants moved significantly faster on surfaces treated with either *Metarhizium* or *Aspergillus* compared with either control treatment, where the ants travelled at the same speed on either half of the Petri dish (Figure 2.3b). This difference was greatest in harvester ants, with the largest difference in speed observed in the *Metarhizium* treatment. Weaver ants did not alter their speed depending on whether they were on treated or untreated halves in any of the treatments. All four species of ants stopped for significantly longer on the untreated side in the fungal trials but not in the control trials (Figure 2.3c). This difference was greatest in the wood ants and weaver ants, which spent around 80% of their inactive time on the untreated sides when fungal conidia were present on the alternative.





**Figure 2.2** Composite tracks from Experiment 2 for four environmental treatments produced from the video analysis of choice trials in four ant species (top left: weaver ant, top right: wood ant, bottom left: leaf-cutting ant, bottom right: harvester ant). Each of the four graphics within each quarter represents an overlay of 10 individual paths. Labels below each graphic show the treatment applied to the right side of the circle, compared to a control treatment on the left side. The track is colour-coded from pink and purple, where the ant travelled fastest, to green and yellow where the ant travelled more slowly. A blue circle is present to represent the point at which an ant stopped, and the larger the circle the longer the time spent stationary

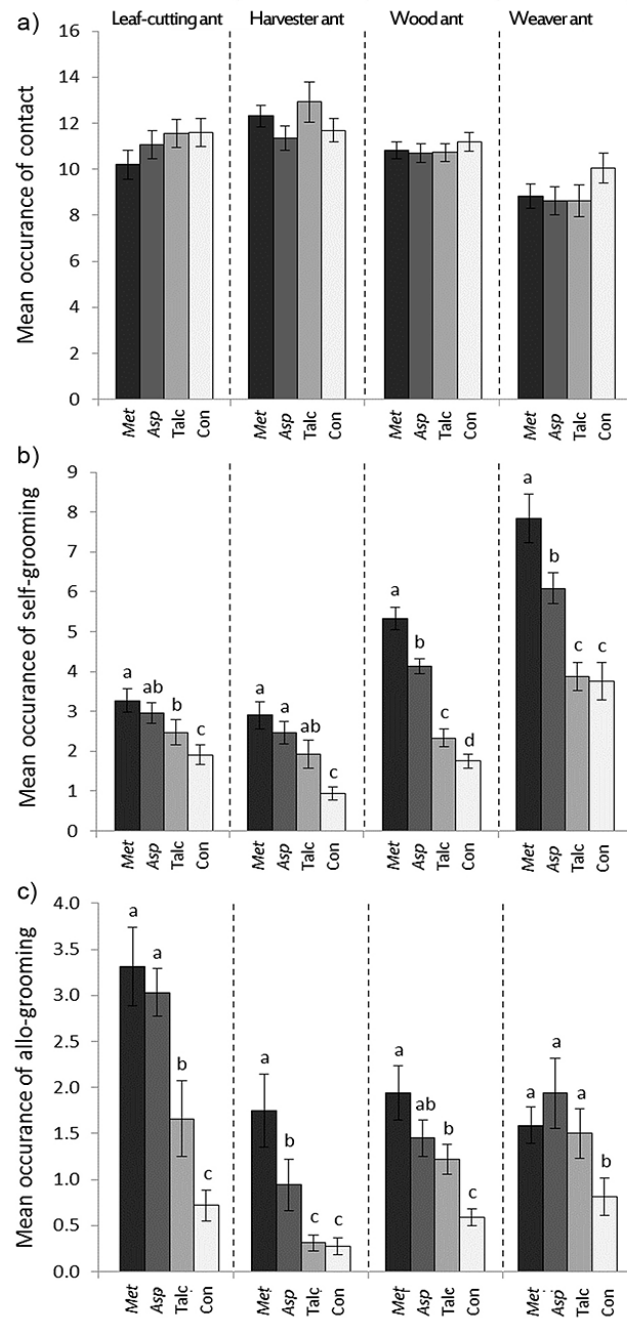


**Figure 2.3** Choice experiment data. The (a) proportion  $\pm$  95% CI of time spent, (b) speed travelled, and (c) time spent inactive, on the treatment side of a choice arena treated with either the *Metarhizium* (Met.) or *Aspergillus* (Asp.) fungal pathogens, talcum powder control (Talc.) or control solution (Ctrl.) in Experiment 2, for leaf-cutting ants (n=64), harvester ants (n=48), wood ants (n=64), and weaver ants (n=32). Proportions with 95% C.I. error bars which do not overlap 0.5 line (\*) show a significant difference between treated and untreated sides. Within each graph, treatments within each species group with different letters differed significantly from one another at  $P < 0.05$

### 2.4.3 Exp. 3: Detection of contaminated nestmates

Contact rates between the contaminated ant and its uncontaminated nestmate showed a significant difference between the four species ( $F_{3,816} = 10.82$ ,  $p < 0.001$ ), with weaver ants showing slightly lower baseline levels of contact compared to the other species, but there was no overall difference between treatments or evidence of interaction ( $F_{3,816} = 2.21$ ,  $p = 0.085$ , and  $F_{9,816} = 1.80$ ,  $p = 0.06$ , respectively; Table S2.7 p.279). When analysed individually only weaver ants showed a significant effect of treatment on contact rates. There was a significant effect of colony on overall contact rates with Mb1 showing consistently higher rates of contact than the other harvester ant colonies ( $Z = 5.95$ ,  $p < 0.001$ ). Harvester ants exhibited the highest contact rates, significantly higher than weaver ants, which had the lowest contact rate (Figure 2.4a; Figure S2.4).

There was a significant interaction between the effect of species and treatment on both self-grooming ( $F_{9,816} = 2.77$ ,  $p = 0.003$ ) and allogrooming rates ( $F_{9,816} = 2.03$ ,  $p = 0.03$ ). Weaver ants had higher baseline levels of self-grooming than any of the other species, which showed similar levels of self-grooming (Figure 2.4b). Ants from all species showed higher frequencies of self-grooming after interaction with nestmates treated with a fungal pathogen. This was significantly different to control treatments for harvester ants and weaver ants in the *Aspergillus* treatment, and in the *Metarhizium* treatment for leaf-cutting ants. Harvester ants allogroomed the least, less than weaver ants, who in turn allogroomed less than leaf-cutting ants (Figure 2.4c). When analysed individually



**Figure 2.4** Contact and grooming rates between ants. The (a) mean  $\pm$  s.e. occurrence of contact and (b) self-grooming by the test ant, and of (c) allogrooming between treated and test ant in Experiment 3 of leaf-cutting ants (n=64), harvester ants (n=48), wood ants (n=64), and weaver ants (n=32) to nestmates that had been treated with either the *Metarhizium* (*Met.*) or *Aspergillus* (*Asp.*) fungal pathogens, talcum powder control (Talc.) or control solution (Ctrl.). Within each graph, treatments within each species group with different letters differed significantly from one another at  $P < 0.05$

each species showed a significant effect of treatment on allogrooming and self-grooming responses, except allogrooming in weaver ants. In all species the control treatment resulted in the lowest incidence of allogrooming, with this being significantly lower than all other treatment groups in leaf-cutting ants and significantly lower than the *Metarhizium* treatment in harvester ants. Harvester ants allogroomed *Metarhizium*-treated nestmates more than those treated with talcum powder or untreated nestmates, but in leaf-cutting ants the talcum powder treatment also resulted in significantly higher rates of allogrooming than the blank control. This result is likely due to colony Ae396 where talcum powder produced dramatically high allogrooming rates. (Figure 2.4c; Figure S2.4c).

## 2.5 Discussion

The results demonstrate the ability of ants to detect fungal pathogens on their food, environment and nestmates. Ants from all the species tested avoided fungal-contaminated surfaces and increased either allo or self-grooming behaviours when they detected contaminants on a nestmate. Treatments of the obligate entomopathogen *Metarhizium* generally resulted in the strongest recognition responses compared to the facultatively entomopathogenic *Aspergillus* and the control treatments. Individual ants from the four species showed different responses depending on the source of contamination.

When presented with contaminated food individual ants were highly discriminatory between food treated with the controls and that treated with either *Metarhizium* or *Aspergillus* fungal parasites. Avoidance of parasites when

feeding may be particularly important. Although fungal conidia can be deactivated in the guts of adult insects, any ingestion will carry a risk of infection if deactivation is not completely effective and the larvae, for which proteinacious food is primarily collected, may lack the deactivation capabilities of adults (Broome et al. 1976; Dillon and Charnley 1988; Siva-Jothy et al. 2005; Chouvenc et al. 2010). Additionally food stored in the crop which is transferred by trophallaxis may still pose a risk of horizontal transmission to other ants, as the fungistatic activity may only be sufficient to retard germination and not completely sterilize conidia (Shah and Pell 2003; Chouvenc et al. 2010).

Ants which did interact with food with fungi present showed significantly higher rates of self-grooming. Similarly the individual ants from all species tested up-regulated selfgrooming, and three out of the four species also allogroomed more frequently, in response to fungal contaminated nestmates. Grooming is an important defence against parasites and is an adaptive behaviour which ants and other social insects can use upon encountering fungal pathogens in various contexts, both to protect themselves, and nestmates (Cremer et al. 2007; Yanagawa et al. 2008; Reber et al. 2011). As well as directly removing parasites from the cuticle, grooming also transfers antimicrobial secretions from the metapleural gland and venom glands (Fernández-Marín et al. 2006; Tragust, Ugelvig, et al. 2013). The relative investment into these different forms of grooming may vary based on the nature of the threat and ant species (Okuno et al. 2011). For example, weaver ants have relatively high levels of self-grooming and low levels of allogrooming, whereas leaf-cutting ants have the opposite pattern (see Figure 2.4b and 2.4c). Allogrooming as a defence may require a greater investment from the colony as it involves the time and activities of two or

more individuals, but it may also be more effective (Hughes et al. 2002; Yanagawa and Shimizu 2006). While preliminary observations of ants encountering uncontaminated food in the Experiment 1 arena when the bridge was left in place (Figure S2.1), confirmed that ants picking up the food then transported it back to the nest, we cannot be certain that this would have been the case for the ants in all our trials, although it seems likely.

It is likely that ants in these experiments were using chemical receptors to detect the presence of contaminants (Yanagawa et al. 2009), though physical detection may have also played a part in the trials where the treatments were applied dry. Ants possess a well-developed ability to detect and communicate information via chemical signals which is fundamental to nestmate recognition and recruitment, trail-building and alarm behaviours (Hölldobler 1978; Hölldobler and Wilson 1990; Hughes and Goulson 2001). Fungi produce small size volatile organic compounds (Morath et al. 2012) which are detectable by insects and can act as signalling molecules (Rohlf et al. 2005). Beetles are attracted to food through detection of volatiles produced by wood-rotting fungi (Drilling and Dettner 2009), pollinators can be deceived by flower-mimic fungi which produce volatiles similar to the real flower (Ngugi and Scherm 2006), and ant queens may, unusually, be attracted to nest sites with entomopathogenic fungi (Brütsch et al. 2014). Conversely invertebrates may be repelled by, or show alarm behaviour in response to, chemical cues from fungi which may indicate a potential threat (Rosengaus et al. 1999; Staples and Milner 2000; Wood et al. 2001; Hussain et al. 2010; Fouks and Lattorff 2011).



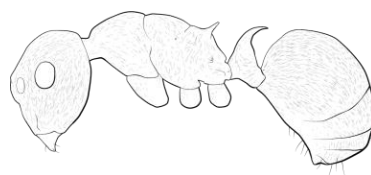
While the results show ants can detect the various parasite threats, they do not reveal whether the differences are due to differences in detection ability or in behavioural response after detection. Further work will be needed to establish this. Additionally, ants may alter their response threshold to a detectable threat based on the costs of avoidance or defence. For social insects in particular this trade-off may be complicated to assess as any benefits and costs need to be considered at both individual and colony levels (Wilson-Rich et al. 2009). In natural conditions avoidance of contaminated food or reduced exploration of unhygienic environments may protect the individual ant from infection (Wisenden et al. 2009), but this benefit may carry a colony-level cost by reducing food harvesting. Avoidance of contaminated nestmates may result in a reduced individual hazard, but overall a much greater threat to the colony as a whole, should a parasitized ant be allowed into the nest without intervention (Wilson-Rich et al. 2009 but see Hughes et al. 2002; Konrad et al. 2012).

Although all four ant species responded to parasites in a qualitatively similar way, there were some interesting quantitative differences in their responses. In particular, leaf-cutting ants and, to a lesser extent, harvester ants were more strongly discriminatory of contaminated food than weaver ants and wood ants. The leaf fragments that leaf-cutting ants retrieve are used as a substrate for their mutualistic fungal crop which is very vulnerable to other fungi, including *Aspergillus* (Luciano et al. 1995; Little et al. 2006; Pagnocca 2012; Tranter et al. 2014), and leaf-cutting ants are well known to scrupulously clean material to protect their fungal crop (Currie and Stuart 2001; Van Bael et al. 2009; Morelos-Juárez et al. 2010; Griffiths and Hughes 2010). Harvester ants store their seed food in granaries which may also be vulnerable to fungal growth, whereas weaver

ants and wood ants possess no equivalent, long-term, within-colony food store to protect. In addition, leaf-cutting ants showed relatively high levels of allogrooming while weaver ants showed relatively high levels of self-grooming. It may be that weaver ants have evolved high rates of self-grooming to compensate for their lack of antibiotic-producing metapleural glands by greater mechanical removal of parasites, or because self-grooming is needed to spread their antimicrobial venom actively over their cuticle (Hölldobler and Engel-Siegel 1984; Graystock and Hughes 2011; Yek and Mueller 2011; Tragust, Ugelvig, et al. 2013). Future comparative studies with more species will be important to establish whether such life-history differences do indeed drive variation in parasite response behaviour, and we may expect the response of these species to be even stronger when presented with fungi that are more dangerous parasites of food stores, such as *Escovopsis* (Crist and Fries 1993; Currie 2001).

In conclusion, the results show that individual ants are capable of recognising fungal threats in various contexts. Host-parasite interaction studies are often conducted on a single host species, but here we use four different ant species to better investigate how individual ants respond to the threat. Ant societies are well known for their organised division of labour and task partitioning, and it will be interesting to see whether ants vary in their ability to detect parasites according to their role in the colony (Anderson and Ratnieks 1999; Vitikainen and Sundström 2011). It will also be interesting to see whether species differences are due to differences in detection ability or behavioural response, whether detection thresholds relate to infectivity thresholds (Rosengaus et al. 1999; Mburu et al. 2009), and if ants are able to recognise and respond to parasites when they are at much lower doses or masked by other environmental cues.

There has been much progress in our understanding of the individual and group-level defences of social insects against parasites, and further comparative studies of different species will be valuable to elucidate the selection pressures that have shaped their evolution.



*Polyrhachis dives*

# 3. Sanitizing the fortress: protection of ant brood and nest material by worker antibiotics

Tranter C, Graystock P, Shaw C, Lopes JFS, Hughes WOH (2014) Sanitizing the fortress: protection of ant brood and nest material by worker antibiotics. *Behav. Ecol. Sociobiol.* 68:499–507

## 3.1 Abstract

Social groups are at particular risk from parasite infection, which is heightened in eusocial insects by the low genetic diversity of individuals within a colony. To combat this, adult ants have evolved a suite of defences to protect each other, including the production of antimicrobial secretions. However, it is the brood in a colony that are most vulnerable to parasites because their individual defences are limited, and the nest material in which ants live is also likely to be prone to colonisation by potential parasites. Here we investigate in two ant species whether adult workers use their antimicrobial secretions not only to protect each other, but also to sanitize the vulnerable brood and nest material. We find that in both leaf-cutting ants and weaver ants, the survival of brood was reduced, and the sporulation of parasitic fungi from them increased, when the workers nursing them lacked functional antimicrobial-producing glands. This was the case both for larvae that were experimentally treated with a fungal parasite (*Metarhizium*) and for control larvae which developed infections of an opportunistic fungal parasite (*Aspergillus*). Similarly, fungi were more likely to grow on the nest material of both ant species if the glands of attending workers were blocked. The results show that the defence of brood and sanitization of nest material are important

functions of the antimicrobial secretions of adult ants, and that ubiquitous, opportunistic fungi may be a more important driver of the evolution of these defences than rarer, specialist parasites.

## 3.2 Introduction

Many species form social groups, and by doing so benefit from greater resource exploitation, anti-predator defence and reproductive fitness (Dornhaus et al. 2010). However, such benefits come at the potential cost of increased parasite exposure (Alexander 1974; Krause and Ruxton 2002). Eusocial insects are one of the pinnacles of sociality, but their vulnerability to parasites is heightened by a homeostatic nest environment and low genetic diversity of individuals within a colony, which will facilitate parasite transmission and evolution (Schmid-Hempel 1998). To counter this, social insects, such as ants, have evolved a suite of behavioural and chemical defences which physically remove or chemically kill parasites that contaminate their cuticle (Boomsma et al. 2005; Wilson-Rich et al. 2009). These first-line defences are important for resistance to specialist entomopathogens and also the more common opportunistic parasites which abound in and around ant colonies (Milner et al. 1998; Schmid-Hempel 1998; Poulsen et al. 2006; Fountain and Hughes 2011; Evans et al. 2011b; Reber and Chapuisat 2012; Anderson et al. 2012). Ants (Storey et al. 1991; Mackintosh et al. 1995; Zelezetsky et al. 2005; Mendonça et al. 2009), bees (Evans et al. 2006; Baracchi and Turillazzi 2010; Baracchi, Mazza, et al. 2012), wasps (Turillazzi et al. 2006; Baracchi, Mazza, et al. 2012), termites (Rosengaus et al. 2000, 2004) and eusocial thrips (Turnbull et al. 2010, 2012), as well as non-social insects

(Bulet et al. 1999; Kuhn-Nentwig 2003; Haine et al. 2008; Stow and Beattie 2008), produce defensive compounds in their haemolymph and venom. In particular, most ants secrete antimicrobial compounds from their metapleural or venom glands on to their cuticle (Hölldobler and Wilson 1990). The secretions from both glands have been shown to inhibit the growth of parasites *in vitro* and adult workers with non-functional glands are more susceptible to parasites (Storey et al. 1991; Blum 1992; Bot, Obermayer, et al. 2001; Poulsen, Bot, Nielsen, et al. 2002; Graystock and Hughes 2011; Tragust, Ugelvig, et al. 2013).

Social insects are characterised by cooperation, with workers acting to maximise the fitness of their colony in spite of costs to themselves on an individual level. As a result, the resistance of social insects to disease consists of individual immunity and group-level responses that produce a form of ‘social immunity’, that can be adaptive and proactive (Rosengaus et al. 1998; Hughes et al. 2002; Traniello et al. 2002; Cremer et al. 2007; Chapuisat et al. 2007; Ugelvig and Cremer 2007; Walker and Hughes 2009; Morelos-Juárez et al. 2010; Reber et al. 2011; Hamilton et al. 2011; Konrad et al. 2012). Social immunity may be particularly important for the more vulnerable aspects of a colony, such as developing brood and nest substrates. Insect brood lack a fully developed physiological immune system (Gillespie et al. 1997; Lavine and Strand 2002; Wilson-Rich et al. 2008), are unable to self-groom, and do not have the important antimicrobial-producing glands (Hölldobler and Wilson 1990). Brood are thus extremely susceptible to disease and may consequently be particularly reliant on social immunity, including potentially the donation of antimicrobial secretions by adult workers. In an elegant study, Tragust et al. (2013) showed that nursing adult workers of *Lasius*

*neglectus* donate venom to brood during grooming, both directly via the acidopore and indirectly through oral uptake, and that this then benefited brood defence against parasites. In addition to brood, the substrate in, on, or with which, ants form their colony is also likely to be vulnerable to contamination, or in some cases infection, by potentially dangerous parasites (Currie, Scott, et al. 1999; Keller et al. 2003; Hughes et al. 2004; Fountain and Hughes 2011; Reber and Chapuisat 2011). This is particularly evident in the attine fungus-growing ants, which cultivate a mutualistic fungal crop that forms the central substrate of the colony and which is very vulnerable to infection by parasites (Mueller et al. 1998; Currie, Mueller, et al. 1999; Little et al. 2006; Gerardo et al. 2006). Consequently fungus-growing ants will mechanically groom their gardens to remove potential threats, have large metapleural glands and apply metapleural secretions onto the fungal crop (Currie and Stuart 2001; Sumner et al. 2003; Fernández-Marín et al. 2006, 2009; Little et al. 2006; Hughes, Pagliarini, et al. 2008). It is likely, therefore, that care, particularly the use of antimicrobial secretions, by worker ants is important to keep colony nest material hygienic.

Here we use the entomopathogenic fungus *Metarhizium anisopliae* with a leaf-cutting ant and a weaver ant to test experimentally if, and how effectively, the antimicrobial secretions produced by the venom and metapleural glands of adult workers are utilised to aid in brood survival, and how worker secretions may be used to keep nest material hygienic.

### 3.3 Methods

We studied two ant species: 1) the Brazilian leaf-cutting ant *Acromyrmex subterraneus subterraneus*, which has large antibiotic-producing metapleural glands (Biggi de Souza et al. 2006) as well as a venom gland, and 2) the south-east Asian weaver ant *Polyrhachis dives*, which lacks the metapleural gland but produces venom with antimicrobial properties (Zenghe 1986; Graystock and Hughes 2011). In both species, the respective glands (metapleural and venom) have been shown to be important in the disease resistance of adult workers (Poulsen, Bot, Nielsen, et al. 2002; Graystock and Hughes 2011). Workers and brood were collected from two colonies of weaver ants (Pd0701, Pd0704) and three colonies of leaf-cutting ants (As085 As086 and As0811) that had been maintained in the lab at 26°C and 80% RH for > 6 months prior to use and showed no apparent signs of decline or infection. Due to the availability of brood at the time of the experiment, all leaf-cutting ant brood were pupae of approximately 5 mm in length, while all weaver ant brood were larvae of approximately 5 mm length. For each species, adult workers were selected of similar size (6-8 mm), cuticle melanisation and location in the colony (and thus inferred age; Armitage and Boomsma 2010). We confirmed in a preliminary experiment that workers of these sizes and ages successfully cared for brood over 14 days when kept in isolation (i.e. a single ant with a single pupa or larva). As our experimental parasite we used a strain of the entomopathogenic fungus *Metarhizium anisopliae* (isolate 144467, CABI; isolated from the soil of a maize field in Canada) which was exotic to both of the ant species. Fungal conidia were harvested from freshly sporulating media plates, and viability was confirmed to be > 92% throughout the experiments by plating the conidia solutions onto



Sabouraud dextrose agar plates, incubating for 24 h and quantifying conidia germination. We applied 0.5 µl doses of species-specific concentrations of conidia in Triton-X, that we had determined in preliminary trials caused 50% mortality to brood (weaver ant:  $1 \times 10^5$  conidia/ml; leaf-cutting ant:  $1 \times 10^4$  conidia/ml).

### **3.3.1 Exp. 1: Brood care**

To determine the importance of adult worker antimicrobial secretions for brood survival, we collected 120 leaf-cutting ant workers and 160 weaver ant workers, split into two cohorts. The leaf-cutting ant cohorts were each formed of 60 ants, with 20 ants from each of the three colonies, whilst the weaver ant cohorts consisted of 80 ants, with 40 ants from each of the two colonies used. Half the ants from each colony had their main antimicrobial-producing glands (the metapleural gland in leaf-cutting ants and venom gland in weaver ants) blocked using nail varnish, and the remaining workers had nail varnish applied to the pronotum as a control (Poulsen et al., 2002; Graystock and Hughes, 2011). After 24 h, we collected 60 leaf-cutting ant pupae and 80 weaver ant larvae, for each of the two cohorts, and surface-treated half of them with the *Metarhizium* parasite and the other half with 0.5 µl of a 0.05% Triton X control solution using a micropipette. Each pupa or larva was then placed in a pot (40 mm diameter) with a single ‘nurse’ worker ant from the same colony to give four combinations of infected/uninfected brood and workers with functional/non-functional glands, in a full factorial design, with a total of 30 leaf-cutting ant and 40 weaver ant replicates of each (Figure S3.1 p.263). Ants were maintained in the pots with moistened cotton wool to supply water and sucrose solution *ad libitum*. Any

workers which died during the experiment were replaced with an identically-treated worker. The survival of the brood was monitored for 14 days. Dead brood were each placed on moistened filter paper in a Petri dish at 26 °C and 80% RH, and checked daily for the appearance of fungal conidia and conidiophores diagnostic of a *Metarhizium* infection. In order to confirm that the blockage treatment did not affect normal brood-care behaviours, we also compared the behaviour of nurse workers for 20 ants of each species. Half the ants in each species had their respective glands blocked and the other half had the control treatment applied to the pronotum. The ants were placed in a Petri dish with a single item of brood (pupae for leaf-cutting ants and larvae for weaver ants) and a) the duration of any non-grooming interaction between nurse and brood (e.g. carrying, antennation), b) the frequency of physical contact between nurse and brood, and c) the frequency of brood-grooming by the nurse ant, was recorded for a 10 minute period.

### **3.3.2 Exp. 2: Nest hygiene**

Sixty weaver ants (30 ants per colony) were collected from within the nest. Half of the ants from each colony had their venom gland blocked with nail varnish and half had a control treatment on the pronotal spines, for a total of 30 replicates per treatment. One hundred and twenty leaf-cutting ant workers (40 ants per colony) were collected from the outer surface of the fungal crop. The ants from each colony were divided evenly into the four blockage treatments as follows: i) varnish applied to the pronotal spines as a control, ii) metapleural gland blocked, iii) venom gland blocked, or iv) both venom and metapleural blocked, with a total

of 30 replicates per treatment. Each ant was placed in a pot with a 10 mm<sup>2</sup> section of either the silk nest material of weaver ants or the fungal garden of leaf-cutting ants, from their original nest, and balls of cotton wool moistened with water and sucrose solution at 26 °C and 80% RH. Thirty further 10 mm<sup>2</sup> sections of nest material were set up identically for each species except no ant was placed in the pot (Figure S3.2 p.264). The nest substrate was monitored for 15 days for the appearance of any foreign fungus and death of the fungal crop. If a worker died during the experiment then it was replaced with an identically treated worker.

To identify the fungi which developed in the leaf-cutting ant trials, three representative samples of each fungal morphotype (based on external morphology, spore structure, and colour) were isolated on malt extract agar (MEA) plates at 30 °C until the fungi produced conidia, and then stored at 4 °C. DNA was extracted from the samples by adding 200 µl of 5% Chelex solution (in 10 mM Tris buffer) and 0.05 g of 0.1 mm silica beads to approximately 0.05g of the sample fungus, and placed in a QIAGEN Tissue Lyser beadbeater for 4 min at 50 oscillations/s. Samples were then incubated at 90 °C before being centrifuged for 30 min at 4 °C. Supernatant from the samples was cleaned with OneStep-96 PCR Inhibitor Removal Kit (Zymo Research) prior to PCR amplification of the internal transcribed spacer regions 1 and 2 with the primers ITS1 and ITS4 (Henry et al. 2000; Foley et al. 2012). PCR products were sequenced and fungi identified by BLASTn searches of the resulting sequences.

### 3.3.3 Statistical analysis

The effects of *Metarhizium* exposure, gland closure, and ant species, on brood survival, and the effects of gland closure and ant species on the appearance of foreign fungi on nest material, were analysed using Cox proportional-hazards regression models. Colony-of-origin and cohort (in Experiment 1), were included in the models to account for the structured nature of the data. Pairwise Kaplan-Meier tests were used to test for pairwise differences between treatment groups. The effects of blockage on the duration of behavioural interactions of nurse ant and brood were examined using Mann-Whitney U-tests, and the survival of the nurses analysed using Cox proportional-hazards regression models. The proportions of brood sporulating with fungi were examined with  $\chi^2$  tests and the proportions of nest material sporulating with fungi were analysed with Fisher's exact tests.

## 3.4 Results

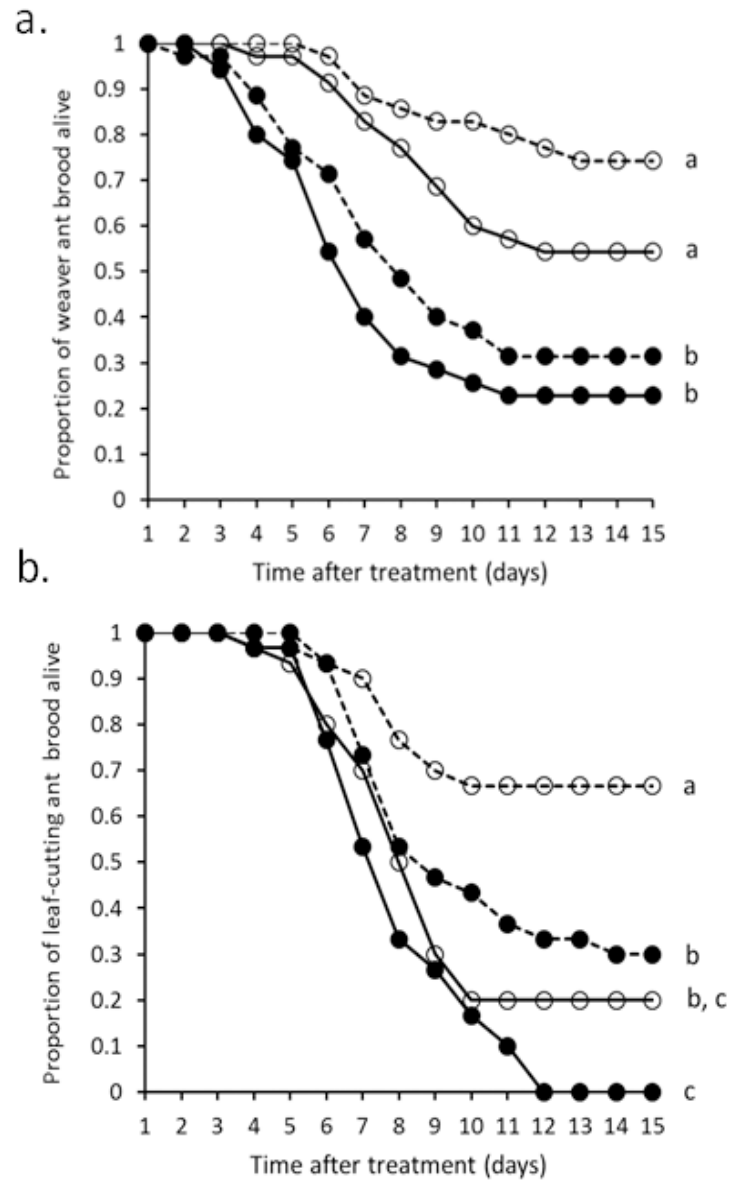
### 3.4.1 Exp. 1: Brood care

Workers of both species tended to the brood throughout the experiment and the survival of brood that were cared for by a replacement worker did not differ from those that were cared for by the same worker ant throughout (leaf-cutting ants: Wald=2.54,  $p=0.111$ ; weaver ants: Wald=0.19,  $p=0.67$ ). Nurse worker ants with blocked or unblocked glands did not differ in their behaviours when

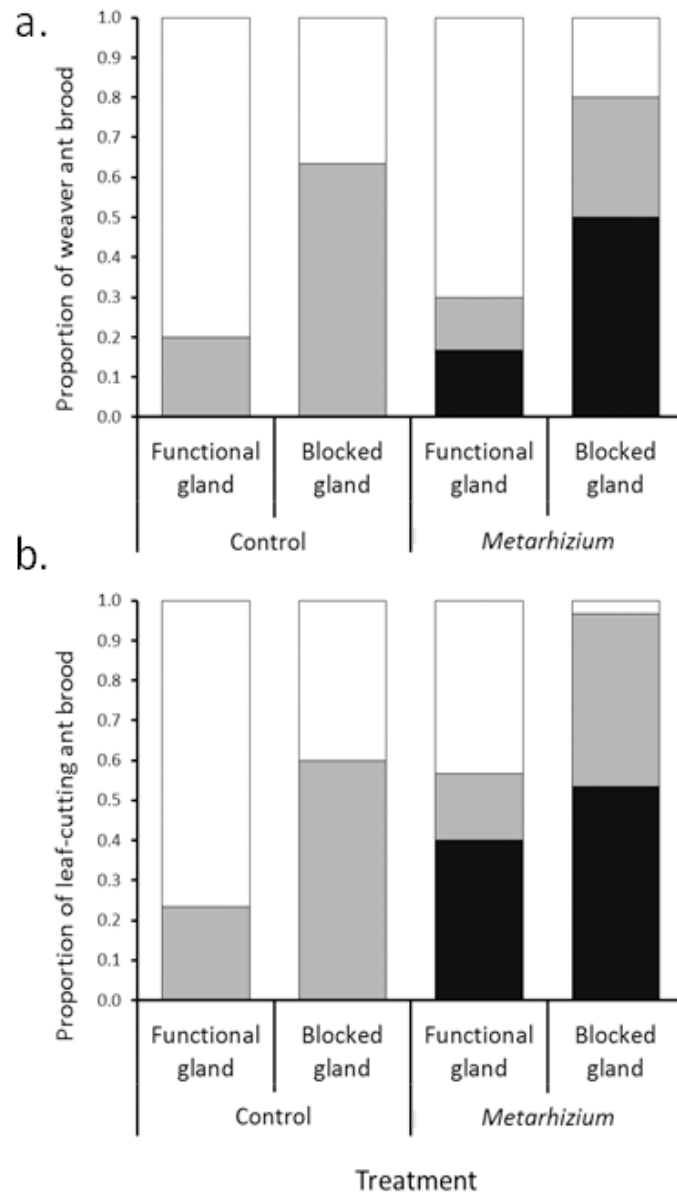
attending to brood or in their survival throughout the experiment; Figure S3.3 p.265. There were significant effects of both exposure to *Metarhizium* and of gland blockage on brood survival, (Wald=17.8,  $p<0.001$ ; Wald=27.2,  $p<0.001$ , respectively), but no overall difference between the ant species (Wald=1.84,  $p=0.1$ ), or significant interactions between these effects ( $p>0.2$  in all cases). There was no difference in brood survival between leaf-cutting ant cohorts (Wald=0.54,  $p=0.817$ ), but mortality was higher in the second, compared with the first, cohort of weaver ants tested (Wald=8.52,  $p=0.004$ ), and there were no significant differences between colonies ( $P>0.1$  in both species). In both ant species, gland blockage reduced brood survival regardless of treatment, while the effect of *Metarhizium* exposure was less consistent (Fig. 3.1). Compared to the control brood cared for by nurse ants with functioning glands, the hazard ratio for the leaf-cutting ant brood was increased to 2.7 by blocking the metapleural gland, to 3.7 by exposure to *Metarhizium* when the metapleural gland was functional, and to 5.5 by both exposure to *Metarhizium* and blocking the gland. For the weaver ant brood, the hazard ratio was increased to 1.9 by exposure to *Metarhizium* with the venom gland of nurse ants functional, to 3.4 by blocking the venom gland, and to 4.7 by both exposure to *Metarhizium* and blocking the gland.

Significantly fewer of the *Metarhizium*-exposed weaver ant brood sporulated with *Metarhizium* when the venom glands of their nurse ants were functional than when the glands were blocked ( $\chi^2=8.25$ ,  $p=0.04$ ), while there was no effect of gland blockage on *Metarhizium* sporulation from leaf-cutting ant brood ( $\chi^2=1.07$ ,  $p=0.3$ ; Fig. 2). A substantial number of brood of both ant species sporulated with the opportunistic fungal parasite *Aspergillus* sp. (Fig. 3.2). The proportion





**Figure. 3.1** Survival ant brood for a) weaver ant pupae and b) leaf-cutting ant larvae that were treated with either *Metarhizium* parasite (solid lines) or control solution (dashed lines) and cared for by workers either with (open circles) or without (black circles) functional antimicrobial glands (the venom gland for weaver ants and the metapleural gland for leaf-cutting ants). For each species, different letters indicate treatments which differed significantly from one another at  $P < 0.05$  in pairwise comparisons with Kaplan-Meier tests.



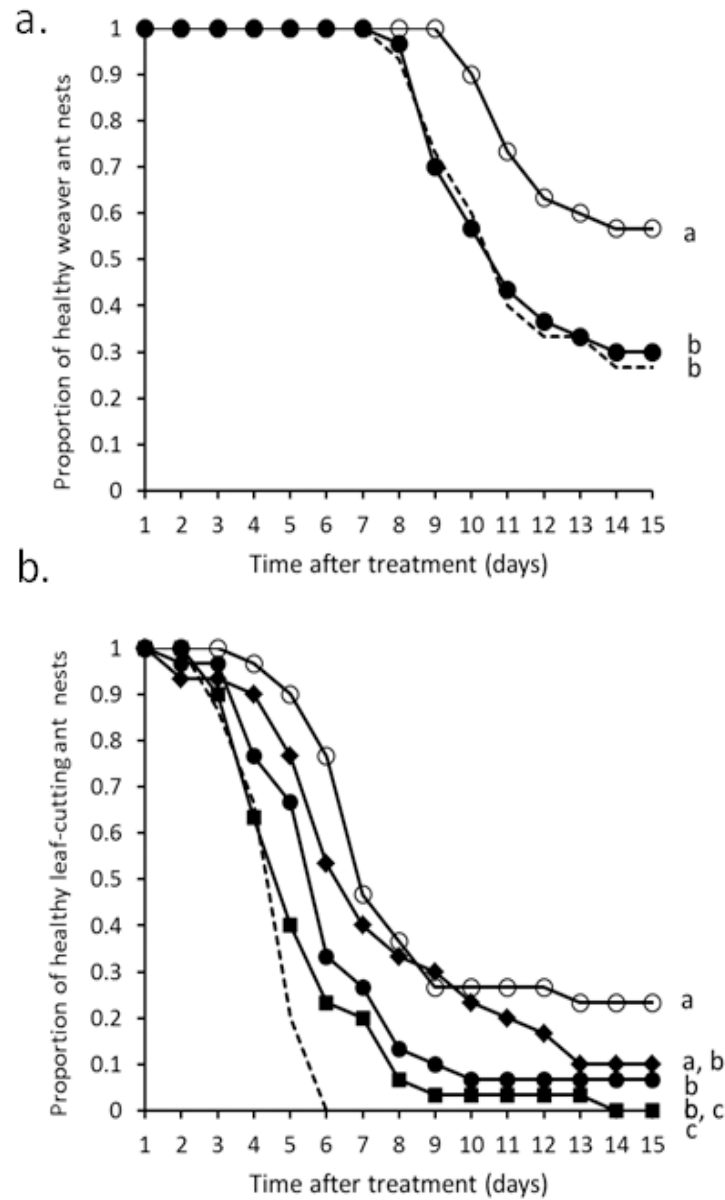
**Figure 3.2** Fungal-sporulating brood. Proportions of a) weaver ant larvae and b) leaf-cutting ant pupae that produced conidia of the *Metarhizium* experimental parasite (black), the opportunistic *Aspergillus* fungus (grey), or remained uninfected (white). Brood were either treated with *Metarhizium* parasite or control solution, and kept with workers either with or without functional antibiotic-producing glands (the venom gland for weaver ants and the metapleural gland for leaf-cutting ants).



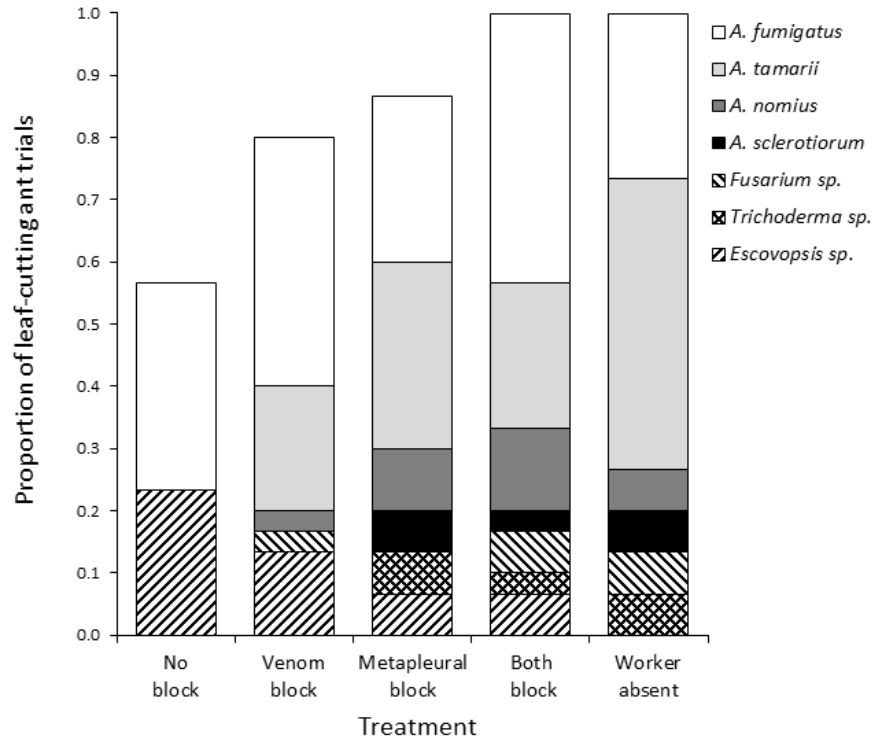
sporulating with this fungus was significantly greater when nurse ants had blocked glands, both for the weaver ants and leaf-cutting ants (respectively,  $\chi^2=12.5$ ,  $p<0.001$ ;  $\chi^2=13.1$ ,  $p<0.001$ ). Few brood sporulated with *Aspergillus* when the nursing workers had functioning glands, but 48% of the weaver ant brood and 50% of the leaf-cutting ant brood did so when the glands were blocked (Fig. 3.2). Gland blockage therefore both significantly increased the proportion of brood exposed to *Metarhizium* that then sporulated with this parasite, and also significantly increased the proportion of brood, either treated with *Metarhizium* or not, that sporulated with opportunistic *Aspergillus* fungi.

### 3.4.2 Exp. 2: Nest hygiene

There was a significant effect of both gland blockage and ant species on the appearance of fungi on nest material, but no interaction between them (Wald=35.9, d.f.=4,  $p<0.001$ ; Wald=55.9, d.f.=1,  $p<0.001$ ; Wald=5.46, d.f.=2,  $p=0.65$  respectively). There were no significant differences between colonies ( $p>0.2$  in both species). Both weaver ants and leaf-cutting ants experienced fungal growth sooner if one or both glands were blocked (Fig 3.3). For leaf-cutting ants, compared to nest material attended by an ant with unblocked glands, the hazard ratio for nest material attended by workers with blocked metapleural glands increased to 1.4, with workers with blocked venom glands it increased to 1.99, when workers had both glands blocked it increased to 2.93, and when no worker ant was present it increased to 5.01. Blocking of the venom gland in weavers increased the hazard ratio to 2.29, and an absence of the



**Figure 3.3** Hygiene of nest material. Proportion of a) weaver ant silk material and b) leaf-cutting ant fungal crop material that was free of contaminant fungal growth when cared for by workers with functional glands (white circles), blocked venom gland (black circles), blocked metapleural gland (black diamonds), both glands blocked (black squares), or where the worker ant was absent (dashed line). For each species, different letters indicate treatments which differed significantly from one another at  $P < 0.05$  in pairwise comparisons with Kaplan-Meier tests.



**Fig. 3.4** Proportion of trials where foreign fungus overgrew leaf-cutting ant nest material grouped by treatment. Foreign fungal species were *Aspergillus fumigatus* (white), *Aspergillus tamarii* (light grey), *Aspergillus nomius* (dark grey), *Aspergillus sclerotiorum* (black), *Fusarium* sp. (leftward diagonals), *Trichoderma* sp. (cross-hatched), *Escovopsis* sp. (rightward diagonals). The appearance of *Escovopsis* was relatively lower, and of other fungi relatively higher, when the glands of the attendant workers were blocked. *Aspergillus fumigatus* was common regardless of whether the ants had functional or blocked glands, while all other fungi grew only when the fungal crop was not tended by a worker with functional glands.

worker ant to 2.39. Both results were significantly different ( $p < 0.05$ ) when compared to nest silk attended to by a worker with a functional gland, but not when compared to each other, in post-hoc pairwise comparisons. Sporulation of fungi on the weaver ant silk resulted in only a sparse emergence of lightly filamentous fungi, which appeared morphologically similar across all trials and

was not successfully isolated and cultured. In those leaf-cutting ant trials where the fungal crop developed other fungi, it was overgrown quickly. *Escovopsis* was found most commonly in the trials where worker ants possessed functioning glands ( $p=0.007$ ; Fig 3.4).

### 3.5 Discussion

Previous work investigating the social immunity and antimicrobial secretions of ants has focused on their protection of other adults ants against parasites. The results presented here show that antimicrobial secretions produced by adult ant workers can also help increase the survival of both control and parasite-treated brood, and reduce fungal growth on nest material. Importantly, the secretions in these contexts appear to be particularly significant for sanitizing against opportunistic fungi. In both leaf-cutting ants and weaver ants, and regardless of experimental exposure to the *Metarhizium* parasite, brood suffered higher mortality and growth of the opportunistic *Aspergillus* fungus when the workers nursing them did not have functional antimicrobial-producing glands. Brood exposed to the specialist fungal parasite *Metarhizium* were also more likely to sporulate with this parasite when nursing workers lacked functional glands. Similarly, in both ant species, nest material was more likely to be overgrown by fungi when tended by workers without functional glands. This effect was most substantial in the weaver ants where blocking the venom gland was sufficient to result in fungal growth on nest material comparable to when no tending ant was present at all. Leaf-cutting ants required the blocking of both metapleural and venom glands to show a similar result. Whilst adult insects, including ants,

wasps, bees, termites and eusocial thrips, utilise antibiotic secretions to protect themselves (Rosengaus et al. 2000; Bot, Obermayer, et al. 2001; Turillazzi et al. 2006; Turnbull et al. 2010; Baracchi et al. 2011; Baracchi, Mazza, et al. 2012), it has recently been shown that *Lasius* ants transfer antimicrobial venom to enhance the resistance of brood to disease (Tragust, Mitteregger, et al. 2013). Our results indicate this is also the case for *Acromyrmex* and *Polyrhachis* ants. There has been much interest in the role of social immunity in the disease resistance of adult social insects, but their lack of individual immunity is likely to make brood the most vulnerable life-stage (Hölldobler and Wilson 1990; Gillespie et al. 1997; Cremer et al. 2007). Social immunity may therefore be especially essential for brood protection.

Surprisingly, there was no significant interaction in either ant species between gland blockage and *Metarhizium* exposure. Antimicrobial secretions have previously been shown to be very important for protecting adult leaf-cutting ants and weaver ants against exposure to the *Metarhizium* parasite (Poulsen, Bot, Nielsen, et al. 2002; Graystock and Hughes 2011) as well as for protecting brood of *Lasius neglectus* ants (Tragust, Mitteregger, et al. 2013). The lack of a significant interaction here between gland blockage and *Metarhizium* exposure is likely to be for two reasons. First, both the probability of parasite infection success and the effects of antimicrobial secretions are dose-dependent (Ebert et al. 2000; Hughes and Boomsma 2004b; Stow et al. 2007; Turnbull et al. 2012). The greater the dose of parasite, the more likely an infection is to be successful, and it may be that the dose of the parasite strain used here was too high for the antimicrobial secretions that were transferred from the adult ants to be fully effective in defending brood against the *Metarhizium* parasite. In addition, lower

doses of antimicrobial compounds are less likely to be effective against a parasite and it may be that the dose of antimicrobial secretions transferred to the brood was too low to fully defend the brood against *Metarhizium*, and thus too low for a strong effect of gland blockage on resistance to *Metarhizium* to be seen. Second, the effect of gland blockage on the mortality of even control brood was relatively high. Both here and in other studies (Poulsen, Bot, Nielsen, et al. 2002; Graystock and Hughes 2011), there has been found to be little impact of gland blockage on control-treated adult ants themselves, but it appears that control-treated brood are far more susceptible to the impact of being with nursing workers with blocked glands. The behaviour of the nursing workers, including their grooming of the brood, was unchanged by gland blockage, and there is no known nutritional role for the glandular secretions, so it seems most probable that this impact relates to the infections by opportunistic fungal parasites which developed.

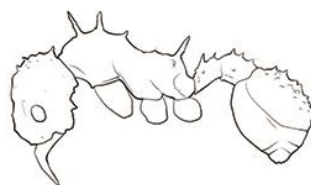
As with all organisms, ant colonies co-exist with a wide diversity of opportunistic microbes that can be parasitic, such as the *Aspergillus* fungus found in this experiment and in ant colonies studied previously (Pereira and Stimac 1997; Schmid-Hempel 1998; Hughes et al. 2004; Poulsen et al. 2006; Lacerda et al. 2010; Fountain and Hughes 2011). Adult ants appear to suffer relatively little from these opportunistic parasites even when their production of antimicrobial secretions is prevented (Poulsen, Bot, Nielsen, et al. 2002; Graystock and Hughes 2011)presumably due to their well-developed immune system and grooming behaviour. We also found this to be the case here for brood and nest material when the antibiotic-producing glands of nurses were functioning. However, when the antimicrobial secretions of nurses were lacking, most brood succumbed to

infection by opportunistic *Aspergillus* fungi and most nest material became overgrown. It cannot be excluded that some of the fungal growth may have been opportunistic growth on larvae that died from another cause. However, even if this is the case, then the results nevertheless demonstrate the importance of antimicrobial secretions from nursing workers for sanitizing the cuticles of larvae. It therefore appears that the antimicrobial secretions of adults ants are essential to protect the vulnerable brood against opportunistic parasites and to prevent nest material becoming overgrown by contaminant fungi. It may indeed be the case that the protection of larvae against ubiquitous opportunistic microbes is of greater importance for ant fitness than protection against more specialist parasites such as *Metarhizium* which tend to be rarer, and may potentially have driven the evolution of antimicrobial secretions in ants.

The leaf-cutting ant nest samples in this study were found to host at least seven species of fungi ranging from generalist, opportunistic *Aspergillus* spp. to *Escovopsis*, which specialises in parasitising the fungal crop of leaf-cutting ants (Currie 2001). Workers with functioning glands reduced both the number and diversity of fungi found compared to treatments with blocked glands. Only *Escovopsis* and the hyperabundant *Aspergillus fumigatus* (Latgé 1999) were found in treatments where the attending workers had functioning antimicrobial-producing glands. Other fungi only occurred when the fungal crop was not tended by workers with functional glands. *Escovopsis* has evolved to be highly successful in natural leaf-cutting ant nest environments (Currie and Stuart 2001; Currie 2001) and, as our results show, is able to grow on the fungal crop even when workers are producing antimicrobial compounds from their metapleural glands. In this antimicrobial-rich setting, *Escovopsis* is then able to exclude most of the

opportunistic fungi found in this study. Interestingly, however, our results suggest the specialist *Escovopsis* may be less dominant if the antimicrobial secretions of the ants are reduced, through blocking of the metapleural gland, in which setting other fungi are far more competitive against *Escovopsis*. Consequently antimicrobial secretions may be more important for protection against more opportunistic fungal pathogens than previously thought.

The results show how social immunity provided by the altruistic provision of antimicrobial secretions from adult ants has evolved to play an important role in brood survival and maintaining hygienic nest conditions, and thus the fitness of their colony. In addition, we show that these social secretions are important, not just to combat specialist parasites like *Metarhizium* and *Escovopsis*, but also in the everyday defence against opportunistic microbes which are ubiquitous in and around nest sites. This not only highlights the vulnerability of brood and nest material to disease but also their reliance on social care, and provides a compelling explanation for how immobile brood with immature immunity, survive in a world abundant with pathogens.



*Acromyrmex subterraneus*



## 4. Acid, silk and grooming: alternative strategies in social immunity in ants?

Tranter C, Hughes WOH (2015) Acid, silk and grooming: alternative strategies in social immunity in ants? *Behav. Ecol. Sociobiol.* 69:1687–1699

### 4.1 Abstract

Parasites are an important force in evolution, driving the need for costly resistance mechanisms. The threat from disease is potentially high in group-living species such as social insects, which have accordingly evolved behavioural and chemical defences that vary between species depending on their life histories. Several ant genera have lost a key exocrine antimicrobial defence, the metapleural gland, and yet are still able to thrive in environments abundant with parasites. We investigate, in species lacking the metapleural gland, how the production of antimicrobial venom, grooming behaviours, and the use of potentially antimicrobial larval silk may have evolved as alternative antiparasite defences. We focus on the Australasian weaver ant *Oecophylla smaragdina*, and compare this to *Polyrhachis* weaver ants. We show that the production of venom by *O. smaragdina* workers is important for disease resistance, but that the presence of larval silk is not, and that workers use their acidic venom to maintain nest hygiene. The grooming defences of *O. smaragdina* differ between castes, with minor workers allogrooming more and major workers showing greater upregulation of grooming in response to parasites. Chemical and behavioural defences differ interspecifically between *O. smaragdina* and *Polyrhachis*, with *O. smaragdina* appearing to rely primarily on its venom while *Polyrhachis* use

higher rates of grooming. The results show how alternative investment strategies can evolve for disease defence, notably the highly effective application of acidic venom by *O. smaragdina*, and highlights the need for targeted comparative studies to understand how organisms respond to the ubiquitous threat from parasites.

## 4.2 Introduction

The threat from disease is an important driver of host biology and population structures (Poulin and Morand 2000; Poulin 2007). Strong parasite pressures can result in the rapid evolution of host defences which are required to reduce the cost of this pressure on host fitness (Brockhurst et al. 2004; Duffy and Sivers-Becker 2007; Decaestecker et al. 2007). This threat has led to the evolution of a complex array of defence mechanisms, ranging from behavioural avoidance strategies to the complex adaptive immune system of vertebrates (Siva-Jothy et al. 2005; Thieltges and Poulin 2008; Wisenden et al. 2009; Tranter et al. 2015). Organisms that live in groups possess the additional benefit of group-level defences, such as allogrooming or shared use of antimicrobial secretions, which function in combination with individual-level defences against the threat from parasites (Krause and Ruxton 2002; Nunn and Altizer 2006). However living in groups also potentially increases the threat of disease, because the greater density of individuals within a group can enhance intragroup transmission, and many social activities such as chemical communication or sharing of food (trophallaxis) put members in close physical proximity (Alexander 1974; Moller et al. 1993;

Rose et al. 1999; Altizer, Nunn, Thrall, et al. 2003; Godfrey et al. 2009).

Defence mechanisms against parasites are often costly (Bowers et al. 1994; Poulsen, Bot, Nielsen, et al. 2002; Rigby et al. 2002), and the great variety of strategies that organisms employ means that comparative studies are key to understanding the evolutionary biology of host disease resistance. In cases where the extensive datasets needed for full comparative analyses are lacking, targeted studies on species that differ in specific life-history traits that are predicted to affect the host-parasite relationship can be highly informative.

In social insects, the selection pressure from the threat of disease has resulted in the evolution of a suite of individual and group-level behavioural and chemical defences (Boomsma et al. 2005; Cremer et al. 2007; Wilson-Rich et al. 2009; Rosengaus et al. 2011). Social insect colonies are based on division of labour and this can apply to disease resistance too, with worker and reproductive castes in leaf-cutting ants show differences in the use and effectiveness of disease resistance mechanisms (Hughes et al. 2002, 2010; Baer et al. 2005; Poulsen et al. 2006). These defences include meticulous self-grooming and allogrooming that are effective, adaptive and proactive in removing parasites (Farish 1972; Basibuyuk and Quicke 1999; Traniello et al. 2002; Yanagawa et al. 2008; Morelos-Juárez et al. 2010). In particular ants and termites will increase grooming when exposed to fungal conidia (Rosengaus et al. 1998; Yanagawa and Shimizu 2006; Yanagawa et al. 2008; Walker and Hughes 2009; Morelos-Juárez et al. 2010; Reber et al. 2011). This grooming can be combined with the use of antimicrobial secretions from exocrine glands by workers to sterilize themselves, their brood, their nest material, and food (Fernández-Marín et al. 2009; Tragust, Ugelvig, et al. 2013; Tragust, Mitteregger, et al. 2013; Tranter et al. 2014).

Similarly ants (Storey et al. 1991; Mackintosh et al. 1995; Zelezetsky et al. 2005; Mendonça et al. 2009), bees (Evans et al. 2006; Baracchi and Turillazzi 2010; Baracchi et al. 2011), wasps (Turillazzi et al. 2006; Baracchi, Mazza, et al. 2012), termites (Rosengaus et al. 2000, 2004) and eusocial thrips (Turnbull et al. 2010, 2012), as well as non-social insects (Lowenberger et al. 1999; Kuhn-Nentwig 2003; Haine et al. 2008; Stow and Beattie 2008), produce defensive compounds in their haemolymph and secretions which can be used in the defence against pathogens. Of particular importance is the antimicrobial-producing exocrine metapleural gland (MG), which is unique to, and ancestral in, ants (Hölldobler and Engel-Siegel 1984; Veal et al. 1992; Ortius-Lechner et al. 2000; Quinet and Vieira 2012; Vieira et al. 2012). Antimicrobial compounds are likely often transferred between nestmates during grooming, and in at least one ant species by trophallaxis (Hamilton et al. 2011).

The most extreme evolutionary transition in antibiotic use, however, is shown by some formicine ant taxa in the genera *Polyrhachis*, *Camponotus* and *Oecophylla* which have secondarily lost the MG entirely, suggesting either a substantial relaxation in parasite pressure negating the requirement for maintaining an energetically costly gland, or the development of alternative forms of defence (Yek and Mueller 2011). The losses of the MG in these genera are correlated with arboreality and the evolution of weaving nests from larval silk, a behaviour which is unique to certain species in these genera (Johnson et al. 2003; Robson and Kohout 2005, 2007). It has been suggested that arboreal ants, such as *Oecophylla* and many *Polyrhachis* species, may be able to invest less in costly defences such as the MG because they are less exposed to the fungal parasites that are abundant in soil (Boomsma et al. 2005), although supporting evidence

for this hypothesis is still lacking (Walker and Hughes 2011). It may also be the case that the silk substrate of weaver ant nests provides an aseptic habitat for these species (Johnson et al. 2003), or that the silk contains antimicrobial compounds, as in other invertebrates (Wright and Goodacre 2012), which may transfer to ants. In the only direct test of this, Fountain & Hughes (2011) failed to find any benefit from silk for defence against pathogenic fungi in the weaver ant *Polyrhachis dives*, with the silk in fact carrying viable opportunistic fungal parasites. However, silk weaving in *Oecophylla* appears to be more derived and complex than in *Polyrhachis* (Crozier and Newey 2010), so it is possible that there may be stronger benefits from silk for *Oecophylla* than for the weaver ants investigated previously (Fountain and Hughes 2011; Graystock and Hughes 2011). Alternatively, the loss of the MG may be associated with the evolution of different defence mechanisms. The venom of many social insects may possess antimicrobial properties, but it is in ants where we see the use of acidic venom diversified beyond stinging behaviours (Moreau 2013). Along with other formicines, *Oecophylla* and *Polyrhachis* produce acidic venom, composed principally of formic acid (Bradshaw 1979; Hölldobler and Wilson 1990; Blum 1992). *Polyrhachis dives*, as well as other formicines, are known to use their venom during grooming, spreading venom on themselves, their brood and their nest material (Graystock and Hughes 2011; Tragust, Ugelvig, et al. 2013; Otti et al. 2014; Tranter et al. 2014). The use of venom in *Oecophylla* and *Polyrhachis* may be particularly important because they have lost their MG and the antimicrobial secretion it produces. Self-grooming also appears to be upregulated in *Polyrhachis* compared to other ants so may be another mechanism to compensate for the lack of a MG (Graystock and Hughes 2011; Tranter et al. 2015). Ants may also alter the amount of trophallaxis, which may reduce the

transmission of pathogens through minimising physical contact, or, in at least one species of ant, transfer antimicrobial proteins to nestmates (Hamilton et al. 2011b Reber and Chapuisat 2012). These behaviours may provide an alternative form of protection in species which lack antimicrobial defences.

In this study we investigate the defences employed for disease resistance by the but little studied green weaver ant *Oecophylla smaragdina*, a species which has complex societies with advanced nest weaving and polymorphic workers, and compare this with three *Polyrhachis* weaver ant species that also lack the MG, but which have monomorphic workers and less advanced nest weaving. Specifically we test: i) the importance of antimicrobial venom and the presence of potentially antimicrobial larval silk for the ability of *O. smaragdina* workers to resist parasite infection; ii) whether polymorphism in *O. smaragdina* allows division of labour in disease resistance, by examining whether the major and minor worker castes of *O. smaragdina* differ in their grooming defence against parasites; iii) how the grooming response of *O. smaragdina* to parasites compares with that of three *Polyrhachis* weaver ant species; iv) the effectiveness of venom in maintaining acidic nest conditions in both *O. smaragdina* and *P. delecta*.

## 4.3 Methods

We used four different species of weaver ant in the experiments. *Oecophylla smaragdina*, was our primary study species and used in all four experiments. This is an ecologically dominant, polymorphic species of weaver ant found across Asia

and Australia which shows the most advanced form of nest weaving, making nests in trees and vegetation using leaves woven together with larval silk (Crozier and Newey 2010). We compared the behavioural and chemical defences of *O. smaragdina* in Experiments 3 and 4 with those of three species of *Polyrhachis* weaver ants: 2) *Polyrhachis (Myrmhopla) dives* (in Experiment 3), a monomorphic arboreal species from Asia and Australia which forms characteristic carton nests from twigs, foliage, and general detritus bound with larval silk; 3) *Polyrhachis (Myrma) foreli* (in Experiment 3), a large monomorphic *Polyrhachis* species which tends to be terrestrial and nest in rotting wood or ground-level epiphytes (Robson and Kohout 2007; Kohout 2012); 4) *Polyrhachis (Cyrtomyrma) delecta* (in Experiments 3 and 4), a monomorphic shiny black weaver ant from Queensland, Australia, which builds carton nests from larval silk between the lower leaves of trees and shrubs (Kohout 2006). Other than in Experiment 2, we used major workers of *O. smaragdina* throughout as they were most similar in size to the *Polyrhachis* workers. Fungi appear to be the most common parasite threat for ants and, while particularly abundant in the soil environment, are also present in the arboreal habitat as well (Boomsma et al. 2005; Griffiths and Hughes 2010). Fungal disease threats to ants include specialist parasites of ants such as *Ophiocordyceps*, generalist entomopathogens such as *Metarhizium*, and opportunistic parasites such as *Aspergillus* (Jouvenaz et al. 1972; Alves and Sosa-Gómez 1983; Humber 1992; Schmid-Hempel 1998; Hughes et al. 2004; Rodrigues et al. 2010; Lacerda et al. 2010; Ribeiro et al. 2012). Specialist ant parasites are likely to show strong coevolution with their specific host species making them unsuitable for comparative experiments, so we here use *Metarhizium* as the experimental parasite because it has been reported parasitizing a wide diversity of ant species (Allen and Buren 1974; Lofgren and

Vander Meer 1986; Sanchez-Pena and Thorvilson 1992; Quiroz et al. 1996; de Zarzuela et al. 2007, 2012; Castilho et al. 2010), and should be less likely to exhibit species-specific coevolution with the ants investigated here.

#### **4.3.1 Exp. 1: Venom gland blockage and silk in *O. smaragdina***

Forty-eight major workers were collected from immediately outside the nest entrances for each of five colonies of *O. smaragdina*. The ants from each colony were divided into eight treatment groups representing a full-factorial combination of trials with ants either: i) treated or untreated with *Metarhizium pingshaense* [MT02\_73 isolated from Panama (Hughes et al. 2004; Pull et al. 2013)] ii) with venom glands blocked with nail varnish or unblocked, and iii) with or without a section of nest silk to test whether ant workers may gain antimicrobial compounds from the silk. The *Metarhizium*-treated ants had 0.5  $\mu\text{l}$  of a  $1.5 \times 10^6$  conidia per ml suspension of *Metarhizium* conidia in a 0.05% solution of Triton-X surfactant applied directly to the mesosoma and gaster of the ant with a micropipette. Similar doses have produced approximately 50% mortality in *Polyrhachis* and other formicine ants (Graystock & Hughes 2011; C Tranter unpublished data). Control ants had 0.5  $\mu\text{l}$  of a  $1.5 \times 10^6$  particle per ml suspension of talcum powder in 0.05% Triton-X solution applied in the same way as a particulate control. Ants were held with soft forceps during the treatment procedure. *Metarhizium* conidia were harvested from freshly sporulating media plates, and viability was confirmed to be  $> 90\%$  throughout the experiments by quantifying conidia germination 24 h after plating onto Sabouraud dextrose agar (Siegel 2012). Venom glands were blocked by placing a drop of quick-dry nail varnish



over the acidopore with a needle, with a drop of nail varnish being applied on to the dorsal surface of the gaster in control ants. The nail varnish was checked daily to ensure that it was still intact, but in all trials remained present for the duration of the experiment. Fresh white nest silk was obtained from the outer sections of the nest-of-origin for each ant, and cut into 1 cm<sup>2</sup> squares, before being paired with each ant. After treatment, ants were kept individually at 22°C in pots (height: 100 mm, diameter: 22 mm), with two small balls of cotton wool soaked in water and 20% sucrose solution, and their survival monitored daily for a period of two weeks.

#### **4.3.2 Exp. 2: Caste differences in *O. smaragdina* behaviours**

In order to investigate whether *O. smaragdina* worker castes may differ in their self-grooming behavioural defences, 10 major and 10 minor workers were collected from each of five colonies of *O. smaragdina*. Half of the ants of each caste were treated with *Metarhizium* and half with a control treatment of talcum powder, as in Experiment 1. Each individual was placed in an individual pot, in the conditions described above, and observed for 15 min, with the length of time the ant spent self-grooming being recorded. In addition, 12 minor and 12 major workers were collected from each of four colonies of *O. smaragdina* to look at differences in allogrooming and trophallaxis between pairs of ants. For this, the ants from each colony were split into three pairings: major-major, major-minor, and minor-minor, with each pair of ants being placed together in a pot. The duration of any allogrooming or trophallaxis between the two ants was then recorded over a period of 15 min. Whilst it was possible to discriminate the

direction of allogrooming e.g. a major grooming a minor, this directionality was not accurately determinable for trophallaxis.

#### **4.3.3 Exp. 3: Behavioural defences of four species of weaver ant**

Ten major worker ants from each of three colonies were collected from the exterior nest surface of each of the four weaver ant species (*P. delecta*, *P. dives*, *P. foreli*, *O. smaragdina*). Half of the ants from each colony were treated with *Metarhizium* and the other half with a talcum powder control suspension, as in Experiment 1, placed in individual pots, and their self-grooming observed for 15 min. Data from *P. dives* was collected prior to the rest of the experiment in 2012 from colonies kept in the UK, using a different stock suspension of the same strain of *Metarhizium*, but with the concentration, delivery methods, and rest of the protocol being identical.

#### **4.3.4 Exp. 4: The use of acidic venom for nest hygiene**

We investigate directly whether worker presence affected the pH of nest material in four ways. First, nest silk from colonies of *O. smaragdina* and *P. delecta* was cut into 1 cm<sup>2</sup> sections, and the pH tested by soaking the nest material samples in 0.5 ml of distilled water and adding universal indicator solution (Fluka Universal indicator solution; pH 4-10). The nest material was sampled broadly from both towards the inside and outside the colony. This was repeated 15 times (five samples from each of three colonies) from each of the two species for: 1) nest material freshly sampled (less than 2 h since colony collection); 2) nest material which had been left on its own in a pot for 24 h; 3) nest material which had been paired with a worker ant and left in a pot for 24 h. *Oecophylla* majors were used

for the pairings, and all ants were sampled from those found immediately external to the nest. Second, the ability of ants to maintain alterations in pH in their environment was investigated by placing ants with a section of pH indicator paper (Fluka indicator paper; pH 0.5-7). Individual ants were placed into a pot with the bottom lined with two sections of indicator paper for 48 h. At time points 2 h, 12 h, 24 h and 48 h both indicator sections of the dish were observed and the pH recorded based on the colour of the indicator paper. At each of the time points the left hand section of the indicator paper was replaced to account for any variation in the result due to time affecting the indicator paper, but both papers gave the same ( $\pm < 0.5$  pH) results throughout. After 48 h the ant was removed and the paper left for a further 24 h before a final recording was taken. This was repeated for five ants from each of three colonies for *P. delecta* and five ants from each of five colonies for *O. smaragdina*. Third, in order to confirm that venom use was responsible for changes in pH we blocked the venom gland of 15 ants with nail varnish and applied nail varnish to the dorsal surface of the gaster for 15 control ants from five colonies of *O. smaragdina*, and recorded the pH of the environment within a pot in which they had been kept for 48 h using pH indicator paper. Fourth, the antifungal effect of pH was tested by measuring conidia viability on media at three acidities. Twenty agar media plates (Sabarose dextrose agar medium plus yeast) were prepared, and 100  $\mu$ l of a  $1.5 \times 10^6$  suspension containing freshly harvested *Metarhizium* conidia in Triton-X was pipetted and spread evenly over the surface of the plate. The plates were left for 10 min for any excess liquid to evaporate before 100  $\mu$ l of one of a series of sequentially diluted pH treatments were applied. Doses were as follows: undiluted formic acid solution (pH 3.7), 1:10 dilution of formic acid:ddH<sub>2</sub>O (pH 4.7), 1:100 dilution of formic acid:ddH<sub>2</sub>O (pH 5.7), and pure ddH<sub>2</sub>O. Plates were then incubated for 16 h

at 28°C before the percentage of viable conidia (counted as those where the germ tube produced was longer than the diameter of the conidia) were counted under a stereo microscope at 200x magnification (Siegel 2012).

## 4.4 Statistical analyses

All data was tested for normality prior to analyses in order to determine the correct test and model distributions were chosen based on the best fit using AIC scores and the structure of the non-normal data. No over-dispersion was observed based on model deviance/df values. The survival of *Oecophylla smaragdina* ants in Experiment 1 were analysed using a Cox regression with fungal treatment, the presence of silk, gland blockage, and colony included as factors. Non-significant interaction terms were removed stepwise to achieve the minimum adequate model. Pairwise comparisons of treatments were made using Kaplan-Meier tests with the Breslow statistic. In Experiment 2, the effect of caste on the duration of self-grooming for *O. smaragdina* workers was examined using a linear mixed model, with caste-pairings and fungal treatment included as fixed factors.

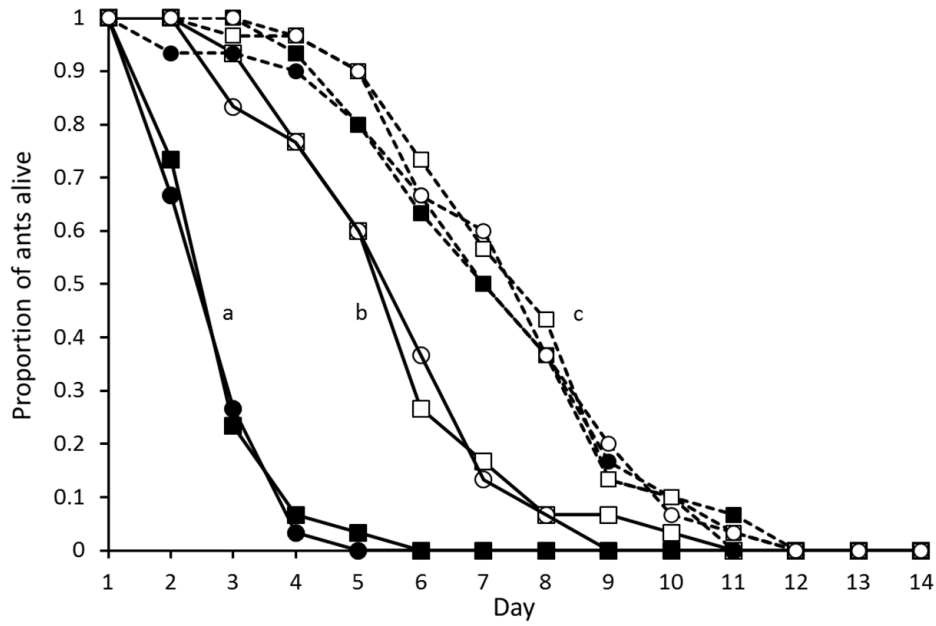
Allogrooming and trophallaxis rates in Experiment 2 and the differences between self and allogrooming rates between the four species of weaver ants with and without fungal treatment in Experiment 3, were analysed using generalized linear mixed models (GLMM) with gamma distributions and log link functions. The pH levels of the environment shared by an individual ant in Experiment 4 was analysed using a GLMM with gamma distribution and log link function, with time and species included as fixed factors. The pH levels of nest silk paired with different ant species for different periods, differences in conidia viability between pH treatments, and the effect of gland blockage on pH, were analysed using linear

mixed model. Post-hoc comparisons for all models were adjusted using the sequential Bonferroni method and colony-of-origin was included as a random factor for all linear mixed models and GLMMs.

## 4.5 Results

### 4.5.1 Exp. 1: Venom gland blockage and silk in *O. smaragdina*

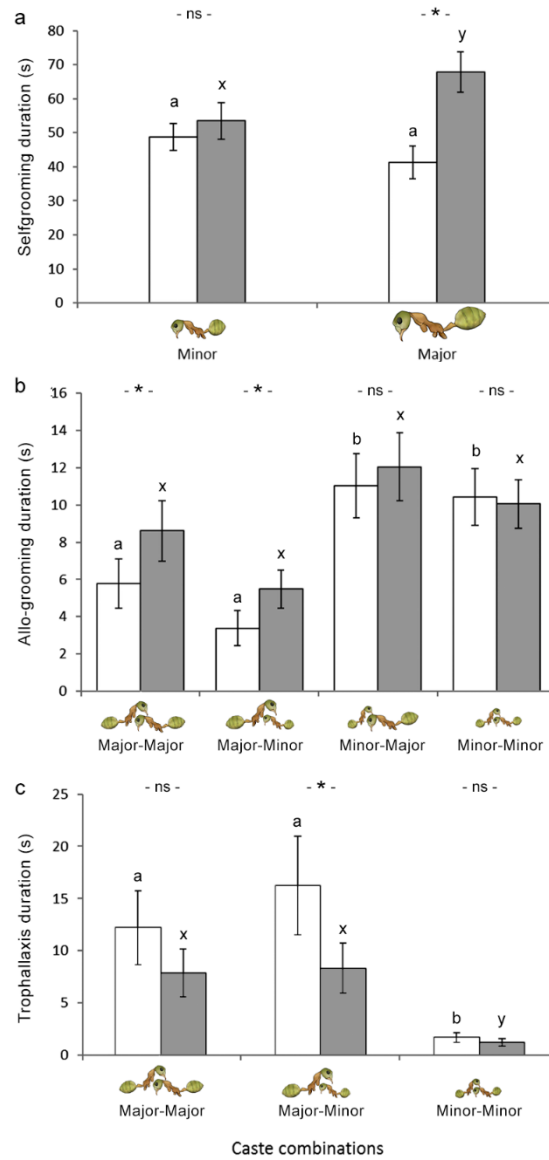
None of the control ants which died sporulated with the *Metarhizium* parasite, whereas approximately 75% of the dead *Metarhizium*-treated ants sporulated with the parasite. There was a significant interaction between the effects of gland blockage and *Metarhizium* treatment on survival (Wald = 44.7, d.f. = 1,  $p < 0.001$ ). Ants treated with control solution survived similarly well regardless of whether their venom gland was blocked and all ants exposed to *Metarhizium* parasite suffered greater mortality, but ants exposed to the *Metarhizium* parasite survived significantly less well when their venom gland was non-functional (Figure 4.1). The presence or absence of nest silk had no effect on worker survival (Wald = 0.42, d.f. = 1,  $p = 0.52$ ). There were differences in the survival rates of ants from different colonies (Wald = 7.08, d.f. = 1,  $p = 0.008$ ) but no interactions between the colony-of-origin and the effects of treatments ( $p > 0.05$  in all cases).



**Figure 4.1.** The survival of individual *O. smaragdina* workers exposed to the *Metarhizium* parasite (solid lines) or control solution (dashed lines), with venom glands blocked (black symbols) or unblocked (white symbols), and kept with nest silk (squares) or without silk (circles). Different letters indicate three sets of treatments which differed significantly from one another in pairwise comparisons ( $p < 0.05$ ).

#### 4.5.2 Exp. 2: Caste differences in *O. smaragdina* behaviours

There was no effect of colony of origin on self-grooming, allogrooming or trophallaxis rates ( $z = 2.12, p = 0.07$ ;  $z = 0.72, p = 0.47$ ;  $z = 0.703, p = 0.48$ , respectively) and therefore data across colonies were analysed together. There was a significant interaction between the effects of caste and fungal treatment on both self-grooming rates ( $F = 5.57, \text{d.f.} = 1, p = 0.02$ ) and allogrooming rates ( $F_{3,88} = 2.85, p = 0.042$ ). Major workers, but not minor workers, significantly increased their self-grooming rates in response to fungal exposure (Figure 4.2a). Minor workers had higher baseline allogrooming levels overall but did not alter their allogrooming rates in response to a fungal threat



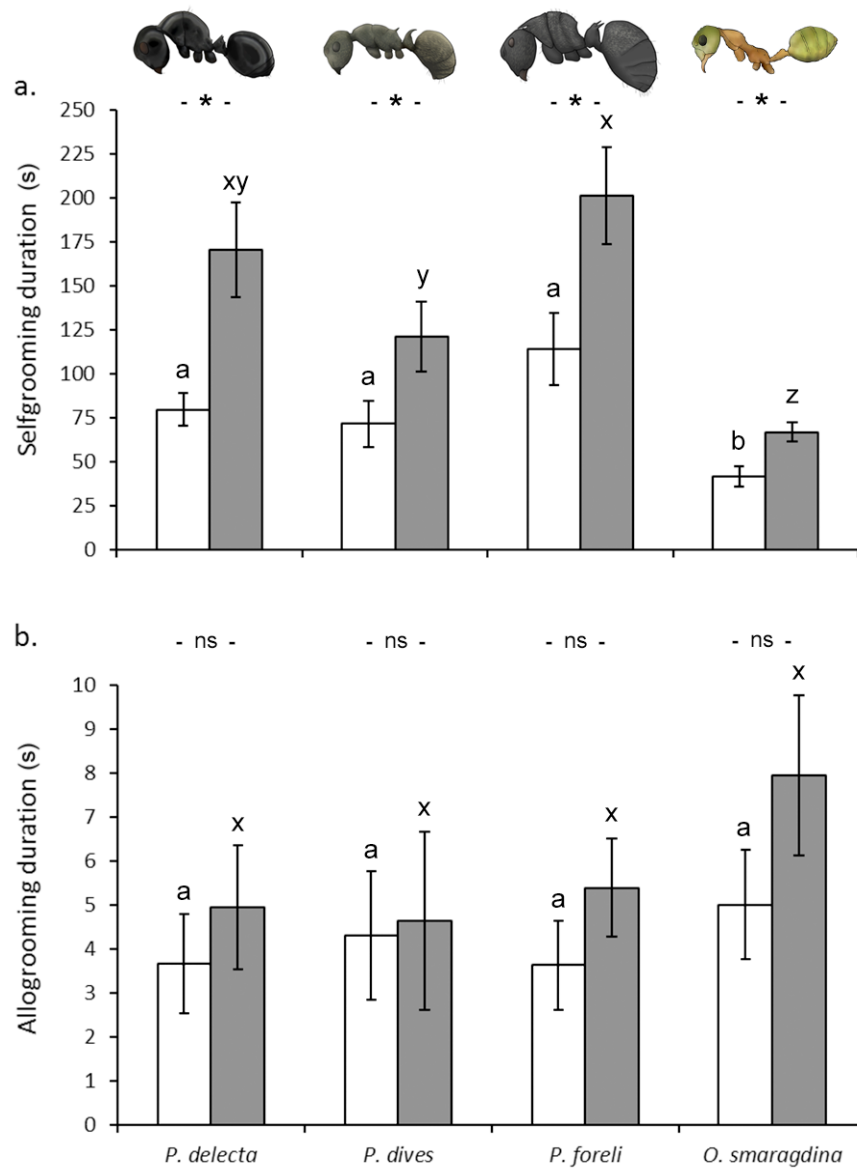
**Figure 4.2.** Grooming duration in *Oecophylla smaragdina*. Either a) self-grooming, b) allogrooming, and c) trophallaxis major or minor workers that were either treated with the *Metarhizium* parasite (grey) or Triton-X control (white). Self-grooming observations were made on individual minor or major workers, whilst allogrooming and trophallaxis rates were quantified for pairs of ants in all four combinations of major and minor castes. In (b), the first caste listed in the x-axis label indicates the ant carrying out allogrooming and the second caste listed indicates the ant receiving allogrooming. It was not possible to accurately determine the direction of trophallaxis. An asterisk above columns indicates a significant difference between the *Metarhizium* and control treatments, while different letters above columns indicate caste combinations which differed significantly from one another (talcum powder: a-c, *Metarhizium*: x-z;  $p < 0.05$ ).

(Figure 4.2b). Major workers, however, significantly up-regulated allogrooming rates, both of major and minor workers, in response to fungal exposure. The increase in allogrooming by majors after exposure to the fungi resulted in levels comparable to minors. Trophallaxis rates differed significantly between the caste combinations, with the presence of majors leading to higher trophallaxis rates ( $F_{2,66} = 61.4, p < 0.001$ ; Figure 4.2c). Overall there was less trophallaxis when an ant had been treated with *Metarhizium* ( $F_{1,66} = 15.4, p < 0.001$ ), although this difference was only significant in the major-minor pairing (Figure 4.2c), and there was no interaction between terms ( $F = 0.25, \text{d.f.} = 2,66, p = 0.77$ ).

#### 4.5.3 Exp. 3: Behavioural defences of four species of weaver ant

The four weaver ant species differed significantly in their baseline self-grooming levels ( $F_{7,112} = 25.6, p < 0.001$ ), and all the species groomed significantly more when exposed to *Metarhizium* ( $F_{1,112} = 58.3, p < 0.001$ ). There was no interaction between factors indicating that they responded in similar ways ( $F_{3,112} = 0.88, p = 0.454$ ), and no effect of colony ( $z = 0.33, p = 0.75$ ). *Oecophylla smaragdina* had significantly lower baseline self-grooming and up-regulation of grooming rates compared with the *Polyrhachis* species, of which *P. foreli* had the highest baseline grooming rates (Figure 3a). Overall there was a significant up-regulation of allogrooming in ants treated with *Metarhizium* compared to those treated with talcum powder ( $F_{1,112} = 4.89, p = 0.029$ ; Figure 3b), but no significant difference between species ( $F_{3,112} = 1.46, p = 0.225$ ), interaction between effects ( $F_{3,112} = 0.515, p = 0.673$ ), or effect of colony ( $z = 0.725, p = 0.469$ ).

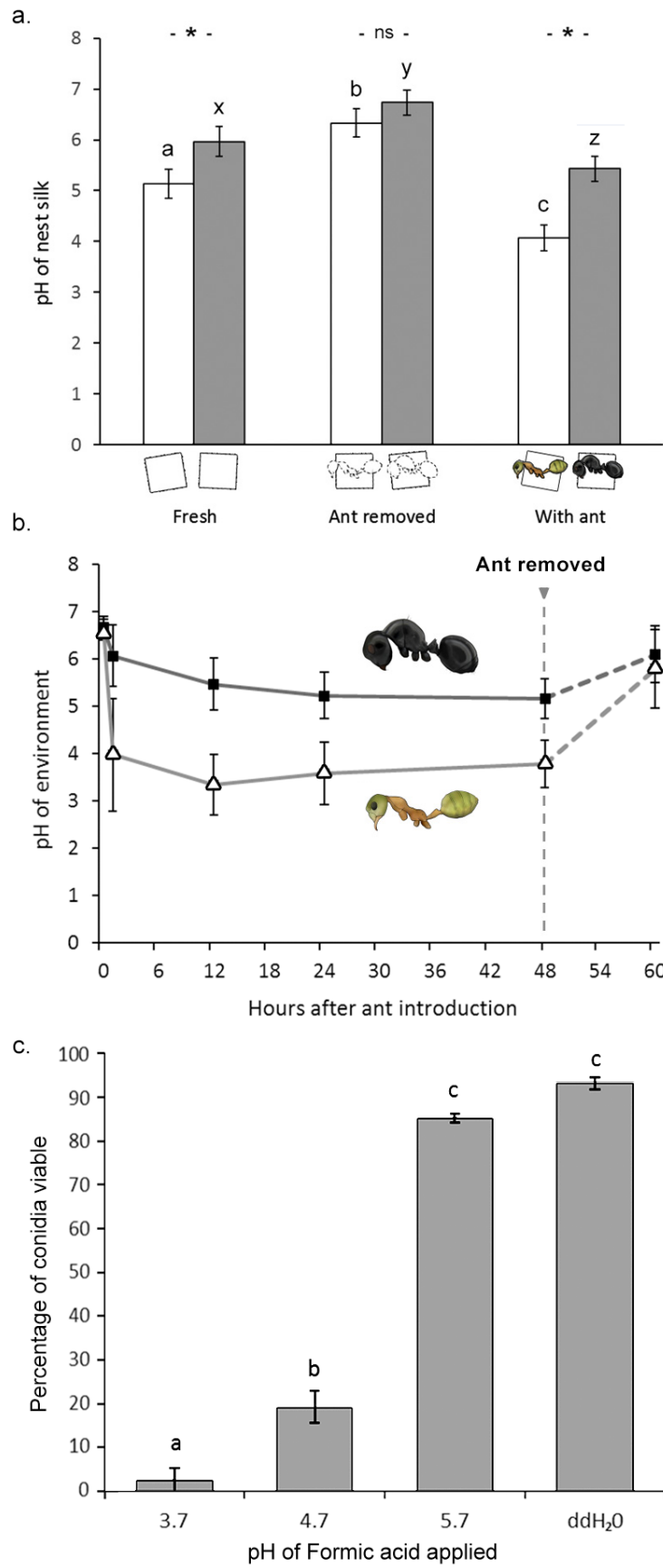




**Figure 4.3.** Grooming durations in four weaver ant species: a) self-grooming and b) allogrooming for works of *Polyrhachis delecta*, *Polyrhachis dives*, *Polyrhachis foreli*, *Oecophylla smaragdina*, after exposure to the *Metarhizium* parasite (grey) or Triton-X control (white), in a 15 min period. An asterisk above columns indicates a significant difference between the *Metarhizium* and control treatments, while different letters above columns indicate caste combinations which differed significantly from one another (talcum powder: a-c, *Metarhizium*: x-z;  $p < 0.05$ ).

#### 4.5.4 Exp. 4: The use of acidic venom for nest hygiene

In the first assay when the pH of silk was measured directly, there was a significant interaction in the effects of ant species and whether the silk had been kept with attending workers on the pH of silk ( $F_{5,84} = 4.41, p < 0.001$ ). The rise in pH when silk was kept without ants was greater for silk from *O. smaragdina* nests than for silk from *P. delecta* nests, and the reduction in the pH of silk when kept for 24 h with an ant was also much greater with *O. smaragdina* than *P. delecta*, becoming more acidic than silk from the nest in the former but becoming identical to silk from the nest in the latter (Figure 4a). The pH of *O. smaragdina* silk was significantly lower when compared to silk from colonies of *P. delecta* for both fresh silk and silk that had been kept with an ant, but there was no difference in the pH of silk from the two species when it had been left without an attending ant for 24 h. In the second assay, the presence of ants resulted in a significant reduction in the environmental pH over time ( $F_{5,228} = 47, p < 0.001$ ). Two hours after introduction of the ants, the pH was substantially reduced to a low level at which it was maintained for the 48 h that the ant was present (Figure 4.4b). After the ant was removed after 48 h, the pH returned to near the initial value by 60 h. The reduction in pH was much stronger with *O. smaragdina* than *P. delecta* ( $F_{1,228} = 203, p < 0.001$ ), but the change over time was similar for the two species ( $F_{3,112} = 0.88, p = 0.454$ ; Figure 4.4b). In the third assay, the pH of the environment within pots was significantly lower when *O. smaragdina* ants had functional venom glands, at  $\text{pH } 4.5 \pm 0.2$ , compared to  $\text{pH } 6.1 \pm 0.1$  when the gland was blocked ( $F_{1,15} = 54.96, p = 0.002$ ) and there was no effect of colony-of-origin on the pH ( $F_{1,4} = 0.28, p = 0.56$ ).



**Figure 4.4.** pH and acid application in weaver ants. a) The pH of nest silk that had been freshly removed from the inside of the colony (left), left for 24 h without an attending ant (middle), or kept for 24 h with an attending ant (right), from colonies of either *O. smaragdina* (white bars) or *P. delecta* (grey bars). An asterisk above columns indicates a significant difference between the *Metarhizium* and control treatments, while different letters above columns indicate caste combinations which differed significantly from one another (talcum powder: a-c, *Metarhizium*: x-z;  $p < 0.05$ ). b) The effect of the introduction of an individual ant on the pH of its environment at six time points over a 60 h period. pH readings were taken at 0, 2, 12, 24, 48 and 60 h and each data point represents a mean  $\pm$  s.e. of 25 individuals of either *Polyrhachis delecta* (black squares) or *Oecophylla smaragdina* (white triangles). At 48 h (indicated by the dashed vertical line) the ant was removed. c) The percentage of *Metarhizium* conidia germinating on media in the presence of three dilutions of formic acid or a control treatment of ddH<sub>2</sub>O. Different letters indicate treatments which differed significantly from one another at  $p < 0.05$ .

Additionally ants were observed spraying acid from their venom glands during trials which could be seen as distinct spots on the pH paper, indicating areas that had undergone significant reductions in their pH. In the fourth assay, the application of formic acid in vitro had a significant effect on conidia viability ( $F_{3,16} = 354.7$ ,  $p < 0.001$ ), with a pH of 4.7 reducing conidial viability by approximately 80% and a pH of 3.7 reducing it to almost to zero (Figure 4.4c).

## 4.6 Discussion

The results shed light on the behavioural and, in particular, chemical defences that *Oecophylla* weaver ants have against disease, and that the strength of these defences varies interspecifically when compared to *Polyrhachis* weaver ants. *O.*

*smaragdina* workers were more resistant to the entomopathogenic fungus *Metarhizium* when they could secrete venom. Their acidic venom was strongly antimicrobial, and application improved the hygiene of their nest material. There was no evidence for *O. smaragdina* workers gaining antimicrobial compounds from silk, although other benefits of silk are possible. The behavioural defences of *O. smaragdina* varied phenotypically, with minor workers showing lower self-grooming, higher allogrooming and lower upregulation in response to fungal threat than major workers. When compared with *Polyrhachis* weaver ant species, *O. smaragdina* exhibited lower grooming rates, but more active use of a more acidic venom.

The use of venom in *O. smaragdina* seems to be very important for disease resistance, with individuals surviving exposure to the *Metarhizium* parasite significantly better when their venom gland was functional, as opposed to being blocked. The presence of *O. smaragdina* workers with functional venom glands also significantly reduced the pH of the ant's environment and nest silk. The original nest material sampled from within the intact colony was found to be maintained at slightly acidic levels of around pH 5 which may represent the natural baseline pH within a colony. When the nest material was removed and left unattended, pH levels rose towards neutral after a short space of time ( $< 24$  h). The acidity of less than pH 4 was rapidly regained when silk was kept with a single attending worker. This acidity appears likely to be key in the general sterilisation of the ant colony against parasitic fungi because the highly acidic environments, generated through venom use in these ants, significantly reduced the viability of *Metarhizium* conidia. Fungi have been previously found to suffer decreased growth and viability at low pH or in the presence of generally acidic

compounds (Do Nascimento and Schoeters 1996; Rousk et al. 2009, 2010), and we confirm this here using formic (methanoic) acid that forms the major component of *Oecophylla*, and other formicine, ant venom (Bradshaw 1979; Blum 1992). Many fungal parasites benefit from a mildly alkaline environmental pH, and *Metarhizium* itself will even raise the pH of its environment in order to promote its own fitness (St Leger et al. 1991; Leger et al. 1999). Thus the use of acidic venom to lower environmental pH may be a beneficial adaptation to help ants combat the efficacy of fungal parasites. However, Tragust et al. (2013a) found that dilutions of both hydrochloric and sulphuric acid set to the same pH (pH 2.5) as formic acid did not result in the same inhibition of fungal germination. Therefore formic acid may be antifungal for reasons other than pH, or the antifungal activity be due to the high concentrations of formic acid used. One other major component of *Oecophylla* venom is undecane which makes up around 40% of the Dufor gland secretions (Bradshaw 1979; Keegans et al. 1991), and when combined with formic acid results in a strong behavioural alarm response in many insects. Undecane can produce antifungal effects synergistically with other gland components, but on its own has no documented antifungal activity (Bradshaw 1979; Dani et al. 2000; Fernando et al. 2005; Tragust, Ugelvig, et al. 2013). Acids such as formic acid when found in combination with other short-chained acids can amplify the effective pH changes, or act as wetting agents promoting the delivery or effect, which may have compounding effects on their antifungal actions (Schildknecht and Koob 1971; Ortius-Lechner et al. 2000; Mendonça et al. 2009). Therefore whilst it seems likely that a large part of the sterilising power of *Oecophylla* venom is due to the presence of formic acid, the interaction of this component with other compounds in the venom and neighbouring glands is likely to be important. Formic acid has also been identified

as a major component of the cephalic exocrine secretions of *Oxytrigona* ‘firebees’, which use the acidic secretions as an effective defence against vertebrates (Roubik et al. 1987), and it would be interesting to discover whether the antimicrobial benefits of formic acid may extend beyond ants to other social insects.

Additionally, within *O. smaragdina* colonies there appears to be some differentiation in grooming and trophallaxis levels, depending on caste. Major workers, which carry out most extranidal work and also predominate at the nest entrances, showed a greater up-regulation in their self-grooming and allogrooming levels in response to treatment with *Metarhizium*, whereas minor workers that stay exclusively within the social environment of the nest had higher baseline allogrooming rates. Trophallaxis levels between minor workers was generally very low, but interestingly, whilst there was no significant difference in trophallaxis rates between majors when exposed to *Metarhizium*, there was a significant reduction between majors and minors. Previously ants have been found to show increases (de Souza et al. 2008; Hamilton et al. 2011), decreases (Aubert and Richard 2008), and no change (Konrad et al. 2012) in trophallaxis rate in response to parasite challenge, so it appears the interaction with this behaviour is quite variable. As minor workers are found almost exclusively within the nest the reduction we see here in trophallaxis may represent a behavioural adaptation to try and stop the spread of parasites via trophallaxis into the colony. Wilson (1984) and Sempo and Detrain (2004), looking at the behavioural repertoires of castes in *Pheidole* found, as in this study, that minor workers performed the majority of allogrooming whilst majors performed more self-grooming. In leaf-cutting ants, it is the minor workers that play the major role in parasite defence, having relatively large metapleural glands, high grooming rates,

and resistance to parasites (Hughes et al. 2002, 2010; Poulsen et al. 2006; Griffiths and Hughes 2010; Abramowski et al. 2011). In *Oecophylla*, in contrast, it appears to be the major workers that may have the major role in defending the colony against parasites on incoming material or ants.

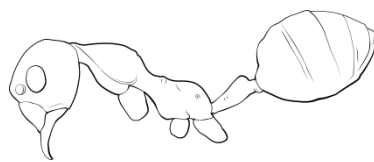
Between the different weaver ant species there were notable differences in grooming rates and venom use. While *Polyrhachis delecta* used its venom in a similar manner to *O. smaragdina*, in order to increase the acidity of nest silk, it did not produce as large a reduction in pH. However, the *Polyrhachis* weaver ants in general exhibited much higher baseline and up-regulated self-grooming rates than *O. smaragdina*, and indeed have higher rates of self-grooming than other ants too (Graystock & Hughes 2011; Tranter et al. 2015). The results of Experiment 4 suggest that pH needs to be reduced to around 4.7 to gain a significant benefit in terms of anti-fungal effect. A reduction to pH 3.7 results in very low conidia viability and is even better for ants trying to prevent the spread of pathogenic fungi. *O. smaragdina* ants were consistently able to lower, and maintain, their environment to less than pH 4, whereas the *Polyrhachis* species tested was not. It appears then that the venom of *Polyrhachis* may be less effective, or used less effectively, when compared with *O. smaragdina*, or alternatively that it is used more sparingly in specific contexts such as to defend brood (Tragust et al. 2013a; Tranter et al. 2014). This may represent a differential investment into parasite defences between the genera, with *Polyrhachis* using behavioural removal of fungal conidia, instead of relying on the chemical sterilisation used by *O. smaragdina*. However, it is difficult to determine which traits are drivers of evolutionary change and which are the result of trade-offs. For example, *Polyrhachis* species may have evolved increased



self-grooming because their venom is less effective, or evolved less effective venom because they do more self-grooming. Conversely, *Oecophylla* may have relaxed investment into grooming as they possess potent venom, or evolved more effective venom as they groom less. It seems likely, however, that there are fewer evolutionary constraints on behavioural self-grooming, making it most probable that there is a constraint on *Polyrhachis* venom production and that this then forces workers to use self-grooming to compensate.

In conclusion, we show that *Oecophylla* weaver ants are able to improve their resistance to the fungal parasite *Metarhizium*, and the hygiene of both themselves and their nest, through application of acidic venom. There was no difference in the survival of ants if they were paired with nest silk and so no evidence that any antimicrobial compounds in silk transfer to adult ants, although it remains possible that silk may have other benefits such as being an antiseptic nest material or blocking the entry of parasites in the environment. There was also evidence of trade-offs between chemical and behavioural defences, with different weaver ant species relying on alternative responses to parasites to protect themselves, and variations in the expression of grooming between castes in *Oecophylla*. It will be interesting to see in future work on more species whether grooming and acid production vary within the diverse *Polyrhachis* and in other genera with weaving habits, whether this is tied to their nesting habits or some other aspect of their biology, and whether venom use in *Polyrhachis* is more sparing or just less effective. We show that ant species which lack the antimicrobial secretions supplied by the metapleural gland, can use the application of acidic venom and grooming to help to defend themselves and their nest material against the threat of fungal parasites which are a ubiquitous and serious threat to

ant societies. This demonstrates how differential investment in parasite defences can occur in species which share many ecological characteristics, further highlighting the complexity of disease resistance mechanisms, and the need for comparative studies to help understand how organisms have evolved in response to the threat of parasites.



*Oecophylla smaragdina*

## 5. Quality and quantity: transitions in antimicrobial gland use for parasite defence

### 5.1 Abstract

Parasites are a major force in evolution, and understanding how host life-history affects parasite pressure and investment in disease resistance is a general problem in evolutionary biology. The threat of disease may be especially strong in social animals, and ants have evolved the unique metapleural gland (MG), which in many taxa produce antimicrobial compounds that have been argued to have been key to their ecological success. However, the comparative importance of the MG in the disease resistance of individual ants across ant taxa has not been examined directly. We investigate experimentally the importance of the MG for disease resistance in the fungus-growing ants, a group in which there is interspecific variation in MG size and which has distinct transitions in life-history. We find that more derived taxa rely more on the MG for disease resistance than more basal taxa, and that there are a series of evolutionary transitions in the quality, quantity and usage of the MG secretions, which correlate with transitions in life-history. These shifts show how even small clades can exhibit substantial transitions in disease resistance investment, demonstrating that host-parasite relationships can be very dynamic, and that targeted experimental, as well as large scale, comparative studies can be valuable for identifying evolutionary transitions.

## 5.2 Introduction

Parasites can inflict considerable costs on host organisms (Sheldon and Verhulst 1996; Rigby et al. 2002; Boots et al. 2004). This can result in a dramatic co-evolutionary reshaping of the genotype, phenotype and overall life-history of both hosts and parasites, and may have been a key influence in major evolutionary transitions including the evolution of sex and sociality (Hamilton et al. 1990; Møller et al. 1993; Boomsma et al. 2005). Understanding how the life-history of hosts affects the selection strength on them from parasite pressure, and how this in turn leads to further changes in the evolutionary investment by hosts in costly disease resistance mechanisms, is an important problem in evolutionary biology.

Group-living is associated with a potential increase in parasite pressure, because a social lifestyle can facilitate the transmission of parasites within the group (Alexander 1974; Cote and Poulin 1995; Møller et al. 2001; Altizer, Nunn, and Thrall 2003). This is compounded in social insect colonies which have highly homeostatic nest environments and low levels of genetic variation within the colony (Schmid-Hempel 1994; Frank 1996; Calleri et al. 2006). However, in addition to individual-level immune defences, social organisms are able to employ social defences that have been termed ‘social immunity’ in the broad sense (Dunbar 1991; Ugelvig and Cremer 2007; Wisenden et al. 2009; Otti et al. 2014). This can include behavioural defences such as grooming, and the production and transfer of antimicrobial compounds (Rosengaus et al. 1998; Fernández-Marín et al. 2006; Yanagawa et al. 2008; Hamilton et al. 2011; Baracchi, Mazza, et al. 2012; Turnbull et al. 2012), which can mitigate or even

outweigh the fitness cost from parasites for group-living animals (Rosengaus et al. 1998; Hughes et al. 2002; Ugelvig and Cremer 2007; Reber et al. 2011).

The threat of disease has led the most diverse group of social insects, the ants, to evolve a unique exocrine structure, the metapleural gland (MG), which varies in size between species and phenotypes, and in many taxa produces an antimicrobial secretion that is spread over the cuticle either passively or, in some species, actively by grooming (Hölldobler and Wilson 1990; Bot and Boomsma 1996; Sumner et al. 2003; Fernández-Marín et al. 2006; Poulsen et al. 2006; Hughes, Pagliarini, et al. 2008; Hughes et al. 2010; Yek and Mueller 2011; Yek et al. 2012). Consequently, ants are able to vary their level of investment in disease resistance both on an evolutionary time-scale, and as a short-term behavioural response to disease threat by active grooming of the secretion on to the cuticle. Whilst the secretion from the metapleural gland can be antibacterial (Iizuka et al. 1979; Veal et al. 1992), effective at inhibiting fungal sporulation and growth, and helping ants resist parasites (Beattie et al. 1985, 1986; Bot et al. 2002; Graystock and Hughes 2011; Tranter et al. 2014; Tranter and Hughes 2015), the gland is energetically costly to maintain (Poulsen, Bot, Nielsen, et al. 2002). Thus its degree of use in different ant species can be used as a measure of their relative investment in disease resistance, and thus to infer the strength of parasite pressure in different species with different life histories (Hughes et al. 2002; Poulsen, Bot, Nielsen, et al. 2002; Hughes and Boomsma 2006). Whilst experimental blockage of the metapleural gland has been shown to increase the susceptibility of ants to fungal parasites (Poulsen, Bot, Nielsen, et al. 2002; Graystock and Hughes 2011; Tranter et al. 2014), the comparative importance of

the metapleural gland in disease resistance of individual ants has not previously been quantified directly.

One group of ants which has proved particularly powerful for comparative analyses of disease resistance are the fungus-growing ants (Attini) which form a monophyletic clade with well-developed MGs (Table S5.1 p.281; Mueller et al. 1998; Currie, Scott, et al. 1999; Currie, Mueller, et al. 1999; Schultz and Brady 2008; Fernández-Marín et al. 2013). Fungus-growing ants have been most extensively investigated with respect to the defence of their fungal crop mutualist against a specialist parasitic fungus, *Escovopsis*. The ants achieve this using a combination of weeding, antimicrobial compounds from the MG, and antimicrobials produced by actinomycete bacteria that the ants culture on their cuticles (Currie, Scott, et al. 1999; Currie and Stuart 2001; Fernández-Marín et al. 2013). There appears to be a trade-off between these two sources of beneficial antimicrobials, with species that possess more of the *Escovopsis*-specific actinomycete defences relying less heavily on the MG to protect their fungal crop (Fernández-Marín *et al.*, 2013). However, while most attention has been focused on the mechanisms by which ants defend their fungal crop against parasites, the ants also need to defend themselves against disease. The antimicrobial compounds produced by the actinomycete bacteria are thought to be specifically active against *Escovopsis* (Currie, Scott, et al. 1999; Little et al. 2006; Pagnocca 2012), and the ants rely for their own defence on mechanisms such as grooming and the MG. The antimicrobial activity of the MG secretion of leaf-cutting ants has been demonstrated *in vitro* (Bot et al. 2002) and the importance of the MG for defending both adults and brood against parasites also demonstrated in leaf-cutting ants experimentally (Poulsen et al. 2006; Tranter et al. 2014). Previous

comparative studies have shown that fungus-growing ants vary substantially in the size of their MG, the chemical composition of its secretion, and their use of active MG grooming to spread the secretion, with the more evolutionary derived *Atta* and *Acromyrmex* leaf-cutting ants which live in larger, more complex societies, having particularly large MG (Fernández-Marín *et al.*, 2006; 2009; 2013; Hughes *et al.*, 2008b; Adams *et al.*, 2012; Vieira *et al.*, 2012b). However whether these evolutionary transitions in MG size and secretion result in differences in the disease resistance of individual attine ants themselves has not previously directly quantified. In this study we use MG use as a measure of investment into disease resistance within the evolutionary framework of the fungus-growing ants. Specifically we test the hypotheses that when compared to more evolutionarily basal species, the more derived species with larger and more complex societies will show 1) greater reliance on functioning MG glands for their own disease resistance, 2) greater active MG selfgrooming rates in response to a parasite threat, and 3) more powerful antifungal components within the chemical makeup of MG secretions.

## 5.3 Methods

### 5.3.1 Study species

We studied six species of Neotropical attine ants spanning the major phylogenetic divisions (Appendix 3): two species of leaf-cutting ant (*Atta colombica*, *Acromyrmex echinator*), three species of higher attines (*Sericomyrmex amabilis*, *Trachymyrmex cornetzi*, *Trachymyrmex sp10*) and one species of lower attine

(*Apterostigma pilosum*; Table S5.1 p.281). Colonies were collected in and around Gamboa, Panama in June 2013, and maintained at 80% relative humidity and 27°C on a 12 h light/dark cycle. Colonies were fed twice per week with fresh privet (*Ligustrum* sp.) leaves for the two leaf-cutting species or chopped flower petals and oat flakes for the other four species, and provided with water *ad libitum*. As the experimental parasite, we used the entomopathogenic fungus *Metarhizium pingshaense* (KVL02-73; which was originally isolated from soil in Gamboa near a leaf-cutting ant nest; (Hughes and Boomsma 2004b)). *Metarhizium* is a ubiquitous, generalist fungal pathogen which parasitizes a wide range of insects, including attine and other ants, but which is unlikely because of its generalist nature to have coevolved to overcome the specific defences of attine ants (Sanchez-Pena and Thorvilson 1992; Quiroz et al. 1996; Hughes and Boomsma 2004b; Castilho et al. 2010; Reber and Chapuisat 2011; de Zarzuela et al. 2012; Ribeiro et al. 2012).

### **5.3.2 Exp. 1: gland blockage and fungal exposure on survival**

Twenty worker ants were selected from just inside the nest entrance of each of six colonies of *S. amabilis*, *T. cornetzi*, *T. sp10*, and *A. pilosum*, 40 ants from six colonies of *A. echinator* and 80 ants from three colonies of *A. colombica*. Trials involving *A. echinator* and *A. colombica* were conducted as two separate cohorts and data later pooled (see Figure S5.1 p.266). Ants used within each species were a similar size, with medium cuticular colouration, and hence inferred age (Armitage and Boomsma 2010). For the polymorphic leaf-cutting ant species, we used workers of similar size to the other attines (0.9-1.4 mm head width). Half of



the ants from each colony had their MG blocked using quick-drying nail varnish, whilst the other half received a control treatment of nail varnish applied to the pronotum. Nail varnish was checked daily and remained intact on all ants treated for the course of the experiment. After 24 h, each of these groups then had either a suspension of *Metarhizium* conidia in 0.05% Triton-X or a control solution of 0.05% Triton-X applied topically to the mesosoma with a micropipette.

Treatment volumes were standardized for body size between species, and conidia concentrations were approximately the LD<sub>50</sub> for the species based on pilot studies (5 x 10<sup>6</sup> conidia per ml for leaf-cutting ant species and 5 x 10<sup>5</sup> conidia per ml for the other species; Table S5.4 p.284). This design involved a total of 120 ants for each of *S. amabilis*, *T. cornetzi*, *T. sp10*, and *A. pilosum* (30 ants per species for each of the four treatment groups), and 240 ants for *A. colombica* and *A. echinator* (60 ants per species for each of the four treatment groups). After treatment, each ant was placed in a plastic pot (diameter: 35 mm, height: 70 mm) supplied with cotton balls soaked in 20% sucrose solution and water and kept at 70% relative humidity and 26°C. Ant mortality was recorded for 14 days. Cadavers were immediately removed and surface sterilised (Siegel, 2012), and then kept in a Petri dish with moistened filter paper for an additional 14 days to allow the sporulation of fungi.

### **5.3.3 Exp. 2: the effect of simulated fungal exposure on grooming rates**

Twelve ants from each species (two individuals from each of six colonies for *A. echinator*, *S. amabilis*, *T. cornetzi*, *T. sp10*, *A. pilosum*, and four individuals from three colonies for *A. colombica*) were observed in a 40 mm Petri dish for 30

min with two nestmates. Incidences of allogrooming, self-grooming and metapleural gland-grooming of the focal ant were recorded (Altmann, 1974). This process was repeated for 12 additional ants per species but with each ant receiving a standardized treatment of dry, unscented, talcum powder (magnesium silicate) applied evenly to the dorsal mesosoma and gaster with a fine brush, to induce grooming, prior to observation for 30 min with two nestmates. We used talcum powder because some of the colonies were small and talcum powder particles, which are similar in size to fungal conidia, act as a non-pathogenic stimulant of anti-parasite defensive behaviour in ants without incurring the mortality that would result from application of fungal conidia (Fernández-Marín *et al.*, 2006; Morelos-Juárez *et al.*, 2010; Tranter *et al.*, 2015).

#### **5.3.4 Exp. 3: chemical inhibition of fungal growth**

Six chemical compounds that have been previously identified as major constituents of attine MG secretions (Vieira *et al.*, 2012b) were tested for their effect on the conidia viability of the entomopathogenic fungus *Metarhizium pingshaense*. The compounds tested were: indole, skatole, methyl oleate (oleic acid), 2-nonanone, phenylacetic acid and methyl-3-indoleacetate (indoleacetic acid; Table S5.2 p.282), as well as acetone solvent control, bleach (NaClO) positive control and ddH<sub>2</sub>O negative control. Each compound was tested 10 times at five different concentrations based on the maximal amounts found in the MG of adult *Atta* workers (Table S5.4 p.284). A conidia solution of  $1 \times 10^5$  conidia per ml was prepared from freshly sporulating *M. pingshaense* plates. 450 agar plates were prepared with selective media (Sabouraud dextrose agar [SDA] with 0.05

g/l streptomycin sulphate and 0.1 g/l chloramphenicol antibiotics, and 0.1 g/l dodin which inhibits the growth of other fungi but not *Metarhizium*; Shah *et al.*, 2005), in 50 mm Petri dishes and stored sealed at 4 °C until use. 500 µl of the *Metarhizium* conidia solution ( $5 \times 10^6$  conidia per ml) was applied evenly over the surface of the Petri dish and left for 10 min to allow excess liquid to dry. A single 6 mm diameter piece of sterile plastic tubing was placed carefully onto the centre of the surface of the agar plates, and 20 µl of test solution applied in the centre of this with the plastic cylinder acting as a well to restrict distribution to a defined area. The cylinder was left in place for 5 min to allow the compound to infuse the media, before the location of the treated area was marked on the underside of the Petri dish and the cylinder removed. The Petri dish was then sealed with parafilm and placed in an incubator at 32 °C overnight. 12 h later the percentage of conidia producing a germ tube longer than the conidia diameter (Siegel 2012; conidia viability) was counted for a standardised area (complete area visible in the microscope eyepiece at 400x magnification) within the section where the compound was applied and also an untreated area outside the marked test area, equidistant with the edge of the Petri dish. A further 60 h later the plates were photographed from above and the average radius of any zone of inhibition, as characterised by an area around the marked test section free from fungal growth, was recorded (Figure S5.2 p267).

### 5.3.5 Statistical analysis

The effects of *Metarhizium* exposure, gland closure, and ant species, on ant survival in Experiment 1 were analysed using Cox proportional hazards regression models. Colony-of-origin, and cohort for the leaf-cutting ant trials, were included in the models to account for the structured nature of the data, but

were not statistically significant ( $p > 0.05$  in all cases). Pairwise comparisons were made with Kaplan-Meier tests using the Breslow statistic. The numbers of cadavers sporulating with *Metarhizium* for ants with blocked or unblocked glands were examined for each species with Chi-squared tests. Grooming rates following exposure to talcum powder in Experiment 2 were analysed using a general linear mixed model with a gamma distribution and log link function; colony-of-origin was included as a random factor but was not statistically significant ( $p > 0.05$  in all cases). Non-significant interaction terms were removed step-wise to obtain the minimum adequate model in each case. Pairwise comparisons were conducted between treatments within each species, and between species for each treatment. The effects of compound and dose in Experiment 3 on the size of fungal inhibition zones were analysed using a generalized linear model with a gamma distribution and log link function. Multiple comparisons were controlled for in all analyses using the sequential Bonferroni adjustment. All analyses were performed in IBM SPSS v21.

## 5.4 Results

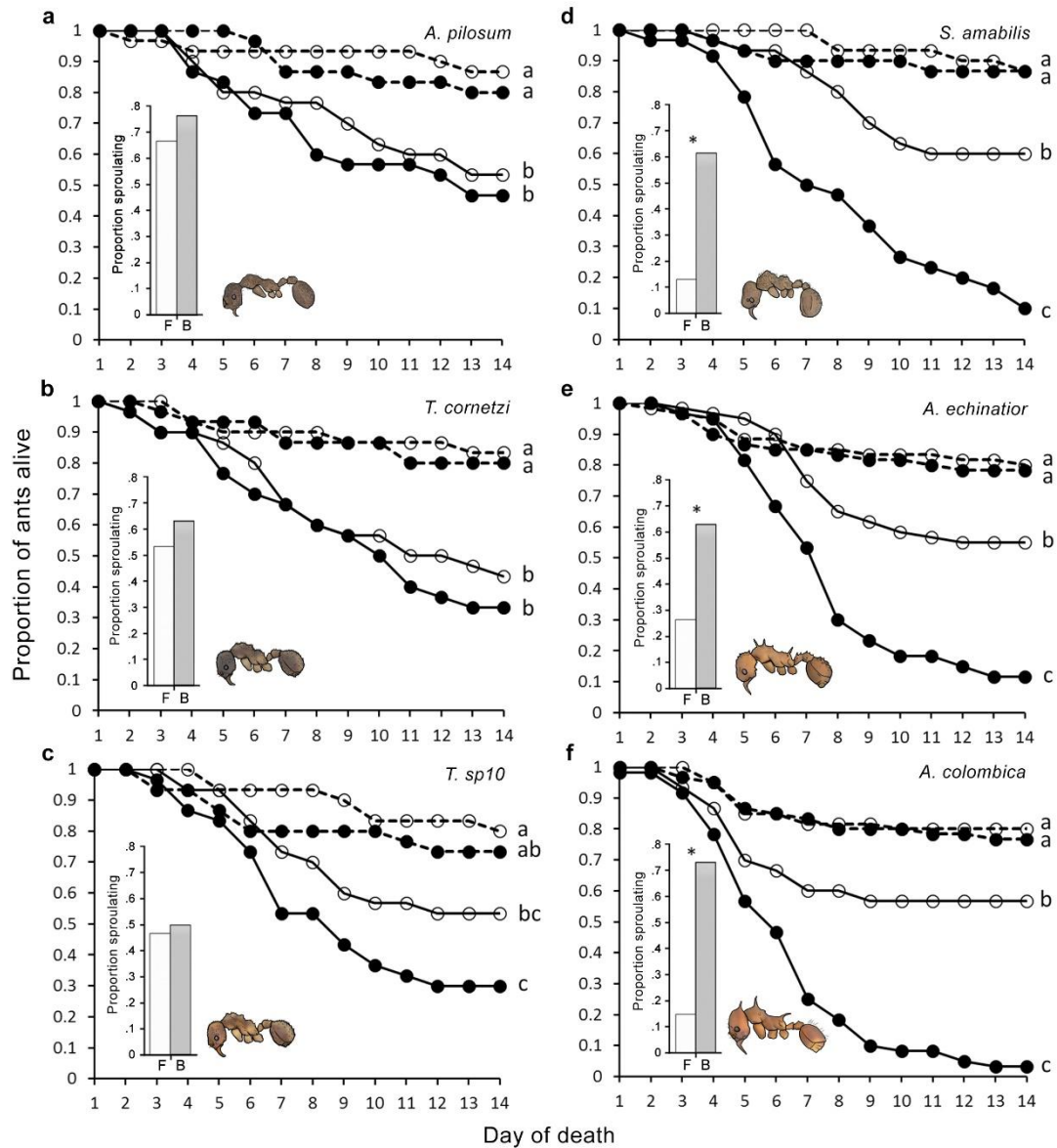
### 5.4.1 Exp. 1: gland blockage and fungal exposure on survival

Overall there were significant effects of species and interaction between blockage and fungal treatments on survival ( $Wald = 17$ , d.f. = 5,  $P = 0.005$ ;  $Wald = 9.98$ , d.f. = 1,  $P = 0.02$ , respectively). Ants from all six species showed a significant reduction in their survival when treated with the *Metarhizium* parasite (Fig. 5.1; Table S5.3 p.283). *A. colombica*, *A. echinator* and *S. amabilis* all

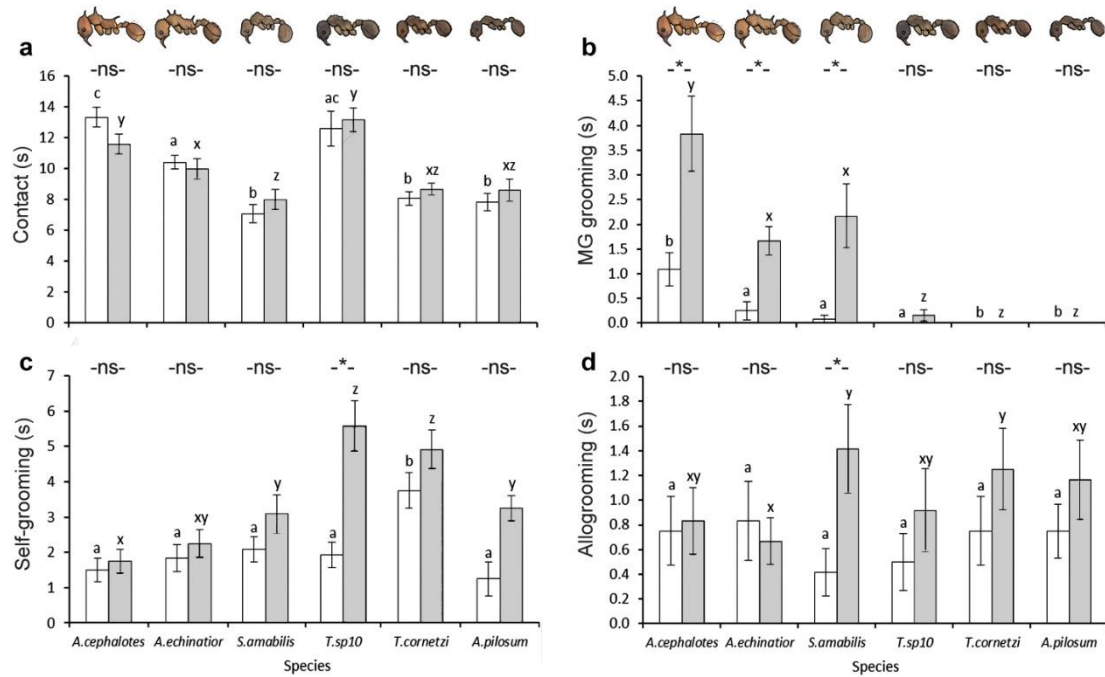
showed a significant reduction in resistance to the parasite when their MG was blocked, while the resistance of ants with blocked and unblocked MG was nearly identical in both *T. cornetzi* and *A. pilosum*, and there was also no significant effect of MG blockage on the resistance of *T. sp. 10* (Fig. 5.1; Table S5.3 p.283). There was no significant effect of colony on survival in any of the species ( $P > 0.05$ ; Table S5.3). None of the control ants sporulated with *Metarhizium*. Of those *Metarhizium*-exposed ants that died, significantly more of the cadavers sporulated with *Metarhizium* when they had blocked glands compared to those where the MG was functional ( $\chi^2 = 47.8$ , d.f. = 5,  $P < 0.001$ ). This difference was present in *A. colombica*, *A. echinator* and *S. amabilis* but not in *T. cornetzi*, *T. sp. 10* or *A. pilosum* (Fig. 5.1).

#### 5.4.2 Exp. 2: the effect of simulated fungal exposure on grooming rates

Contact rates between ants differed between species ( $F_{1,137} = 20.8$ ,  $P < 0.001$ ), reflecting interspecific differences in the general activity levels of the ants, but there was no effect of talcum powder application or interaction between effects on activity ( $F_{1,132} = 0.42$ ,  $P = 0.52$ ;  $F_{5,132} = 1.12$ ,  $P = 0.36$ ; Fig. 5.2a). There was, however, a significant interaction between the effects of ant species and talcum powder treatment on MG grooming ( $F_{5,132} = 9.67$ ,  $P = 0.014$ ). *A. colombica* exhibited the highest rate of MG grooming by far, with *A. echinator* and *S. amabilis* also conducting higher levels of MG grooming compared to the *Trachymyrmex* and *Apterostigma* species which exhibited little or no MG grooming (Fig. 5.2b).



**Figure 5.1.** Ant survival over two weeks of a) *A. colombica*, b) *A. echinator*, c) *S. amabilis*, d) *T. sp. 10*, e) *T. cornetzi* and f) *A. pilosum* attine ants after treatment with either *Metarhizium pingshaense* fungal parasite (solid line) or control solution (dashed line), and with their antimicrobial-producing metapleural glands either experimentally blocked (closed circles) or functional (open circles). Different letters beside lines indicate treatments which differed significantly from one another within species at  $P < 0.05$ . Inset graphs show the proportions of cadavers of *Metarhizium*-exposed ants that sporulated with *Metarhizium* for ants of each species with either their glands blocked ('B': dark bars) or functional ('F': light bars). Species in which the frequency of sporulation differed significantly between ants with blocked and functional glands at  $P < 0.05$  are marked with an asterisk (\*).



**Figure 5.2.** Grooming rates. The mean  $\pm$  s.e. frequencies of (a) contact, (b) metapleural gland grooming, (c) self-grooming and (d) allogrooming in 30 min for six species of attine ants (*A. colombica*, *A. echinator*, *S. amabilis*, *T. sp10*, *T. cornetzi* and *A. pilosum*) either treated with talcum powder (dark bars) or untreated (light bars). Significant differences between treated and untreated ants at  $P < 0.05$  for each species are indicated with an asterisk. Species which differed significantly from one another at  $P < 0.05$  are indicated by different letters, a, b, c for untreated ants, or x, y, z for treatment ants.

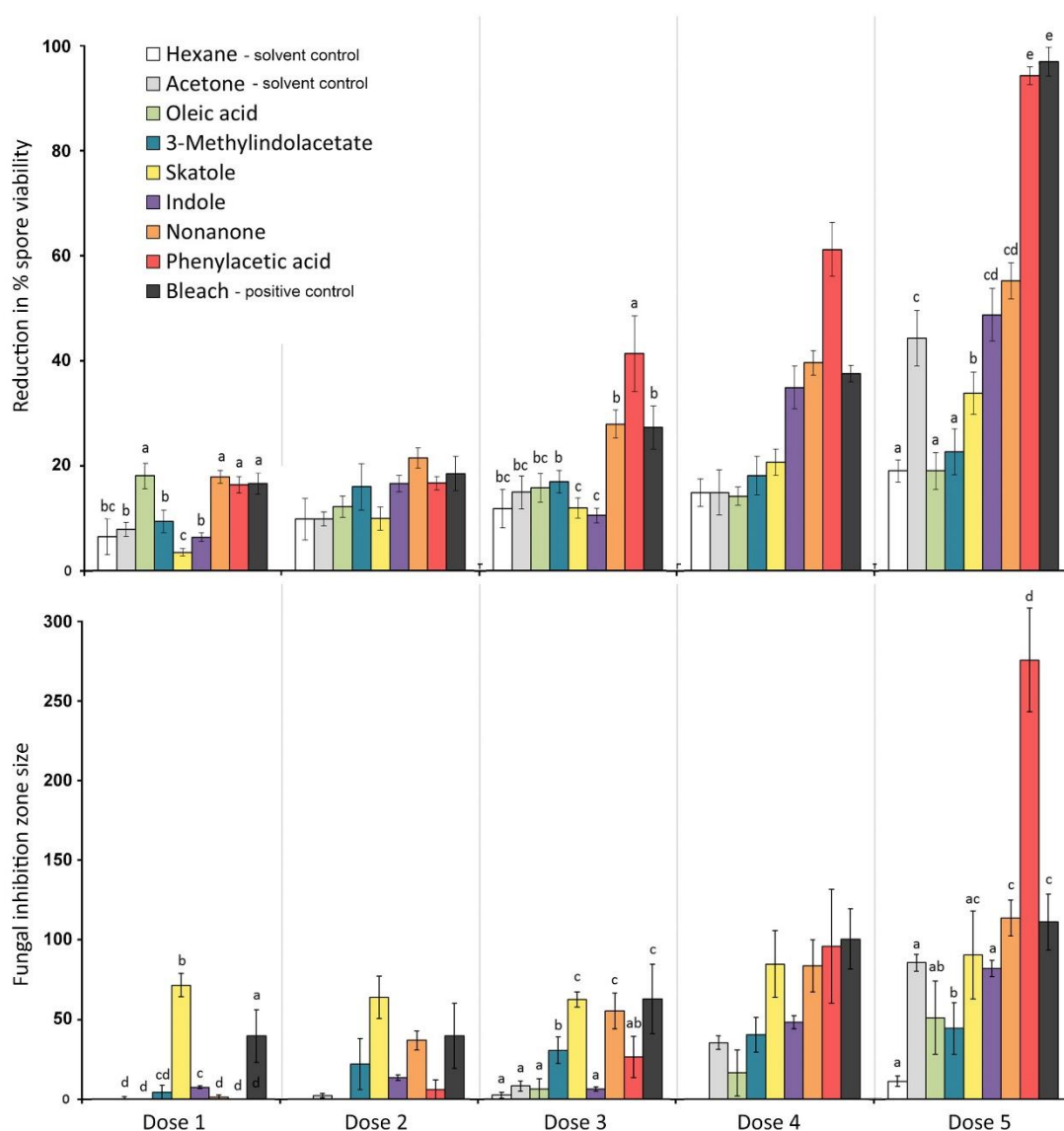
There was also a significant interaction between species and talcum powder treatment on the rates of self-grooming ( $F_{5,132} = 3$ ,  $P = 0.014$ ), with *T. sp10* and *A. pilosum* both self-grooming significantly more when talcum powder was applied to them and baseline levels of self-grooming being highest in *T. cornetzi* (Fig. 5.2c). Only *S. amabilis* allogroomed significantly more when exposed to talcum powder treatment ( $F_{1,132} = 5.5$ ,  $P = 0.02$ ; Fig. 5.2d), and there was no

overall effect of species or interaction between factors for allogrooming ( $F_{5,132} = 0.4$ ,  $P = 0.86$ ;  $F_{5,132} = 0.79$ ,  $P = 0.56$ ).

### 5.4.3 Exp. 3: chemical inhibition of fungal growth

There was a significant interaction between the compound tested and the dose applied on the size of the zone in which *Metarhizium* fungal growth was inhibited ( $\chi^2 = 194.5$ , d.f. = 32,  $P < 0.001$ ) and on the number of fungal conidia that were viable ( $\chi^2 = 575.4$ , d.f. = 32,  $P < 0.001$ ). Phenylacetic acid consistently produced the largest reductions in spore viability, especially at higher doses (Fig. 5.3a). It also produced the largest inhibition zone in the highest dose, where it was generally comparable to, or even more effective than, bleach in its antifungal activity (Fig. 5.3b).



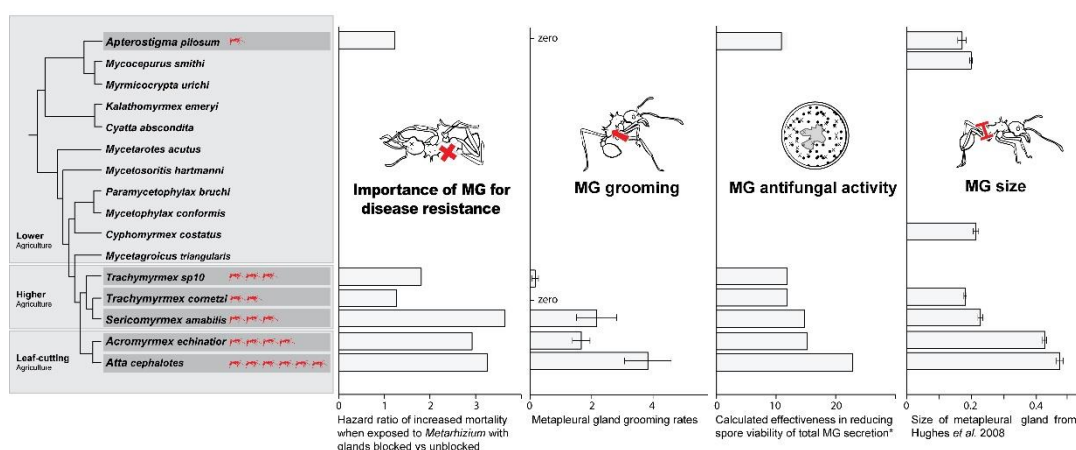


**Figure 5.3.** The effect of MG chemicals on fungal spores. The mean  $\pm$  s.e. reduction in viability of conidia of the *Metarhizium pinshaense* fungal parasite (a) and size of growth inhibition zone produced (b), for six chemicals from the metapleural gland secretion (phenylacetic acid, 2-nonanone, indole, skatole, methyl-3-indolacetate, methyl oleate), and bleach positive control, acetone and hexane solvent control, with each compound applied at five concentrations (1 lowest dose - 5 highest: see Table S5.4 p.284 for details). A ddH<sub>2</sub>O negative control produced negligible effect and was omitted from the graph. For doses 1, 3 and 5 where pairwise comparisons were performed, different letters indicate chemicals which differed significantly from one another at  $P < 0.05$ .

## 5.5 Discussion

Exposure to the fungal parasite *Metarhizium pingshaense* significantly reduced the survival of the six species of ants in this study, and there were species-specific differences between the resistance of ants when their MGs were blocked, compared with when they were left functional. There were also differences in MG grooming rates and the antifungal activity of chemical components of the MG secretions between species. The leaf-cutting ants *A. colombica* and *A. echinator*, as well as the higher attine *S. amabilis* which also has relatively large colony sizes, all demonstrated a consistently greater reliance on MG use for protection against fungal pathogens compared with the more basal *Trachymyrmex* and *Apterostigma* species (Fig. 5.4). The resistance of leaf-cutting ants and *Sericomyrmex* to the parasite was significantly reduced when their MG glands were blocked. Additionally, of those ants that died, significantly more of the cadavers sporulated with the parasite when the MG was blocked. This highlights the importance of the gland in sterilising the ant's cuticle and promoting their survival through fungistatic or fungicidal effects. The leaf-cutting ants and *S. amabilis* all also showed higher rates of MG grooming behaviour and their secretions contained chemicals with stronger antifungal activity, including more acidic compounds phenylacetic acid and methyl oleate (Do Nascimento and Schoeters 1996; Yek et al. 2012). This was particularly evident in *Atta colombica*, which was the only species in which the highly antifungal phenylacetic acid has been identified (Kim et al. 2004; Quinet and Vieira 2012; Fernández-Marín et al. 2015). Additionally the strength of this effect, and reliance on the MG, seems to be compounded by the efficacy of some of the MG secretions themselves; not only do the leaf-cutting ants and *S. amabilis* use their glands

more actively, but the compounds in their secretions are more effective antifungal agents as well. These findings support our predictions that the more derived leaf-cutting ant species and *S. amabilis* with their larger and more complex societies are considerably more reliant on their MG compared to the more basal species.



**Figure 5.4.** Phylogenetic patterns of metapleural gland usage in the attine ants. Graphs show for the six species studied here (highlighted in dark gray boxes): 1) the hazard ratio from blockage of the antibiotic-producing metapleural gland for ants exposed to the *Metarhizium* fungal parasite; 2) the mean  $\pm$  s.e. frequency of active grooming of the metapleural gland in a 30 min period; 3) the effectiveness of the overall metapleural gland secretion at reducing *Metarhizium* conidia viability, based on the sum of the average effectiveness of each chemical weighted by its representation in the secretion (Table S5.4, S5.5); 4) the size of the metapleural gland reservoir (bulla width) relative to body size (pronotum width; data from Hughes *et al.*, 2008 and includes data for *A. collare*, not *A. pilosum* as in our study). The number of ant symbols shown next to species name represents the typical number of workers per colony in orders of magnitude.

The importance of the MG in resisting disease varied across the attine phylogeny providing evidence for the existence of a series of four evolutionary transitions in MG use (Fig. 5.4). First, between *Apterostigma* and *Trachymyrmex sp10* we see a small increase in the use of the MG and consequent increased importance of the

MG in resisting disease. Second, there is then a small increase in MG size and antifungal activity, and a large increase in MG grooming and the importance of the MG for disease resistance, between *Trachymyrmex* and *Sericomyrmex*. Third, there is a substantial increase in MG gland size between *Sericomyrmex* and the leaf-cutting ant species. Fourth, there is a transition between *Acromyrmex* and *Atta*, with *Atta* producing a more powerful suite of antifungal chemicals within their MG secretions, which they produce more of and groom much more actively than *Acromyrmex* (Fig. 5.4). There is no evidence of a trade-off between different aspects of individual self-directed MG use, but rather a general trend across the phylogeny towards the MG becoming more effective and important in disease resistance.

These differences in MG use, in combination with transitions in other morphological and behavioural attributes such as gardening of the fungal crop or hitchhiking on harvested leaf material, may reflect changes in host-parasite interactions within the attine clade, notably, as a result of specialization of the fungal mutualism and the appearance of polymorphic workers (Hughes, Pagliarini, et al. 2008; Schultz and Brady 2008; Fernández-Marín et al. 2009; Griffiths and Hughes 2010; Quinet and Vieira 2012; Vieira et al. 2012). It may be that these derived characters have allowed for greater investment into costly disease resistance through improved resource acquisition; for example the fungal gardens cultivated on fresh vegetation by *Atta* and *Acromyrmex* ants may provide a more nutritious and reliable food source. Additionally, MG use within the group maps broadly with colony size between the species (Fig. 5.4; Table S5.1 p.281). Larger colonies have a greater workforce, greater resource acquisition, and are a more robust entity that can provide more stable nest conditions (Kunz 1982;

Rosengren et al. 1987; Jeanne and Nordheim 1996; Anderson and Ratnieks 1999; Jones and Oldroyd 2006; Jeanson et al. 2007). Similarly, morphological and behavioural specialization will allow for more efficient disease resistance strategies. Within the Attini, the leaf-cutting species, and *Atta* especially, are the most morphologically and behaviourally specialised taxa (Hart et al. 2002; Evison and Ratnieks 2007), and, as we demonstrate here, are especially well adapted for parasite defence. The presence of discrete castes allows for a differentiation in MG morphology in the higher attine species with the smallest castes having relatively larger MG reservoirs (Hughes, Pagliarini, et al. 2008). This may aid in parasite defence through allowing investment into MG defence in those individuals that may benefit from them the most, i.e. in leaf-cutting ants the minors which tend the fungus, clean leaf fragments, and brood (Currie and Stuart 2001; Hughes, Pagliarini, et al. 2008; Griffiths and Hughes 2010). However, larger group size may also result in increases in parasite pressure through the same stable and favourable nest conditions and increased colony longevity, which promote parasite transmission (Schmid-Hempel, 1998; Poulin, 2007). Also larger colonies ‘sample’ more of the environment which increases the chance of contracting parasites (Wilson 1971; Rosengren et al. 1987; Sherman et al. 1988; Hölldobler and Wilson 1990; Tschinkel 1991; Schmid-Hempel 1998; Zahn 1999; Poulin 2007). An alternative explanation is therefore that the larger colonies of leaf-cutting ants may be exposed to greater parasite pressures than lower attine species and thus invest more in disease resistance in order to mitigate this increased (Hart and Ratnieks 1998, 2001; Hughes et al. 2002; Naug and Camazine 2002; Fernández-Marín et al. 2006, 2009; Poulsen et al. 2006; Tranter et al. 2014).

Our results show how changes in the antifungal activity and use of MG secretions have evolved to protect individual ants from parasites, and demonstrate that even relatively small clades can exhibit substantial transitions in investment into disease resistance mechanisms. This highlights how dynamic the evolutionary relationships between host and parasite can be, and demonstrates the value of targeted experimental comparative studies for identifying and understanding evolutionary transitions in host-parasite relationships.



*Apterostigma pilosum*

## 6. Simple societies and hygienic personalities: species and individual differences in ant social immunity

### 6.1 Abstract

Living in groups has many benefits, but can increase the threat from parasites. This threat may increase with group size, and species with larger societies are expected to invest more in disease resistance and potentially express greater variation between individuals in behavioural defences ('hygienic personalities'). We examine the social immunity of dinosaur ants and leaf-cutting ants, which respectively have simple and complex societies, and describe for the first time the presence of hygienic personalities in individual behavioural defence. We find that leaf-cutting ants show higher levels of social immunity, and more consistent personalities, than dinosaur ants, and ants with more hygienic personalities resist infection with a fungal parasite significantly better. This suggests that complex societies can possess higher levels of social immunity and more developed hygienic personalities than simple societies, which together may have contributed the ecological and evolutionary success of group-living species.

## 6.2 Introduction

Animals, such as social insects, live in groups that vary in size and composition (Dornhaus et al. 2010). Part of the success of social insects is their ability to organize themselves within these groups (Naug and Camazine 2002). This has resulted in the evolution of complex societies that through cooperation and specialisation can perform tasks beyond the scope of individual members (Hart et al. 2002). However, as colony size increases so too does the potential risk of parasite transmission (Wilson et al. 2002). Thus, larger and more complex societies would be expected to invest more into disease resistance, and there is evidence that disease resistance strategies may be better developed in species with larger colony sizes (Hughes, Pagliarini, et al. 2008; Turnbull et al. 2010). The evolution of more complex societies may also allow for the expression of defensive specialisation and variation between individuals in behavioural defences, which may evolve as a strategy to mitigate the increased cost from parasites (Boomsma et al. 2005).

Morphological and behavioural specialisation between castes can perform a key role in the maintenance of colony hygiene. The highly complex societies of leaf-cutting ants, for example, operate extensive task partitioning and division of labour to stop intracolony pathogen transmission (Hart et al. 2002). Individuals of the same caste in several social insect species have also been found to show consistent individual differences in behaviours, termed ‘personalities’ or ‘behavioural syndromes’, including aggression, learning and foraging (Jandt et al. 2014). However besides some variation in undertaking behaviour (Diez et al. 2011), and genetic variation in the hygienic behaviour of honeybees (Arathi et al.



2000), there has been little investigation of individual-level variation in consistent disease resistance behaviours in social insects, despite the importance of parasite defences for the evolutionary success of their societies.

Grooming behaviours can be highly effective in parasite defence (Reber et al. 2011; Tranter et al. 2015), and we use them here to test whether there are differences in the social immunity of an ant species with a larger more complex society that is potentially more at risk from parasites, compared with a species with a relatively simple society. Additionally we investigate whether social insects may possess ‘hygienic personalities’, in the form of consistency in grooming, and test if this has survival benefits when challenged with a fungal parasite.

## 6.3 Methods

We used ten colonies of *Dinoponera quadriceps*, a monomorphic, queenless, ponerine ant species with small, simple societies of monomorphic workers, and four colonies of *Acromyrmex echinator*, a leaf-cutting ant with large, complex societies and highly polymorphic workers (see Supplementary Material).

### 6.3.1 Social immunity and personalities

We investigated the levels of social immunity by measuring selfgrooming and allogrooming of 40 workers from both *D. quadriceps* (4 ants from 10 colonies) and *A. echinator* (10 ants from 4 colonies). These measurements were repeated seven times for each individual over 25 days for the dinosaur ants and 7 days for

the leaf-cutting ants. For each observation the individual ant was placed in a 100 x 75 mm container lined with Fluon and left for 5 min to acclimatize. For self-grooming trials, the individual focal ant was treated with Triton-X control solution and the total duration spent self-grooming recorded for 15 min. The ant was then treated with a  $5 \times 10^6$  particles/ml suspension of talcum powder in 0.05% Triton-X solution, and selfgrooming was observed for a further 15 min. Volumes were adjusted for body size between species (leaf-cutting ants: 0.5  $\mu$ l; dinosaur ants: 5  $\mu$ l; both delivering  $\sim 320$  particles/ $\text{mm}^2$  body area. For allogrooming the same protocol was followed, but a second ant from the same colony was placed in the container and treated with talcum powder suspension. Allogrooming of this second ant by the focal ant was recorded for the 15 min period.

### **6.3.2 Personalities and disease resistance**

As a parasite we used the entomopathogenic fungus *Metarhizium pingshaense* (KVL02-73 isolated from soil in Panama; (Pull et al. 2013)), which is a natural parasite of ants (Boomsma et al. 2005), but a generalist insect pathogen and thus unlikely to have coevolved with either ant species used here (see supplementary material). After the grooming-observation period, each of the leaf-cutting ants (35 in total) was exposed to the *Metarhizium* parasite by placing the ant into a 60 mm Petri dish lined with filter paper on to which an even coating of 150  $\mu$ l of  $1.5 \times 10^6$  conidia/ml *Metarhizium* suspension had previously been applied and left to dry for 10 min. The ants were left in the Petri dish for 1 h, before being transferred into individual 70 x 35 mm pots, supplied with water and sugar *ad libitum*, and then monitored daily for survival for 14 days. Cadavers of ants that

died during this period were surface sterilised, placed in a sterile Petri dish with moistened filter paper, and observed for two weeks for *Metarhizium* sporulation.

### **6.3.3 Statistics**

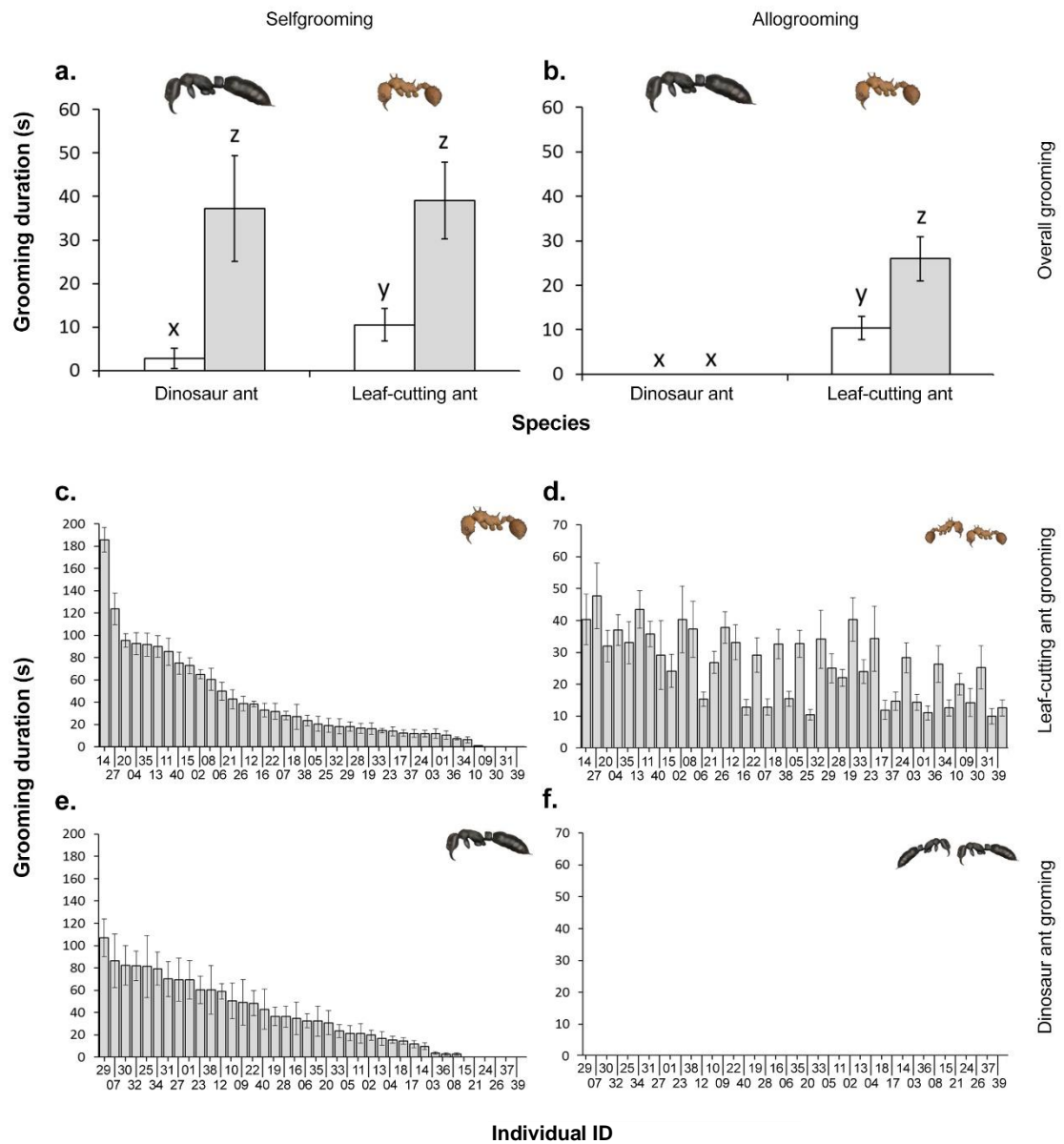
Data was tested for normality prior to analyses and distributions were chosen based on AIC scores data structure. To test overall grooming rates, a GLMM with gamma distribution and log link function, with species and treatment included as factors, was run on both self-grooming and allogrooming data averaged for individuals over the time points. Post-hoc pairwise comparisons were adjusted using the sequential Bonferroni method, and colony-of-origin was included as a random factor for all GLMMs. To examine individual consistencies in the selfgrooming and allogrooming behaviour of ants, individual GLMMs were run for both species with ant identity and treatment included as factors. A gamma distribution with log link function was used for selfgrooming data, and a negative binomial distribution and log link function for allogrooming data. A Mann-Whitney U test compared the intraindividual variances in grooming durations between the species, for both treatments. Pearson's correlation coefficients were used to investigate the relationships between grooming behaviour in response to treatment, between selfgrooming and allogrooming durations in both species, and between grooming duration and survival in leaf-cutting ants. Statistics were calculated using SPSS v22.

## 6.4 Results

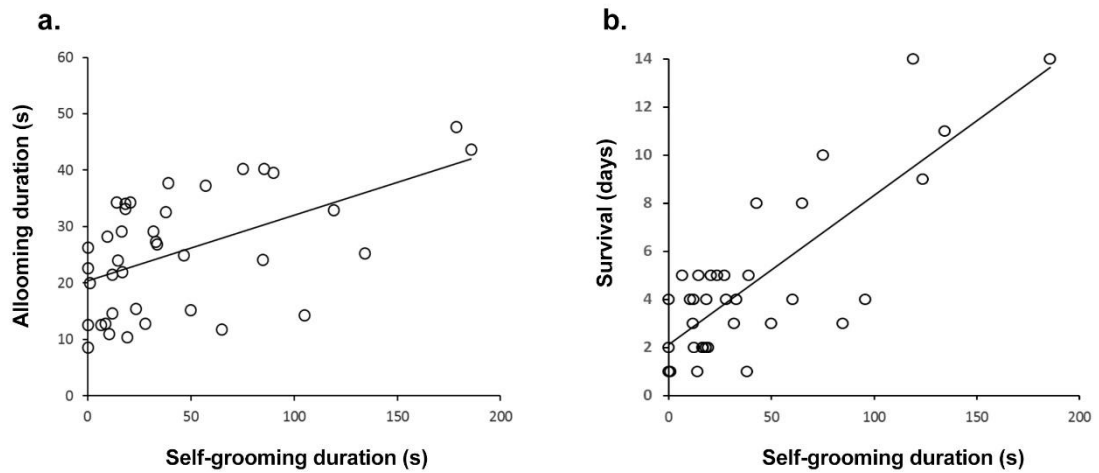
### 6.4.1 Social immunity and personalities

There was a significant interaction between treatment and species on overall selfgrooming and allogrooming ( $F_{1,156} = 9.19$ ,  $p = 0.003$ ;  $F_{1,156} = 61.71$ ,  $p < 0.001$ ; respectively). Leaf-cutting ants had higher baseline selfgrooming levels compared with the dinosaur ants, and both species up-regulated selfgrooming in response to talcum powder (Figure 6.1a; supplementary material). Leaf-cutting ants also significantly up-regulated allogrooming in response to talcum powder, but dinosaur ants performed no allogrooming in either treatment (Figure 6.1b).

Individual leaf-cutting ants and dinosaur ants differed in their selfgrooming duration ( $F_{39,402} = 3.58$ ,  $p < 0.001$ , and  $F_{39,394} = 4.53$ ,  $p < 0.001$ , respectively; Figures 6.2c and 6.2e), but leaf-cutting ants did not differ significantly in their allogrooming (Figure 6.2d). The intraindividual variation in selfgrooming duration was larger in dinosaur ants compared with leaf-cutting ants in the talcum powder treatment ( $U = 573$ ,  $n1 = n2 = 40$ ,  $p < 0.01$ ). In leaf-cutting ants there was a significant positive correlation between selfgrooming and allogrooming durations ( $r = 5.25$ ,  $n = 40$ ,  $p = 0.001$ ; Figure 6.2a; supplementary material).



**Figure 6.1.** The mean  $\pm$  s.e. grooming durations of selfgrooming (a) and allogrooming (b) for dinosaur ants and leaf-cutting ants, treated with a control (white bars) or talcum powder (grey bars), and the mean  $\pm$  s.e. selfgrooming and allogrooming durations for individual leaf-cutting ants, (c, d) and dinosaur ants (e, f), treated with talcum powder. Bars with different letter codes within each graph differ significantly at  $p < 0.05$ .



**Figure 6.2.** Correlation plots. The relationships between (a) allogrooming and selfgrooming durations after talcum-powder treatment, and (b) ant survival and self-grooming duration after treatment with the *Metarhizium* parasite, in leaf-cutting ants.

#### 6.4.2 Personalities and disease resistance

The survival of leaf-cutting ants exposed to the *Metarhizium* parasite was positively correlated with self-grooming duration ( $r = 0.80$ ,  $n = 35$ ,  $p < 0.001$ , Figure 7.2b). There were also significant correlations with allogrooming (see supplementary material). *Metarhizium* sporulated within two weeks of death from 97% of the cadavers, confirming that the parasite was the major cause of death.

## 6.5 Discussion

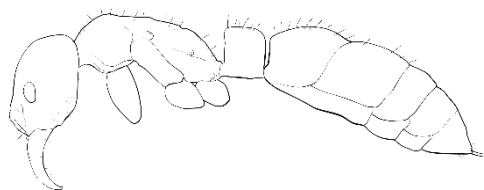
We show that dinosaurs ants, with simple societies, have lower levels of social immunity compared to leaf-cutting ants, with larger, more complex societies. Both species showed individual differences in selfgrooming that were consistent across time. These ‘hygienic personalities’ were more consistent in leaf-cutting ants, and their strength also positively correlated with allogrooming, and resistant to the fungal pathogen *Metarhizium*.

Larger, more complex colonies may be under increased threat from parasite transmission or acquisition, and therefore require the evolution of more effective resistance strategies. Alternatively larger colonies may be more productive and able to invest more in defences. For example fungus-growing ant species with larger, more complex colonies seem to possess more active antimicrobial defences than related species (Hughes, Pagliarini, et al. 2008; Fernández-Marín et al. 2009). Other social insects have also shown differences in investment in chemical defences with group size and social complexity (Traniello et al. 2002; Stow et al. 2007; Turnbull et al. 2010), and our results demonstrate that a similar change can be present for behavioural defences too. Leaf-cutting ants have previously been shown to have comparable grooming levels to other ant species with similar colony sizes (Tranter and Hughes 2015; Tranter et al. 2015), so it seems less likely that other aspects of their biology (e.g. protection of their vulnerable fungal crop) may explain the differences seen here.

Variation in disease resistance can be seen at multiple levels of social insect organisation, including between species, colonies, and castes (Tarpy 2003; Hughes, Pagliarini, et al. 2008; Vitikainen and Sundström 2011). Here we show

for the first time consistent individual differences in selfgrooming which indicate the existence of ‘hygienic personalities’. These personalities correlated positively with the resistance of leaf-cutting ants to disease, and were also more consistent than dinosaur ants. This may suggest that some degree of general hygienic specialisation which may be a prerequisite, or by product, of the task-partitioning seen in leaf-cutting ants, possibly in response to increases in parasite pressure due to their large colony size.

The response of social insects to disease can be highly dynamic both between and within species. This study demonstrates that social immunity and hygienic personalities can be more highly expressed in species with more complex societies. Future comparative investigations of disease resistance mechanisms between both individuals within colonies and also between social insect species with varying degrees of sociality will be vital in exploring these ideas.



*Dinoponera quadriceps*



# 7. Sanitary slaves and slovenly slavemakers: disease resistance of social parasites and their hosts

## 7.1 Abstract

Disease represents a considerable fitness cost to organisms and hosts have consequently evolved a suite of defensive adaptations against pathogens. Understanding how host life-history affects the selection pressure from pathogens, and how this in turn affects investment by hosts in disease resistance, is a key issue in evolutionary biology. In social insects, certain ‘social parasite’ species have evolved to exploit the efforts of other colonies for routine tasks like foraging and brood care. Given that disease resistance is often costly, social parasites, especially those that are obligate, may be selected to also exploit their hosts for defence against pathogens. Here we test this hypothesis using a targeted experimental comparison of the behavioural, chemical and physiological immune resistance of closely related formicine ant species that are an obligate social parasite, a facultative social parasite, or free-living host species. We find that socially parasitic ant species have significantly reduced disease resistance when compared to closely related free-living species, and that a facultative social parasite was intermediate in disease resistance. There was no evidence of any trade-offs between different defence mechanisms, with social parasites instead having reduced levels of all defences. This highlights the considerable reliance social parasites have on their hosts and demonstrates how the evolutionary

transitions between free-living, facultative, and obligate socially parasitic modes of life, may alter investment into disease resistance. Further targeted comparative studies like this will be valuable to develop our understanding of how the evolution of disease resistance mechanisms influences, and has been influenced by, changes in life history.

## 7.2 Introduction

Nature is rife with examples of parasitism, and the diversity and prevalence of parasitic interactions is testimony to the evolutionary success of this mode of life (Windsor 1998; Poulin and Morand 2000). The coevolutionary dynamics between host and parasite have produced an array of adaptations as hosts evolve to better defend themselves against a constantly evolving threat from parasites (Poulin 2007). Across taxa and environments, parasite pressure has been influential in shaping the evolutionary trajectory of species and communities (Boots et al. 2004; Schmid-Hempel 2011). However not all parasitic interactions and influences are obviously parasitic. For example, brood parasitism, which has been well studied in cuckoos, involves parasites exploiting not organisms as physical hosts, or as a direct source of sustenance, but rather for their investment in brood rearing (Payne 1977; Rothstein 1990). Similarly, the group-living eusocial insects include some ‘social parasite’ species of ants, bees, and wasps that have evolved the ability to live within colonies of closely related taxa and exploit the workers of their host colony for routine tasks such as foraging and brood care (Buschinger 1986, 2009; D’Ettorre and Heinze 2001). By unloading work onto their hosts, social parasites are then able to focus their energies on alternate ways to increase their fitness (Stearns 1992).

Sociality is often associated with considerable costs in terms of an increased risk of disease (Frank 1996; Altizer, Nunn, Thrall, et al. 2003; Schmid-Hempel 2005), but this threat is exacerbated in social insects, where crowded and homeostatic nests, combined with low levels of genetic diversity between eusocial individuals, provide ideal conditions for the transmission and evolution of parasites and pathogens (Schmid-Hempel 1994; Liersch and Schmid-Hempel 1998; O'Donnell and Beshers 2004; Calleri et al. 2006). Consequently ants and other social insects have evolved effective disease resistance strategies (Boomsma et al. 2005; Cremer et al. 2007; Wilson-Rich et al. 2009). These include behavioural responses such as selfgrooming and grooming, chemical defences such as the production of antimicrobial gland secretions and venom, and internal innate immune responses including pathogen encapsulation by haemocytes and the phenoloxidase (Siva-Jothy et al. 2005; Wilson-Rich et al. 2009; Otti et al. 2014). The presence of these advanced, conspicuous, and energetically costly (Poulsen, Bot, Nielsen, et al. 2002), defence mechanisms makes ants good models to study how life-history affects the evolution of disease resistance. The facts that investment in disease resistance mechanisms is costly and that social parasites can rely on their host colonies for many normal functions, leads to the hypothesis that social parasites may invest less in disease resistance because they can rely on the workers of their host colonies for this (Stearns 1992; Michod 2006). An example of this is the reduction of the energetically costly antimicrobial-producing metapleural gland in many ant social parasites. The leaf-cutting ant social parasite *Acromyrmex insinuator* has reduced disease resistance as a result of its smaller metapleural gland (Sumner et al. 2003), and roughly three quarters of obligate socially parasitic ant species show a loss of the metapleural gland compared to less than 10% of ant species

that are ‘temporary’ social parasites (Yek and Mueller 2011). This is likely because the temporary parasites would only benefit from host defences for initial colony foundation and once the original host workers have died the parasitic species must be sufficiently able to defend against disease on their own. Thus, investment in disease resistance cannot be relaxed as much as in obligate social parasites in which host workers are present for the whole of the life cycle. This idea broadly predicts a negative correlation between the dependency of a social parasite on its host and investment in disease resistance. Facultative social parasites are thus expected to show a greater investment (or less of a reduction) in disease resistance than more obligate social parasites.

In this study we use a selection of closely related species within the tribe Formicini (Subfamily: Formicinae; Agosti 1994) to study experimentally the relationship between different modes of social parasitism and disease resistance. This group provides an ideal comparative framework within which to study the evolutionary relationship between social parasitism and disease resistance as it contains a range of free-living and socially parasitic species (Topoff 1990; Agosti 1994). Specifically, we examined for four free-living species (*Formica cunicularia*, *F. fusca*, *F. rufibarbis* and *F. rufa*), a facultative social parasite (*F. sanguinea*) and an obligate social parasite (*Polyergus rufescens*): i) the disease resistance of ants and the benefit for this of their production of antimicrobial venom, ii) self- and allogrooming behavioural defences against parasites, iii) the quantity and quality of the primary chemical defence mechanism, the production of antimicrobial venom, and iv) the level of a key physiological immune defence mechanism, the phenoloxidase pathway (Armitage and Siva-Jothy 2005; Cerenius et al. 2008). Given the reliance of socially parasitic species on their

hosts for other tasks, we would expect the obligate social parasite *Polyergus*, and, to a lesser extent, the facultative social parasite *F. sanguinea*, to have lower disease resistance, behavioural, chemical and immune defences, compared to free-living species. Additionally, because we test a suite of different defence mechanisms, we may be able to see if there are trade-offs in defences employed by the different species.

## 7.3 Methods

### 7.3.1 Study species

We used six sympatric species of European formicine ants (see supplementary online material): the obligate, socially parasitic slavemaker *Polyergus rufescens*, the facultative, socially parasitic slavemaker *Formica sanguinea*, and four free-living species (non-parasitic), *F. rufibarbis*, *F. fusca*, *F. cunicularia* and *F. rufa*. Within the free-living species, *F. rufibarbis*, *F. fusca*, and *F. cunicularia* are all commonly taken as ‘slave’ hosts by both of the social parasites. Colonies were maintained in the lab at 23°C and 60% relative humidity for at least three months prior to use and were in good health at the time of the experiment. All species were fed twice per week with chopped *Tenebrio molitor* mealworm larvae and supplied with water *ad libitum*. Ants for the experiments were collected from just outside the nest entrance, and were selected to be of similar size and cuticular colouration within each species, representative of an adult worker at least four weeks old (Table S7.2 p.287). Workers of *P. rufescens*, *F. cunicularis*, *F. fusca* and *F. rufibarbis* are monomorphic, and for the polymorphic *F. rufa* and *F.*

*sanguinea* we used smaller workers which more closely matched the size of workers from the other species.

As a parasite for the experiments, we used the generalist entomopathogenic fungus *Metarhizium pingshaense* (strain KVL02-73 isolated from the soil in Panama; Hughes et al. 2004; Pull et al. 2013). Microparasitic fungi are one of the most common parasitic threats encountered by ants and are highly abundant in the soil environment (Keller et al. 2003; Boomsma et al. 2005; Reber and Chapuisat 2011). Fungal threats to ants include specialist parasites, generalist entomopathogens, and opportunistic parasites, but specialist parasites of ants are likely to show close coevolution with their specific hosts which renders them unsuitable for comparative experiments. *Metarhizium* infects a very wide range of insects, including many ants (Allen and Buren 1974; Lofgren and Vander Meer 1986; Sanchez-Pena and Thorvilson 1992; Quiroz et al. 1996; de Zarzuela et al. 2012), and will therefore be unlikely to exhibit species-specific coevolution with the ants investigated here.

### **7.3.2 Exp. 1: Pathogen resistance and antimicrobial venom**

Twenty ants were used from each of four colonies for each of the five *Formica* species (*F. fusca*, *F. cunicularia*, *F. rufibarbis*, *F. sanguinea*, *F. rufa*; 80 ants total per species), and eight ants from each of four colonies of the obligate slavemaker *Polyergus rufescens* (32 ants total). Half of the ants from each colony had their venom glands blocked using quick-drying nail varnish, whilst the other half received a control treatment of nail varnish applied to the pronotum. After 24

h, half of the ants in each case had a suspension of  $5 \times 10^7$  *Metarhizium* conidia per ml in 0.05% Triton-X applied topically to the mesosoma with a micropipette and half were treated with a control solution of 0.05% Triton-X. Treatment volumes were standardized for body-size between species (Table S7.2 p.287). After treatment, each ant was placed in a plastic pot (diameter: 35 mm, height: 70 mm) supplied with cotton balls soaked in 20% sucrose solution and water, and kept at 50% RH and 21 °C. Ant mortality was recorded for 14 days, with cadavers surface sterilised (Siegel 2012), and then kept in a Petri dish with moistened filter paper for a further 14 days to allow for the sporulation of the *Metarhizium* pathogen. This full factorial design resulted in 20 replicates per treatment per *Formica* species and eight replicates per treatment for *P. rufescens*.

### **7.3.3 Exp. 2: Allogrooming and self-grooming behavioural defences**

To assess the behavioural defensive response of ants, 30 workers were used from each of four colonies for each of the five *Formica* species (*F. fusca*, *F. cunicularia*, *F. rufibarbis*, *F. sanguinea*, *F. rufa*; 120 ants in total per species), plus ten workers from four colonies of *P. rufescens* (40 ants in total; Table S7.1 p.286). Each ant was placed in a pot (diameter: 35 mm; height: 70 mm) and left to acclimatize for 5 min. Half the ants from each colony were then treated with a talcum powder suspension ( $5 \times 10^7$  particles per ml) in 0.05% Triton-X solution whilst the other half were treated with just Triton-X solution as a control, with volumes adjusted for species as in Experiment 1. Talcum powder particles are comparable in size to fungal conidia and act as a non-pathogenic stimulant of anti-parasite defensive behaviour in ants (Morelos-Juárez et al. 2010; Tranter et al.

2015). The solutions were applied topically to the dorsal surface of the mesosoma and gaster using a micropipette, with amounts standardized for body size between species (Table S7.2 p.287). For each trial the frequency and total duration of self-grooming were recorded for 30 min. To assess allogrooming, 20 pairs of ants were collected from each of four colonies for each of the *Formica* species (80 pairs in total per species), plus four pairs from each of five colonies of *P. rufescens* (20 pairs in total). Following set-up and acclimatization as above, one ant was treated with a talcum powder suspension in half of the pairs, whilst one ant in the other half of the pairs was treated with control solution (as above). The frequency and total duration of allogrooming performed by the attending ant on the treated ant were recorded for 30 min.

In addition, in order to examine whether there is a relationship between the slave:slavemaker ratio in colonies and the grooming rates of the slavemakers, we recorded the self-grooming rates of ten *F. sanguinea* workers from each of ten colonies for a period of 15 min, using the same procedure as above. The numbers of *F. sanguinea* workers and slave workers (either *F. fusca* or *F. rufibarbis*) in each colony were counted, and the slave:slavemaker ratio calculated. For four colonies the ratio was then increased or decreased through manipulations of the colony populations. The ratio was increased by either the experimental introduction of slave brood from a different colony or the experimental removal or natural death of the *F. sanguinea* slavemakers, and the ratio of slaves to slavemakers was decreased by either removing slaves or the natural eclosion of *F. sanguinea* slavemaker workers. These manipulations were performed twice, each two weeks apart, to provide three different colony ratio compositions at different time points. The grooming rates of ten *F. sanguinea* workers per colony were



recorded using the method described above at each time point, giving three measures of colony grooming at different slave:slavemaker ratios for each colony.

#### **7.3.4 Exp. 3: Use and antimicrobial effectiveness of ant venom**

We tested the antifungal activity of ant-produced venom on both the conidia viability and hyphae growth of *Metarhizium*. Ants were chilled on ice for 20 min, and the venom gland and reservoir then dissected out by anchoring the ant at the petiole and pulling from the acidopore with fine forceps. This resulted in the clean liberation of the venom gland and reservoir from the gaster, leaving other organs such as the gut intact. The head of each ant was photographed using a stereomicroscope at fixed magnification, with the head width of each ant measured (Image J) and used as an estimate of body size (Figure S7.2 p.272). The venom gland and reservoir from three ants from the same colony were pooled together and immediately stored at -20 °C in 20 µl Phosphate Buffered Saline. Venom was collected from 30 ants (with venom from 3 ants pooled to give 10 pooled replicates) from each of four colonies of each of the *Formica* species (40 pooled replicates in total per species), and 30 pooled replicates (again, each pooled from 3 individual ants) in total from seven *Polyergus* colonies (see Table S7.1 p.286 for colony selection). 500 µl of a solution containing  $1 \times 10^5$  *Metarhizium* conidia per ml was applied evenly over the surface of 50 mm Petri dish media plates (Saboraud dextrose agar with 0.1 g/l dodin , 0.05 g/l streptomycin sulphate and 0.1 g/l chloramphenicol) and left for 10 min to dry. A sterile segment of 6 mm diameter plastic tubing was placed onto the centre of the surface of the agar plates to form a well, and 20 µl of venom gland test solution placed in

this. The well was left in place for 5 min to allow the compound to infuse and dry, before the location of the treated area was marked on the underside of the dish and the well removed. The Petri dish was then sealed with parafilm and placed in an incubator at 24 °C. After 14 h the percentage of conidia producing hyphae longer than the germ tube (conidia viability; Siegel 2012) was counted for a standardised area (complete area visible in the microscope eyepiece at 400x magnification), both within the section where the compound was tested, and in an untreated area outside the marked test area, equidistant with the edge of the Petri dish. A further 60 h later the plates were photographed from above and the average radius determined based on three measurements made using Image J software of any zone of inhibition around the marked test section that was free from hyphal growth .

In addition, ten ants from each of *P. rufescens*, *F. cunicularia* and *F. rufa* were observed for their venom use. Each ant was anaesthetised with a standardised burst of CO<sub>2</sub> gas and then placed in a small pot (14 mm x 32 mm) with a 7 x 14mm rectangle of pH indicator paper (Whatman pH 0.5-5.5). After 3 h, a photograph of the indicator paper was taken, and the pH value calculated from the colour of the pH paper (rounded to the nearest 0.5). Finally, the gasters of 30 ants from each of the same three species were removed from the rest of the ant, pooled into groups of three (10 replicates per species), and crushed in 250 µl of ddH<sub>2</sub>O. The pH of the gaster solutions were then tested using indicator solution (Fluka universal indicator solution pH 0-7). The pH of a solution of formic (methanoic) acid and pure ddH<sub>2</sub>O were also tested as a positive and negative control.

### **7.3.5 Exp. 4: Physiological immunity**

To assess investment by the ant species in general physiological immunity, we quantified the number of haemocytes, and the levels of the phenoloxidase and prophenoloxidase immune enzymes. Ants were chilled on ice for 20 min before being placed dry under a stereo dissecting microscope. The head and gaster were removed and 0.2  $\mu$ l haemolymph was extracted by placing a calibrated microcapillary against the wounds. Samples were collected from five ants from each of four colonies for each of the ant species (20 replicates per species) for measurement of the phenoloxidase (PO) and prophenoloxidase (PPO) immune enzymes, and the same number again for haemocyte counts. PO and PPO was measured using methods detailed in Armitage and Boomsma (2010) with minor modifications. Both PO and PPO measurements from each haemolymph sample was performed at the same time on the same plate. Samples from five ants for each species were pooled and used as a sample standard to control for any variation between plates. Haemolymph was immediately mixed with 12  $\mu$ l of ice cold sodium cacodylate buffer (0.01M NaCac, 0.005M CaCl<sub>2</sub>, pH 6.5) after collection and snap frozen in liquid nitrogen. Samples were then defrosted on ice prior to measurement. For the measurement of PPO, 5  $\mu$ l of sample and 5  $\mu$ l of Bovine Chymotrypsin (5mg/ml in phosphate buffered saline) were added to each well. For the measurement of PO, 5  $\mu$ l of sample and 5  $\mu$ l of ddH<sub>2</sub>O were added to each well. A master mix containing 20  $\mu$ l filtered L-DOPA (11mg/ml in PBS), 20  $\mu$ l of PBS and 100  $\mu$ l of ddH<sub>2</sub>O, was prepared on ice and added to each cell. Each plate was run with a negative control well consisting of the master mix but without a sample, and a blank well with only ddH<sub>2</sub>O. Two positive control wells were also run per plate using a fixed volume of Sudan black dye in one well and

the pooled sample haemolymph run as above in another, in order to confirm that readings were consistent between plates. Immediately after the plate was prepared it was placed into a VersaMax plate reader spectrophotometer which was pre-heated to 25°C and light absorbance readings recorded every 15 s for 2 h at a wavelength of 490 nm using Softmax Pro (Molecular devices) software. The Vmax values of the linear phase, which usually occurred after an initial lag period of 200 s, were recorded for each sample. Five samples which had  $r^2 < 0.7$ , or where no reading was given by the software, were repeated. To count the number of haemocytes in haemolymph, 0.1 µl of defrosted haemolymph (20 replicates per species) was vortexed and added to 20µl Ringer solution and mixed on parafilm (Armitage and Boomsma 2010). 2 µl of this mix was added to a microscope slide cover slip treated with Poly-D-Lysine. The cover slip was allowed to dry out overnight and was viewed under a fluorescence microscope at 400x and all cells in the field of view counted. Use of frozen haemolymph resulted in comparable cell counts to using freshly extracted unstored haemolymph samples (Figure S7.1p 271)

### **7.3.6 Statistical analyses**

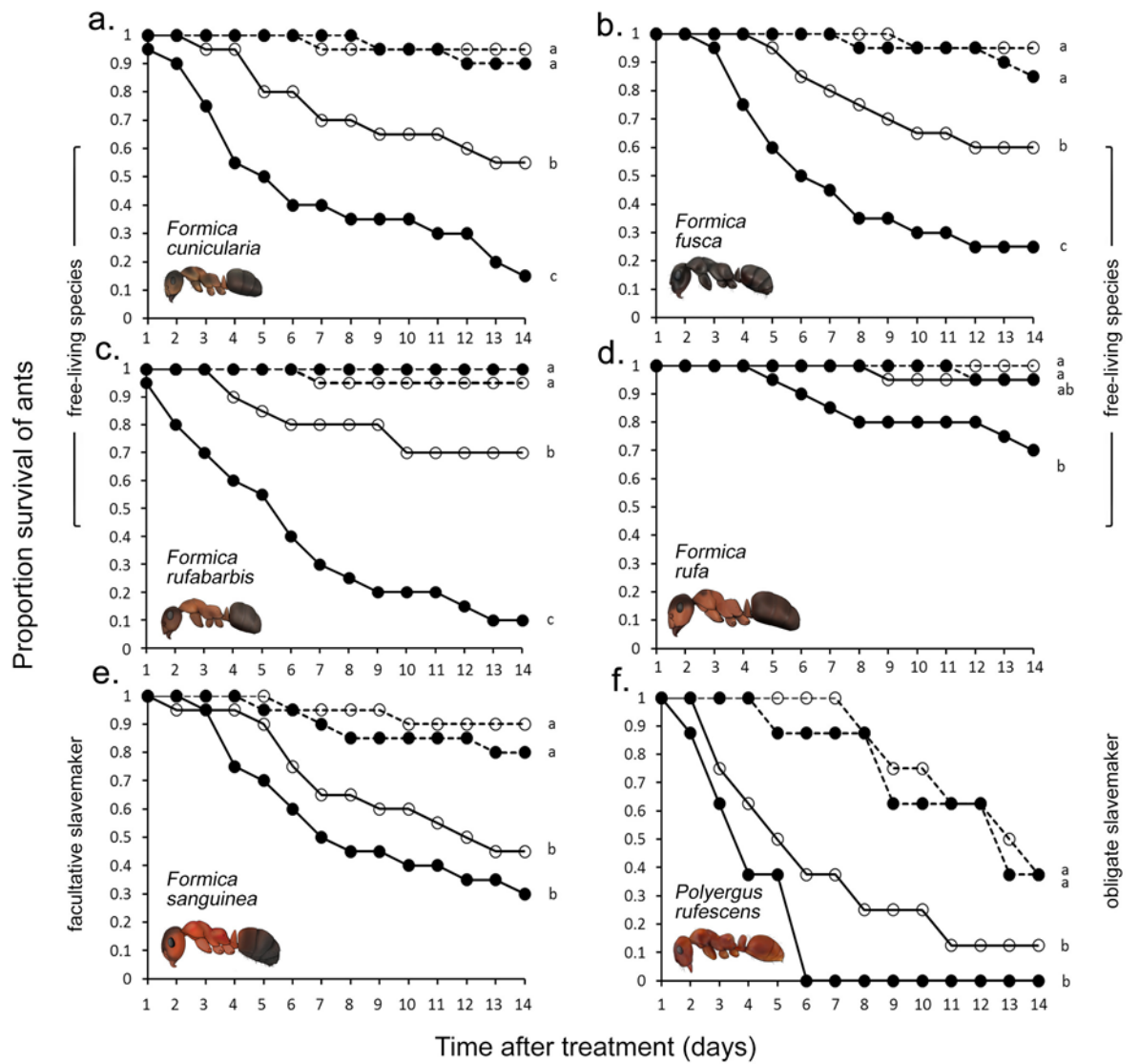
The overall difference in the survival of ants in Experiment 1 was analysed using a Cox regression model, including species, blockage treatment, fungal treatment and their interactions. The data was then split based on species and the survival of ants analysed with respect to their colony-of-origin. Pairwise comparisons between treatment groups were made using Kaplan-Meier analyses with the Breslow statistic. The data from Experiments 2 and 3 were analysed using generalised linear mixed models (GLMM) where data from experiments were non-normal, with model distribution selected based on AIC scores and the structure of

the non-normal data. No overdispersion was observed based on model deviance/df values. Non-significant interaction terms within the models were removed sequentially based on likelihood-ratio tests to achieve the minimum adequate models. Self- and allogrooming durations in Experiment 2 were analyzed using a GLMM with gamma distribution and log link function, with the data analysed first overall to compare species. The relationship between the grooming duration of *F. sanguinea* and the ratio of slaves:slavemakers was analysed using a quadratic regression. The antifungal action of ant venom on conidia viability and hyphae growth in Experiment 3 was examined using a GLMM with gamma distribution and log link function. Differences in the pH of the environment and gaster of species were analysed using a general linear model. In Experiment 4, PO and PPO levels, and haemocyte counts, were compared between species using GLMMs with gamma distribution and log link function. All GLMM models included colony as a random factor nested within species and pairwise post-hoc comparisons were adjusted using sequential Bonferroni adjustments to control for multiple comparisons. All analyses were performed in SPSS v.21 (IBM).

## 7.4 Results

### 7.4.1 Exp. 1: Pathogen resistance and antimicrobial venom

There was a significant three-way interaction between species, fungal treatment, and gland blockage (Wald = 17.7, d.f. = 5,  $p = 0.003$ ), but no effect of colony ( $p > 0.05$  in all species). Ants from all of the *Formica* species survived well when treated with the control solution, while those of *P. rufescens* survived less well (Figure 7.1). Exposure to the *Metarhizium* pathogen significantly reduced the survival of ants from all species except for the free-living *F. rufa*, and blocking of the venom gland resulted in a significant reduction in the resistance of ants to *Metarhizium* for *F. fusca*, *F. cunicularia* and *F. rufibarbis* (Figure 7.1; Table S7.3 p.288). *F. rufa* was highly resistant to *Metarhizium*, with the survival of *Metarhizium*-exposed *F. rufa* ants being the same as control-treated ants when their venom glands were functional, and only moderately reduced even when the venom gland was blocked. In the cases of *F. fusca*, *F. cunicularia* and *F. rufibarbis* ants, *Metarhizium*-exposed ants had reduced survival compared to control-treated ants when their venom glands were functional, and much lower survival still when the venom glands were blocked. In the two social parasites, *F. sanguinea* and *P. rufescens*, there was no effect of venom gland blockage on the survival of *Metarhizium*-exposed ants, with it being significantly lower than control-treated ants in both species and particularly low in *P. rufescens*.

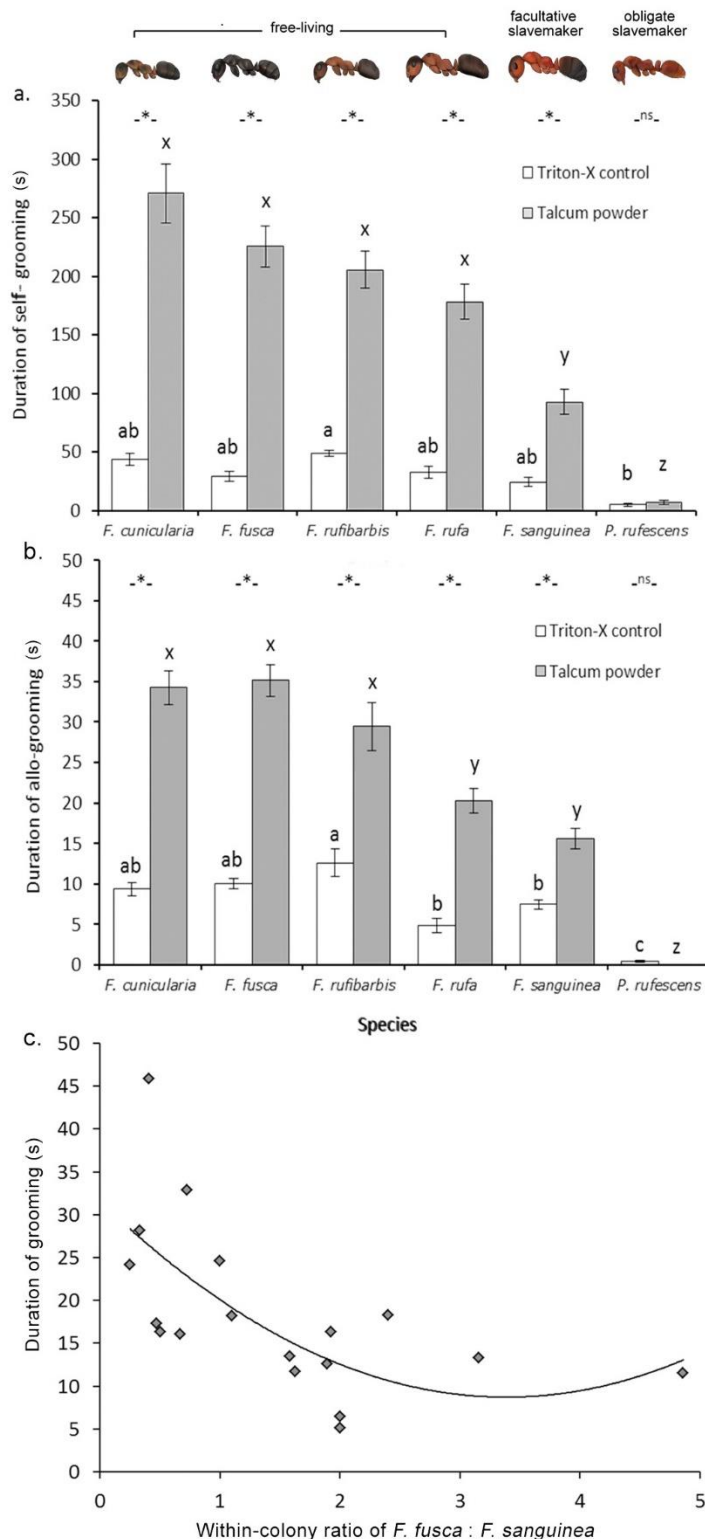


**Figure 7.1.** Pathogen resistance and the importance of antimicrobial venom. The survival over two weeks of a) *Formica cunicularia*, b) *F. fusca*, c) *F. rufibarbis*, and d) *F. rufa* free-living ant species, e), the *F. sanguinea* facultative social parasite, and f) the *P. rufescens* obligate social parasite, after treatment with either the *Metarhizium pingshaense* fungal pathogen (solid line) or control solution (dashed line), and with their venom gland either experimentally blocked (closed circles) or functional (open circles). Different letters beside lines indicate treatments within species that differed significantly from one another at  $p < 0.05$ .

#### 7.4.2 Exp. 2: Allogrooming and self-grooming behavioural defences

There was a significant interaction between the effects of species and treatment on the duration of self-grooming ( $F_{5,668} = 10.4$ ,  $p < 0.001$ ), while for allogrooming there were significant effects of both treatment and species, but no interaction between them ( $F_{5,408} = 14.9$ ,  $p < 0.001$ ;  $F_{1,408} = 32.2$ ,  $p < 0.001$ ;  $F_{5,408} = 1.74$ ,  $p = 0.124$  respectively). Colony-of-origin had a significant effect on self-grooming but not allogrooming rates ( $Z = 2.06$ ,  $p < 0.001$ ;  $Z = 0.97$ ,  $p = 0.33$ , respectively), and there was no interaction between colony and treatment ( $p > 0.05$  for both allogrooming and selfgrooming). All species, except for *P. rufescens*, allogroomed and selfgroomed for significantly longer when treated with talcum powder compared with the control treatment (Figure 7.2a, b). The four free-living *Formica* species selfgroomed significantly more than the two social parasite species when treated with talcum powder, while there was only a difference between the selfgrooming of *F. rufibarbis* and *P. rufescens* when treated with control solution (Figure 7.2a). The free-living *F. cunicularia*, *F. fusca* and *F. rufibarbis* allogroomed for longer than *F. rufa* and the facultative slavemaker *F. sanguinea*, and these in turn groomed for significantly longer than the obligate slavemaker *P. rufescens* which showed almost no allogrooming (Figure 7.2b). There was a significant negative correlation between the rates of selfgrooming by *F. sanguinea* and the ratio of slaves:slavemakers in their colony, with *F. sanguinea* from colonies with fewer slaves tending to self-groom more ( $r^2 = 0.1$ ,  $F = 9.66$   $p < 0.001$ ; Figure 7.2c)

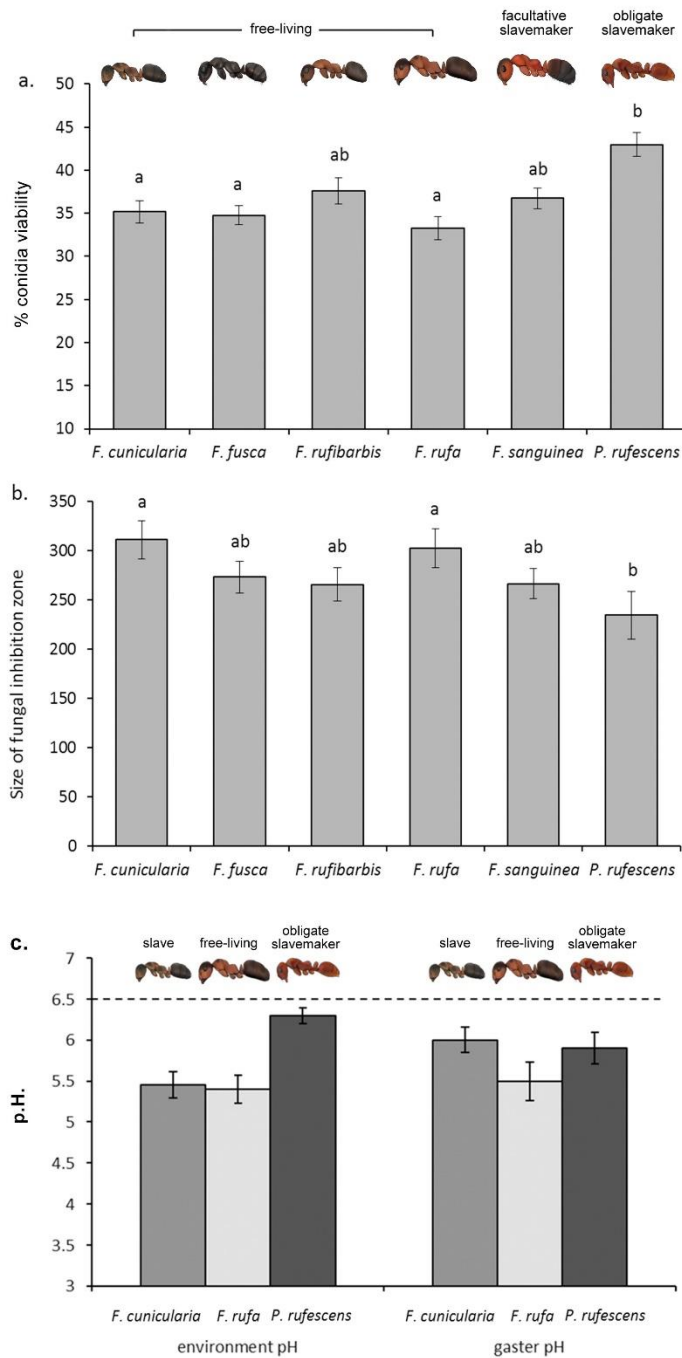




**Figure 7.2.** Allogrooming and selfgrooming defences. The mean  $\pm$  s.e. duration of a) self-grooming and b) allogrooming by ants from the free-living species *Formica cunicularia*, *F. fusca*, *F. rufibarbis* and *F. rufa*, the *F. sanguinea* facultative social parasite, and the *P. rufescens* obligate social parasite after treatment with either a fungal pathogen mimic (talcum powder; grey) or control solution (white). Asterisks above columns indicate species for which the talcum powder and control treatments differed significantly in the grooming response stimulated ( $p < 0.05$ ), and different letters above columns indicate species that differed significantly from one another in grooming for that treatment (control solution: a-c; talcum powder: x-z). Also shown is c) the relationship between the mean duration of self grooming by ants of the facultative social parasite *F. sanguinea* and the ratio of *F. fusca* host workers to *F. sanguinea* workers in their colony.

### 7.4.3 Exp. 3: Use and antimicrobial effectiveness of ant venom

Application of venom significantly reduced the viability of *Metarhizium* conidia from its baseline level of  $88 \pm 6\%$ , with the strength of the effect differing between ant species ( $F_{5,234} = 2.47$ ,  $p = 0.033$ ). Venom from the obligate slavemaker *P. rufescens* did not reduce the viability of conidia as much as venom from *F. cunicularia*, *F. fusca* and *F. rufa* (Figure 7.3a). There was no overall difference between species in the zone of inhibition of *Metarhizium* hyphal growth ( $F_{5,234} = 1.98$ ,  $p = 0.082$ ), but there were significant differences between species in pairwise comparisons with venom from *P. rufescens* producing smaller zones of inhibition than *F. cunicularia* and *F. rufa* (Figure 7.3b). There was a significant effect of colony-of-origin on both conidia viability and the inhibition zone size ( $Z = 1.14$ ,  $p = 0.048$ ;  $Z = 1.49$ ,  $p = 0.037$ , respectively). There was no significant difference in the acidity of whole-gaster extracts from *F. cunicularia*, *F. rufa* and *P. rufescens* ( $F_{2,27} = 5.35$ ,  $p = 0.069$ ), but there were significant differences in the pH of the environment after the presence of an ants from the different species ( $F_{2,27} = 13.22$ ,  $p < 0.001$ ), with the obligate slavemaker *P. rufescens* reducing the pH of the environment much less than the free-living ant species (Figure 7.3c). However it seems that the venom gland in *P. rufescens* may not be any smaller, relative to body size, than the other free-living species, but may be enlarged in *F. rufa* (Figure S7.2 p.272).



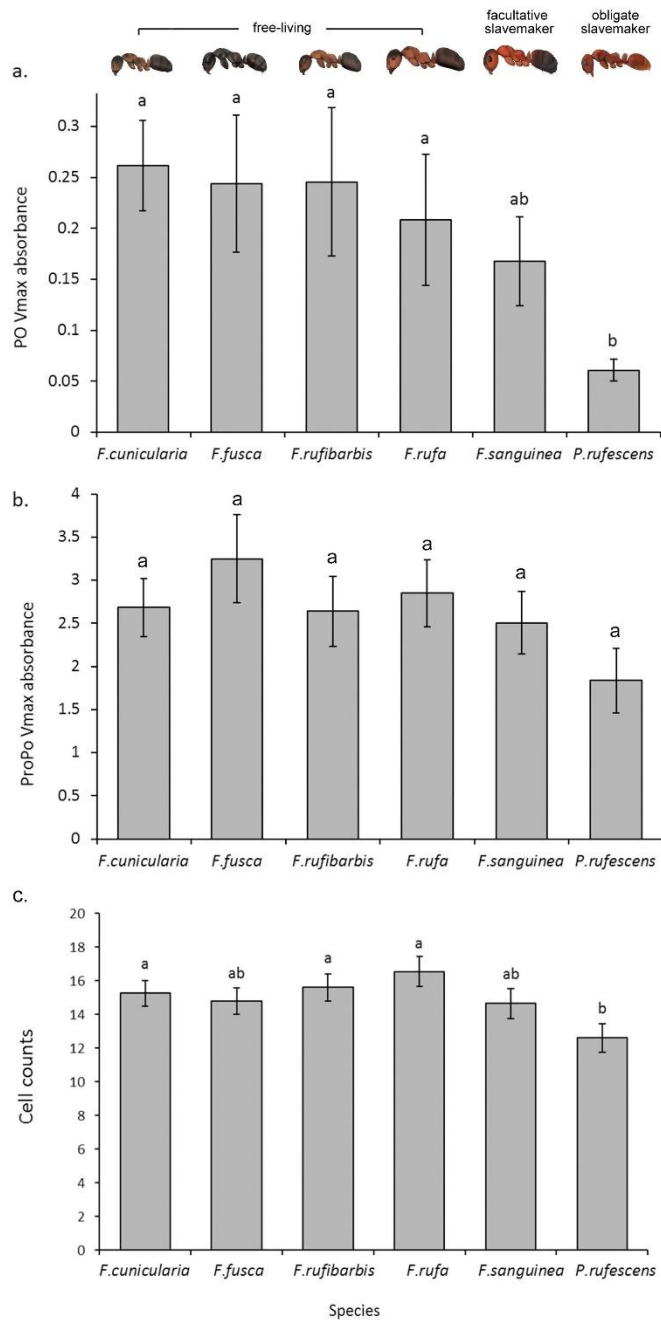
**Figure 7.3.** Use and antimicrobial effectiveness of ant venom. The mean  $\pm$  s.e. a) percentage viability of conidia and b) zone of inhibition of hyphal growth of the *Metarhizium* fungal pathogen when exposed to venom from the free-living species *Formica cunicularia*, *F. fusca*, *F. rufibarbis* and *F. rufa* ant species, the *F. sanguinea* facultative social parasite, and the *P. rufescens* obligate social parasite. Bars with different letter codings differ significantly at  $p < 0.05$ . Chart c) shows the mean  $\pm$  s.e. pH of the environment and of ant gasters for the free-living *F. cunicularia* (dark grey bars), and *F. rufa* (medium grey bars), and the obligate social parasite *P. rufescens* (light bars). The dashed line represents the pH of blank control trials.

#### 7.4.4 Exp. 4: Physiological immunity

There was a significant difference between the ant species in their baseline levels of the phenoloxidase (PO) immune enzyme in their haemolymph ( $F_{5,114} = 2.97$ ,  $p = 0.015$ ), with *P. rufescens* having lower levels than the *Formica* species (Figure 7.4a). However there was no difference between ant species in their levels of constitutive prophenoloxidase (PPO;  $F_{5,114} = 0.92$ ,  $p = 0.47$ ; Figure 7.4b). We trialled two methods for counting haemocytes and these produced comparable results (Figure S7.1). There were significant differences in the number of haemocytes between species ( $F_{5,114} = 2.31$ ,  $p = 0.048$ ), with the obligate slavemaker *P. rufescens* having fewer haemocytes than *F. cunicularia*, *F. rufibarbis* or *F. rufa* (Figure 7.4c). There was no effect of colony on PO, PPO or haemocyte counts ( $Z = 1.24$ ,  $p = 0.214$ ;  $Z = 1.96$ ,  $p = 0.05$ ;  $Z = 0.734$ ,  $p = 0.16$ , respectively).

### 7.5 Discussion

The results show that the two socially parasitic species examined here have reduced disease resistance compared to related free-living species, and consequently were more susceptible to infection by a fungal pathogen. Between the socially parasitic species, the obligate social parasite *P. rufescens* generally had lower disease resistance than the facultative social parasite *F. sanguinea*. Both of the socially parasitic species had lower behavioural defences than the four free-living *Formica* species, and *P. rufescens* also had less effective chemical defences and reduced physiological immunity.



**Figure 7.4.** Physiological immunity. The mean  $\pm$  s.e. levels (Vmax absorbance) of a) phenoloxidase and b) prophenoloxidase immune enzymes, and c) number of haemocytes (see also Supp mat S7.2 p286), for the free-living species *Formica cunicularia*, *F. fusca*, *F. rufibarbis* and *F. rufa*, the *F. sanguinea* facultative social parasite, and the *P. rufescens* obligate social parasite. Bars with different letter codings differ significantly at  $p < 0.05$ .

Our findings support the hypothesis that socially parasitic species have reduced disease resistance compared to related free-living species. Other free-living formicine ants, including representatives of *Lasius*, *Camponotus*, *Polyrhachis*, and *Oecophylla*, have been found to have effective individual-level disease resistance mechanisms in the form of antimicrobial venom and grooming, as well as cooperative social defences as well (Chapuisat et al. 2007; Ugelvig and Cremer 2007; Graystock and Hughes 2011; Hamilton et al. 2011; Walker and Hughes 2011; Reber and Chapuisat 2011; Konrad et al. 2012; Tragust, Mitteregger, et al. 2013; Tranter et al. 2014, 2015; Tranter and Hughes 2015). The evolutionary history of these disease defences therefore indicates that they have probably been secondarily reduced in *F. sanguinea* and *P. rufescens* in parallel with their evolution of a socially parasitic lifestyle. This mirrors a socially parasitic species of leaf-cutting ant, *Acromyrmex insinuator*, in which the size of the antibiotic-producing metapleural gland has been secondarily reduced, with a consequent decrease in individual-level disease resistance (Sumner et al. 2003). It has been predicted that the evolution of a group-living lifestyle is associated with greater pressure from pathogens (Alexander 1974), and there is some evidence for a positive relationship between sociality and investment in ants, bees and social thrips (Stow et al. 2007; Hughes, Pagliarini, et al. 2008; Turnbull et al. 2010, 2012; Fernández-Marín et al. 2013). The finding of secondary reductions in disease resistance in socially parasitic species suggests that this evolutionary relationship may also go in reverse.

The various different defences exhibited by social insects are often traded-off with one another (Lochmiller and Deerenberg 2000; Schmid-Hempel and Ebert 2003), with certain species investing heavily in one mechanism, but not another (Sheldon and Verhulst 1996; Fernández-Marín et al. 2013; Tranter and Hughes 2015).

The facultative slavemaking ant, *F. sanguinea*, demonstrated normal physiological immune defences when compared with the free living species, but a reduction in the levels of grooming behaviours, placing it intermediate between the obligate slavemaker and the free-living species in terms of behavioural disease defence. Additionally, *F. sanguinea* seemed able adjust its grooming rates depending on the number of slaves present, and was flexible over relatively short time scales of a few months. It is likely that this finding may be exclusively observable in facultative social parasites such as *F. sanguinea*, which retain enough of their behavioural anti-parasite repertoire (Snelling and Buren 1985). This suggests that the more plastic behavioural defences, like the flexible grooming seen in *F. sanguinea*, may be reduced first during evolution, and that the less plastic defences such as acid production and physiological immunity are then reduced later. This is supported by *P. rufescens*, which shows not only significant reductions in behavioural grooming and the use of venom, but also in physiological immunity and the antimicrobial effectiveness of venom.

The relaxation of disease resistance may be unsurprising when you consider that they are often very energetically costly (Poulsen, Bot, Nielsen, et al. 2002; Schmid-Hempel 2005), and that behaviours also potentially increase the individuals own risk of exposure (Rosengaus 2000; Hughes et al. 2002; Fefferman et al. 2007). However the significance of these costs for host fitness and evolution is relatively poorly understood. The finding that both obligate and

facultative slavemaker species have reduced investment into disease resistance, in addition to similar reductions in *Acromyrmex insinuator* (Sumner et al. 2003), provides evidence that these costs are significant enough for evolution to reduce them when the need for them is lower. The production of antimicrobial secretions from the metapleural gland in leaf-cutting ants can be very costly (Poulsen, Bot, Nielsen, et al. 2002), and it seems reasonable to assume that the production of antimicrobial venom is costly in the formicines. But the reduction in grooming in *F. sanguinea* indicates that behavioural defences likely incur significant costs as well, perhaps in terms of energy and time costs, or even in the cost of maintaining neural tissue governing these behaviours (Giorgi et al. 2001; Reber et al. 2011; Sulger et al. 2014). These reductions in the more plastic behavioural defences may have evolved over just a few million years, as *F. sanguinea* is estimated to have diverged from the other *Formica* subgenera around 6 mya (Jansen et al. 2010), whereas the physiological responses to a more obligate socially parasitic lifestyle may have taken longer (40 mya ca. divergence between *Formica* and *Polyergus*; Goropashnaya et al. 2012).

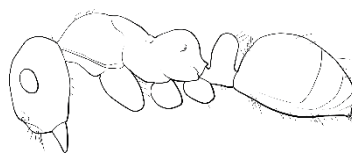
We also found that the free-living species, *F. rufa*, is more resistant to disease than the other free-living *Formica* species, despite having lower levels of grooming. Although there was no difference in the potency of their venom or physiological immunocompetence, *F. rufa* does seem to possess much larger venom glands for its body size, which may explain its greater disease resistance. *F. rufa* has much larger, more complex colonies, than the other free-living *Formica* species we examined (Yarrow 1955; Domisch et al. 2009), which may increase parasite pressures through promoting horizontal transmission and greater ‘sampling’ of the environment (Tschinkel 1991; Schmid-Hempel 1998;



Poulin 2007). Thus it is possible the size, and ecological dominance, in *F. rufa* allows or necessitates greater investment into disease resistance. This may be similar to how larger, more complex, societies in fungus-growing ants possess more developed antimicrobial defences (Fernández-Marín et al. 2006; Hughes et al. 2008). Alternatively, the greater disease resistance of *F. rufa* may be a consequence of greater investment in predator defence. Unlike the defensive metapleural secretion, the venom of Formicines, large-colony wood ant species like *F. rufa* in particular, is often used as an antipredator defence (Blum 1978, 1992). This predator defence strategy may therefore have driven the evolution of the larger venom glands found in *F. rufa*, with a secondary benefit in terms of disease resistance.

Although slave-making social parasites have been studied for over 100 years the drivers of the evolution of this interesting trait are still debated (Wheeler 1910; Buschinger 1986). We suggest this study provides interesting evidence of how, in part, the evolutionary transition from non-parasite, to facultative parasite, to obligate parasite, may have evolved or been maintained; given increasing care from, and reliance on, their host species for parasite defence (Buschinger, 1986). Additionally, whilst there has been considerable work looking at how parasites affect the evolution of host defences, there are few studies looking at the defence responses of the parasites themselves to other threats, which can have considerable influences on the evolution of life-histories (Schmid-Hempel and Ebert 2003; de Roode and Lefèvre 2012). This is despite the not uncommon occurrence of hyperparasitism (parasites parasitizing parasites) and disease in parasitic species (Sullivan 1987; Foitzik et al. 2001). We show how the evolutionary transition between free-living, facultative and obligate socially

parasitic modes of life may influence or be influenced by pathogen parasite pressures and in turn alter investment into disease resistance and reliance on their hosts. Further targeted comparative studies like this will be vital to understanding how the evolution of immune function and parasite resistance has been influenced by a parasitic mode of life, and ultimately how these pressures may have influenced the evolution and persistence of social parasitism and other transitions in life history.



*Polyergus rufescens*

## 8. Evidence for cocladogenesis of *Wolbachia* symbionts and their hosts in a tropical ant assemblage

### 8.1 Abstract

Intracellular reproductive parasites such as *Wolbachia* are common in invertebrates and can have diverse and substantial effects on host biology. These parasites are usually transmitted vertically from, mother to daughter, but can also be transferred horizontally. By examining the phylogenies of both host and parasite within a system it is possible to reveal the modes of infection present. Here we screen species from a tropical ant assemblage in order to reveal the infection prevalence of *Wolbachia* and to explore the genetic relationships between hosts and parasites, and between parasite strains. Of 73 host ant species, 26% were found to be infected with *Wolbachia*. Congruence in the topologies of host and *Wolbachia* phylogenies, based on both *wsp* and MLST sequencing, indicated cocladogenesis of *Wolbachia* and its hosts, supporting the current paradigm that *Wolbachia* is predominantly vertically transmitted. The substantial diversity in *Wolbachia* strains found also provides the possibility for future phylogenetic trait-mapping analyses which will allow the exploration of how host life-history affects may affect transmission dynamics of reproductive parasites in social insect societies.

## 8.2 Introduction

Microbial endosymbiosis, where one partner lives within its host, is an important driver of evolutionary change, and has resulted in the formation of both mitochondria and chloroplasts (Cavalier-Smith 1987). Some endosymbionts which are transmitted vertically from one generation to the next, can alter the biology of their hosts in order to increase their own transmission, and in so doing act as ‘reproductive parasites’ (Duron et al. 2008). *Wolbachia* is the quintessential intracellular reproductive parasite, and is widespread in arthropods and nematodes. Although usually vertically transmitted, *Wolbachia* can also transmit horizontally between species at least occasionally, which has resulted in a global distribution within a diverse array of invertebrate hosts (Baldo et al. 2008). *Wolbachia* can feminise hosts, induce mating incompatibilities or asexuality, result in the death of males, and be required for oogenesis (Duron et al. 2008; Werren et al. 2008). These manipulative behaviours are interesting examples of how complex the evolutionary dynamics between host and parasite can be, but also have important effects on the ecology and evolution of the hosts themselves (Poulin 2007). Reproductive parasites can drive changes in sex ratio or even reproductive strategies in their hosts which can have drastic impacts on their populations, as hosts undergo rapid selection due to these pressures (Duron et al. 2008). In addition, *Wolbachia* can have either positive and negative direct fitness costs in terms of increased or reduced host immunocompetence, and provides strong benefits in other cases such as the protection of *Drosophila melanogaster* and mosquitoes against several RNA viruses and nematodes (Gerth et al. 2011; Martinez et al. 2014)

In most horizontally transmitted parasites, transmission rates are heavily influenced by host density (Mccallum et al. 2001; Riesa and Amazineb 2001). Therefore in group-living species, especially social insects, which live in very high-population nests with very little genetic diversity, the threat from parasites is often considered to be potentially high (Schmid-Hempel 1998; Calleri et al. 2006; Fefferman et al. 2007). However the dynamics of vertically transmitted parasites like *Wolbachia* within social insects are especially complex (Baldo et al. 2008). *Wolbachia* can only vertically transmit through the founding of new colonies by queens, as all worker females are unable to produce daughters and the parasite does not transmit through males (Riesa and Amazineb 2001; Baldo et al. 2008). In vertical transmission, parasite and host fitness are closely intertwined, as effective transmission relies on the fecundity of its host (Bull et al. 1991). Phylogenetic studies of *Wolbachia* and their hosts can be useful for exploring the transmission modes and rates of endosymbiosis. Cocladogenesis between host and symbiont phylogenies is indicative of vertical transmission, while incongruence in phylogenies can indicate horizontal transmission (Vavre et al. 1999; Baldo et al. 2005, 2008; Werren et al. 2008; Russell et al. 2009; Frost et al. 2010). Here we investigate the relationships between *Wolbachia* and its hosts in an assemblage of sympatric, tropical ants from the same ecosystem but which are diverse both in phylogeny and in life history. Our aim was to determine the occurrence of *Wolbachia* and to reveal the genetic relationships between it and the ant hosts, to explore the extent and dynamics of *Wolbachia* infection in this system.

## 8.3 Methods

### 8.3.1 Sampling

In order to sample an array of ants, representing a diverse tropical assemblage of species with varied life histories, we collected ants from Barro Colorado Island (BCI), Panama, in July of 2012 and July of 2013. BCI is home to over 400 species of ants (D. Donoso, in prep). Ants were sampled using active searching, baiting, leaf-litter sifting and pitfall traps, and were immediately stored in 100% ethanol after collection. Ants were identified from morphological characteristics using keys by Longino (2007) and Bolton (1994; 1995) under a stereomicroscope, and were compared with reference samples from collections where possible. This resulted in a collection of 84 species, 80 of which were represented by at least 4 individual worker samples and were screened in this study. Samples were stored at -20°C until use.

### 8.3.2 Screening for *Wolbachia*

DNA from 878 individual ants (Table 8.1) was extracted by removing a single leg, or multiple legs for smaller species, which were then placed in 100µl of 5% Chelex 100 (Biorad) suspended in 10µM Tris buffer, with 5 µl of Proteinase K (5µL/mL). This mixture was then incubated for 4 h at 56°C before boiling for 15 min at 99°C. The mixture was centrifuged at full speed for 25 min and 40 µl of the supernatant removed and frozen at -20°C. To screen samples for the presence of *Wolbachia* we used the primers *wsp\_f* and *wsp\_r* (Baldo et al. 2005) to amplify a 603-bp fragment of the *wsp* gene (Table 2). One individual from each species that screened positive for *wsp* was used for MLST sequencing using five housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) distributed across the

genome, following standard procedures (Baldo et al. 2005; [www.mlst.net](http://www.mlst.net)). Each *Wolbachia* strain can then be characterized by the allelic profile of the five alleles at the MLST loci, producing its sequence type. Additionally the primers LCO and HCO were used to amplify a 710-bp fragment of the *cytochrome oxidase 1* (*cox1*) mitochondrial gene, commonly used for DNA barcoding in many species (Folmer et al. 1994). This was used as a positive host control and to generate a host phylogeny. Primer details and PCR conditions are listed in Supplementary Table S8.1 p.289. PCR products were run on a 1% agarose gel and visualized under UV. Positive and negative samples were added with each complete run. Samples which positively amplified were purified with a QIAquick PCR Purification kit (QIAGEN), DNA concentrations checked with a NanoDrop 2000 (Thermo Sci.) and sequenced (Genbank: KT783613-KT783662). Samples were sequenced using both forward and reverse primers to ensure accuracy of the sequence results.

### 8.3.3 Phylogenetic analyses

Sequences were first aligned and trimmed using the CLUSTALW2 algorithm. *Wsp*, *CO1* and concatenated MLST phylogenies were produced using a Maximum Likelihood criterion. Maximum likelihood model selection analysis based BIC scores suggested the use of a GTR+I model for *wsp* data and GTR+G+I for the MLST and *CO1* data. The robustness of the inferred ML tree topology was assessed by performing 10000 bootstrap replicates in in MEGA6 (Tamura et al. 2013). Bayesian analyses were performed using MrBayes v3.2.5 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) using a Markov chain

Monte Carlo analysis using a GTR+G model with Inverse gamma rates, performed for 1,000,000 generations with a sampling frequency of 100 generations and a 25% burn in. All other settings were as default. Cophylogenetic trees were converted using FigTree v1.4.2 (Rambaut and Drummond 2012) and produced using TreeMap3. Coclادogenesis between host and parasite phylogenies was tested using tree reconciliation in TreeMap 3. The probability of obtaining the observed number of cospeciation events was then calculated by 1000 Markov randomizations of parasite trees to test if reconciled host and parasite trees demonstrate significantly higher levels of speciation events than chance (Page 1998; Page and Charleston 1998).

## 8.4 Results

### 8.4.1 Infection status

Of the 80 ant species collected, DNA was successfully extracted, and CO1 fragment amplified from at least 4 replicate samples in 73 species (Table 8.1). Of these, the amplified *CO1* fragment was successfully sequenced from 51 species. In total 19 out of 73 (26%) species had at least one individual test positive for *Wolbachia* infection. No species from the subfamilies Ectatomminae, Pseudomyrmicinae, Ecitoninae, Dorylinae, Ponerinae or Paraponerinae (total 28 species), screened positive for *Wolbachia*. In contrast, 10 of 29 (34%) myrmicine species, and 6 of 10 (60%) of dolichoderine species screened positive. All of the *Azteca* and *Crematogaster* species screened had very high proportions of workers infected. However there were also genera with mixed infection statuses, for example *Camponotus* and *Pheidole* in which only a single species was found to be

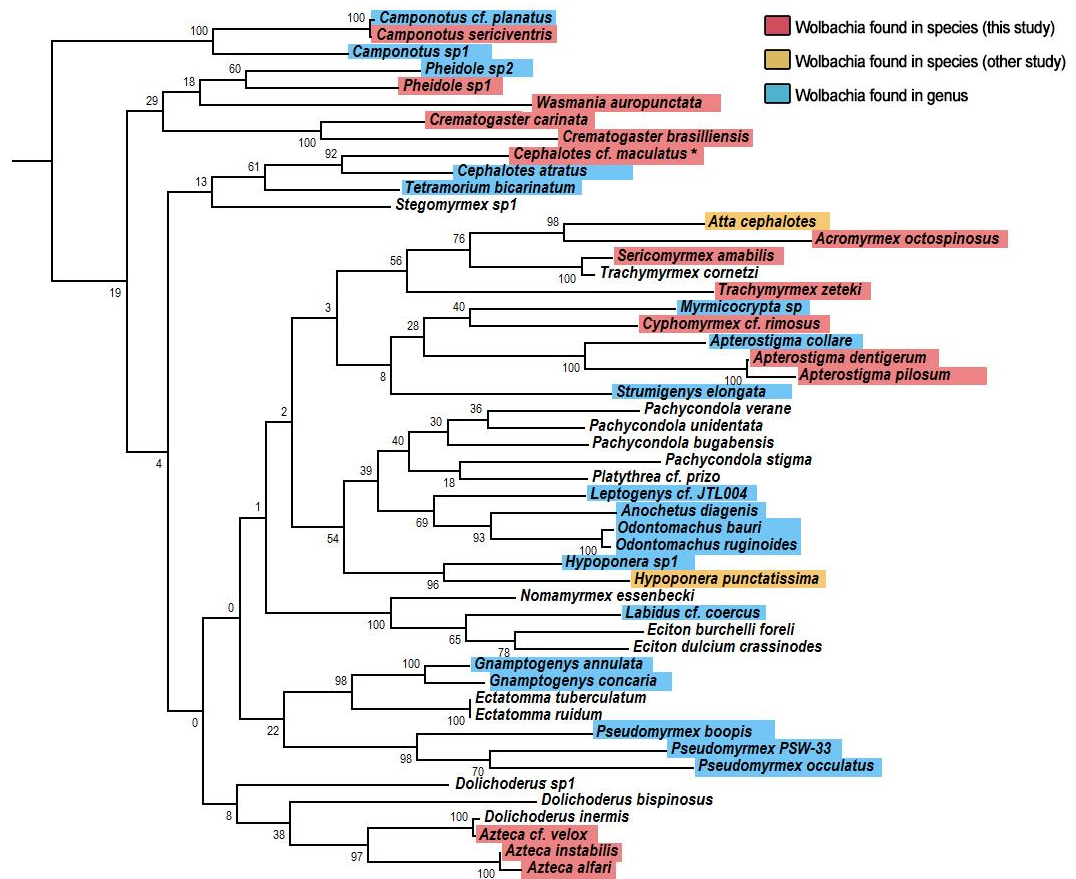




infected in each case. Samples from single colonies of *Cyphomyrmex rimosus*, *Crematogaster brasiliensis*, *Crematogaster limata*, *Solenopsis* sp2, and *Azteca trigona*, showed evidence of multiple *Wolbachia* infections based on the sequencing traces. Where possible multiple colonies were screened to ascertain the distribution of infection within a species. *Wsp* samples were only successfully sequenced from multiple colonies in *Crematogaster carinata* and *Azteca trigona*, in both cases the colonies possessed the same *wsp* sequence type. There was a wide range of species infection intensities, from ~100% in *Azteca spp.* to 7% in *Camponotus sericeiventris*, but broadly similar infection intensities between colonies where species had multiple samples collected

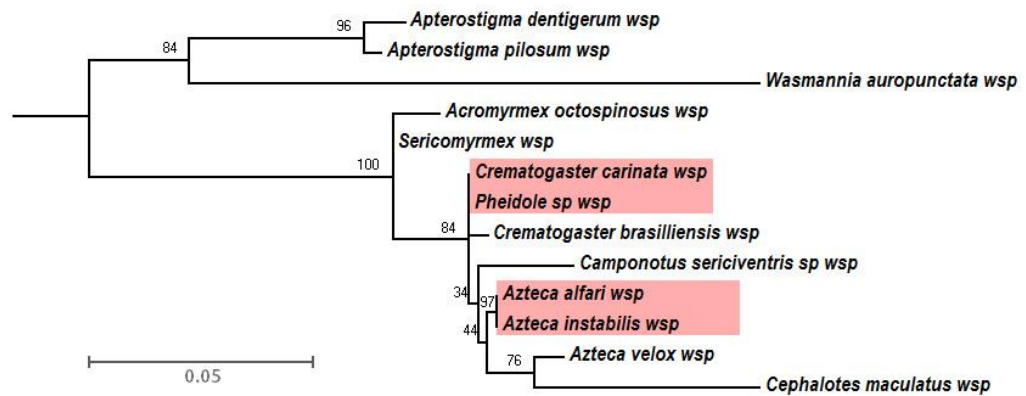
#### 8.4.2 Phylogenetics and MLST profiling

The overall host tree produced from *COI* sequences (648 bp; Figure 8.1) mapped to the *Wolbachia* parasite *wsp* and MLST phylogenies (Figures 8.2 and 8.3). ML and Bayesian analyses produced *wsp* and *COI* topologies that were not significantly different from one-another, and only ML results are shown (Figure 8.2). For the concatenated MLST sequences there was less consistency in topologies produced between the two analyses (Figure 8.3). Generally bootstrap values for early branching patterns were low, which suggests some of the more broad phylogenetic patterns may be poorly resolved, but the comparisons at terminal nodes are largely well defined.

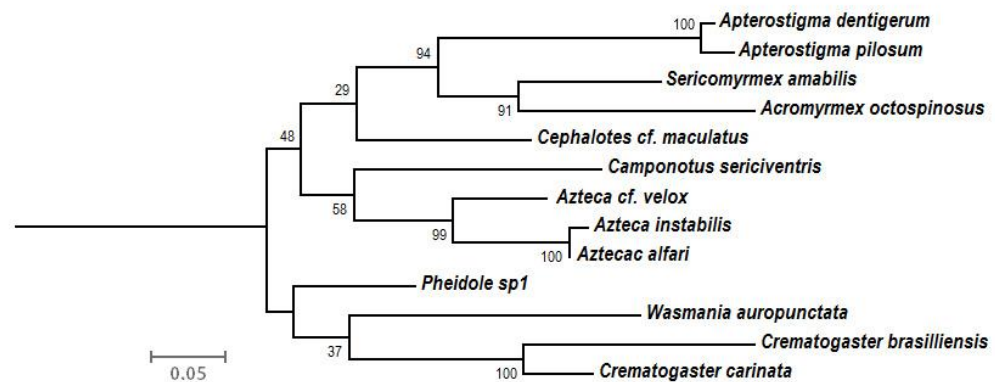


**Figure 8.1.** Phylogeny based on COI sequences of 51 species from a tropical ant assemblage that were screened for infection by the *Wolbachia* symbiont. Maximum likelihood bootstrap 1000 values are shown at each node. Host species are labelled based on the presence of *Wolbachia* after *wsp* screening. Red: *Wolbachia* found in this species from this study. Yellow: *Wolbachia* found in this species from previous studies. Blue: *Wolbachia* found in ants from this genus in previous studies.

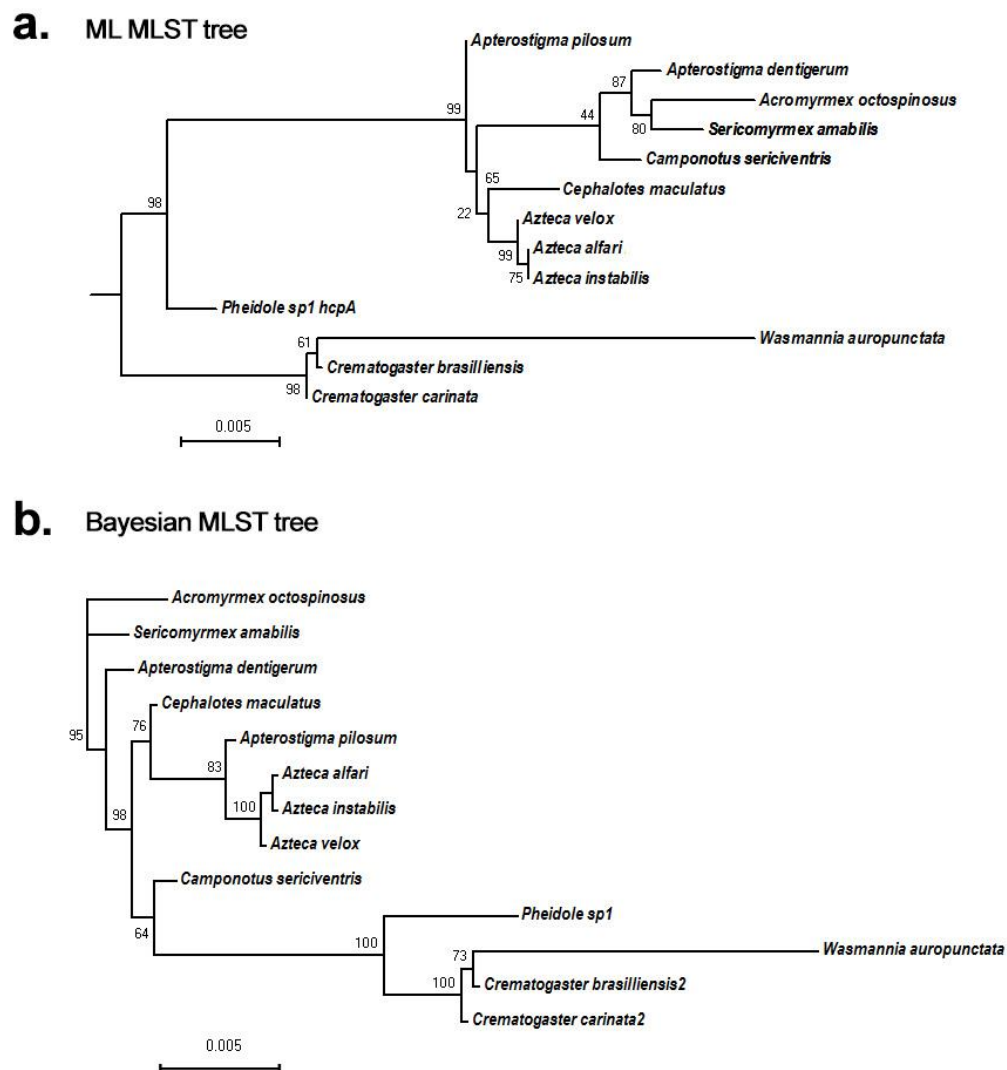
**a.** *Wolbachia* wsp tree



**b.** Host+ CO1 tree

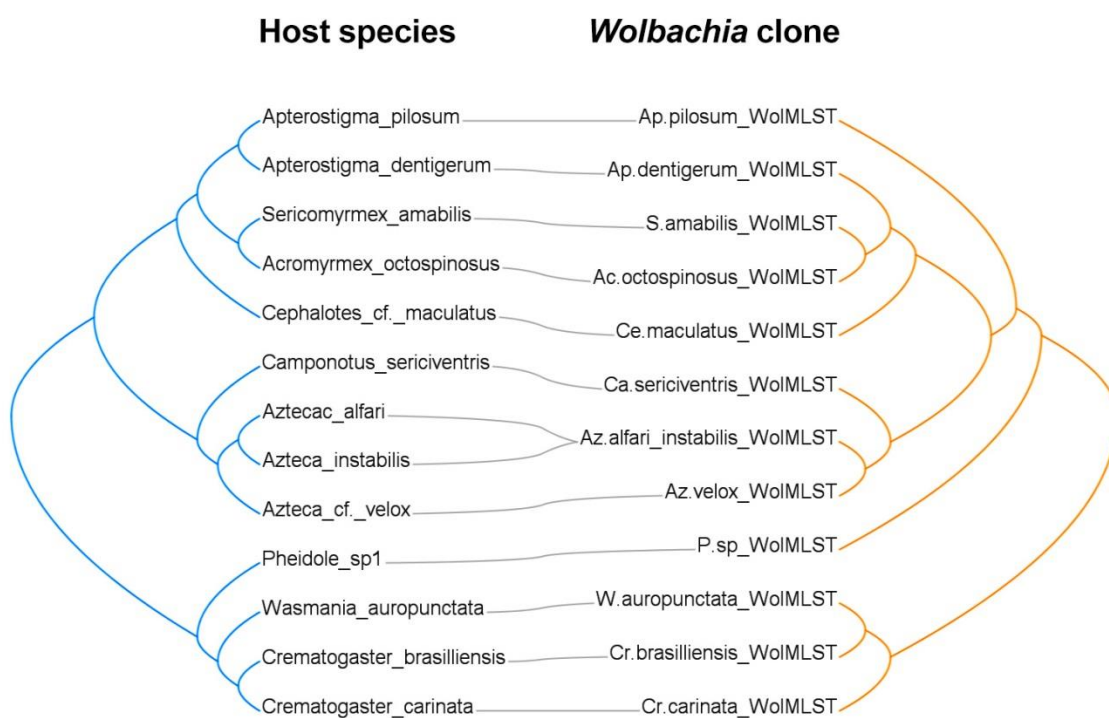


**Figure 8.2.** Host and *Wolbachia* phylogenies. a) Maximum likelihood phylogeny of 13 *Wolbachia* strains found in the tropical ant assemblage studied based on wsp sequences, and b) a maximum likelihood phylogeny based on CO1 sequences of the 13 host ant species of these *Wolbachia* strains. Host name are given at terminal nodes, and bootstrap 10000 iterations values are given at each node. Red highlight indicates identical sequences.



**Figure 8.3.** MLST phylogenies. Maximum likelihood a) and Bayesian b) phylogenies of 13 *Wolbachia* strains found in the tropical ant assemblage studied, generated from concatenated MLST sequences. Host name is given at terminal nodes and bootstrap 10000 iterations (ML) and clade credibility (Bayesian) values are given at each node.

Comparing host and *wsp* *Wolbachia* trees showed significant evidence for cocoladogenesis, with the observed number of speciation events extremely unlikely to have occurred by chance, but still different from if the parasite had undergone complete cocoladogenesis ( $p = 0.030$ , 95% CI [0.019, 0.041]; 19 codivergence, 6 duplication, 11 loss, 0 host switch, 16 non-codivergence, events; Figure 8.4). For example the *Apterostigma*, and *Crematogaster* species share highly similar *Wolbachia* strains with other species of their genus, and the *Camponotus*-*Azteca* clade shows complete matching.



**Figure 8.4.** Tanglegram. The concatenated MLST phylogeny of *Wolbachia* strains (right) mapped to the phylogeny of their host ants (left; based on *COI* sequences). Mappings are untangled to minimise line intersections.

Sequencing of MLST gene fragments produced many alleles not found in the online MLST database and were therefore assigned unique identifiers for the purposes of this study (Table 8.2). One *Wolbachia* sample, from *Apterostigma pilosum*, did produce a match for each of the 5 alleles, however the overall allelic strain profile did not match that of any other *Wolbachia* sample published. *A. cf. alfari* and *A. instabilis* had identical strain profiles. *A. velox* matched for 4/5 alleles (1 bp difference) the other *Azteca* species, and the strain profiles for the two *Crematogaster* species matched for 4/5 alleles.

**Table 8.2.** MLST allele profiles of *Wolbachia* symbiont strains identified in 13 host species from a tropical ant assemblage

Ant species	gatB	coxA	hcpA	ftsZ	fbpA
<i>Apterostigma dentigerum</i>	CT5	20	32	154	CT1
<i>Apterostigma pilosum</i>	75	20	32	154	46
<i>Acromyrmex octospinosus</i>	CT1	20	32	CT1	CT4
<i>Sericomyrmex amabilis</i>	3	20	CT2	154	CT2
<i>Cephalotes c.f. maculatus</i>	CT6	20	207	154	46
<i>Crematogaster brasiliensis</i>	19	CT2	CT4	CT2	CT3
<i>Crematogaster carinata</i>	19	CT3	CT4	CT2	CT3
<i>Wasmannia auropunctata</i>	CT2	CT1	210	CT2	CT5
<i>Pheidole sp 1</i>	CT3	CT3	CT5	154	380
<i>Camponotus sericeiventris</i>	CT4	CT5	CT3	46	46
<i>Azteca c.f. alfari</i> <sup>a</sup>	75	CT4	CT1	46	46
<i>Azteca instabilis</i> <sup>a</sup>	75	CT4	CT1	46	46
<i>Azteca c.f. velox</i>	75	CT4	207	46	46

## 8.5 Discussion

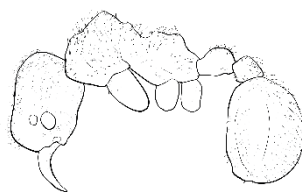
We found that 26% of the 73 host species sampled from a tropical ant assemblage were infected with the reproductive parasite *Wolbachia*, with substantial intraspecific and interspecific variation in infection prevalence. Unique MLST sequence types were found from all hosts where the complete set of genes were sequenced, except for *Azteca* c.f. *alfari* and *Azteca instabilis*, which shows that even closely related species can harbour different *Wolbachia* strains. However, the similarity of *Wolbachia* and host phylogenies, suggests that although different, *Wolbachia* strains from related hosts are themselves also generally related.

This study provides the first wide-scale evidence cocladogenesis between *Wolbachia* and its hosts in social insects. However, this cocladogenesis was not complete, which overall supports the current model of *Wolbachia* being predominantly vertically transmitted in social insects but with occasional horizontal transmission events (Baldo et al. 2008; Frost et al. 2010). Studies on *Wolbachia* in ants have found infection rates of 50% (Wenseleers et al. 1998), 29% (Russell et al. 2009) , 29% (Russell et al. 2012), 22% (Kautz et al. 2013) and 26% (this study). The prevalence in ants is broadly in line with infection frequencies found in termites (27%; (Lo and Evans 2007), but lower than bees and wasps (54% and 66%; (Gerth et al. 2011), and insects more generally (ca. 40% (Russell et al. 2012; Martinez et al. 2014). Across the ant subfamilies there seemed to be strong evidence for infection heterogeneity, with the majority of *Wolbachia*-infected ants being from the Myrmicinae and Dolichoderinae. In the dolichoderines, we only screened samples from two genera, but there was a



distinct difference, with *Azteca* being nearly uniformly infected and *Dolichoderus* samples being uninfected. We found no *Wolbachia* in ponerines and only one of the formicine species screened was infected. The *Wolbachia* strain from *Wasmannia auropunctata* was the most highly divergent from the other strains. Although found natively in Panama, this species is a widespread invasive species that can reproduce both clonally and sexually. Clonal populations of this species have been found to harbour very specific strains of *Wolbachia* which have been repeatedly inherited through exclusively vertical clonal reproduction, which may explain the significant divergence from other strains (Rey et al. 2013)

The results provide evidence for cocladogenesis between *Wolbachia* and its hosts. Additionally we found a considerable diversity in *Wolbachia* strains between even closely related species, and show that infection status can be highly heterogeneous between taxa. The identification of multiple unique strains in our study suggests the possibility that as more ants are screened for *Wolbachia*, and as we continue to build knowledge of the host's natural history, it may be possible for trait-mapping to be used to see how *Wolbachia* infection is influenced by the life-history of lineages over and above phylogenetic or geographic transmission patterns. This will allow us to build a more complete picture of the dynamics of reproductive parasites such as *Wolbachia* in social insects.



*Sericomymex amabilis*

# 9. General Discussion

Work on the evolutionary ecology of infectious disease has received increasing interest as host-parasite interactions continue to demonstrate important insights into key biological phenomena, including the evolution of sexual reproduction, the maintenance of genetic diversity, and the sculpting of community structure (Hamilton et al. 1990; Prenter et al. 2004; Poulin 2007; Schmid-Hempel 2011). This is built on a fundamental framework underlying the evolutionary theories for the evolution of parasite emergence, virulence, and their consequent effects on host biology and populations (Frank 1996; Schmid-Hempel 1998; Poulin 2007; Watson 2013). Identification and quantification of host defences and their role in combating disease is an important part of this process. In this thesis I show that ants possess a variety of defence mechanisms to protect themselves against the threat of parasites, and demonstrate how investment into these important defences can vary between individuals and species, and may depend on context, type of parasite, and life-history of the host.

Current understanding of host-parasite relationships now includes a conceptual division between direct reduction of parasite loads, termed parasite resistance, and a reduction in the cost of a given parasite load, termed parasite tolerance (Miller et al. 2005; Chambers and Schneider 2012; Ayres and Schneider 2012; de Roode and Lefèvre 2012). Resistance and tolerance are two alternative but complementary host defense strategies, with host fitness representing the result of these two components, and thus hosts that are good at parasite resistance may still show considerable fitness costs if they are nonetheless poor at tolerating

disease (Chambers and Schneider 2012). There are key difference between these components from an evolutionary ecology perspective, with resistance protecting the host at the expense of the parasite, while tolerance reduces the cost to the host without direct negative effects on the parasite (Miller et al. 2005). Thus resistance and tolerance may result in different evolutionary outcomes for both host and parasite. Increased host resistance should be observed to reduce parasite prevalence in host populations, and may strongly drive antagonistic host-parasite coevolution (Miller et al. 2005; Poulin 2007). This is unlike tolerance, which may have little effect on parasite populations, and limited selection to overcome tolerance defences in the parasite (Miller et al. 2005). In this work we have tended not to make this explicit distinction, rather relating to the two collectively as a combined defence. This is partly because successful infections of the entomopathogenic fungi *Metarhizium* has very high rates of mortality and hence resistance defences will be the most important (St Leger et al. 1991; Boomsma et al. 2005). However, mechanisms such as grooming, which I measured extensively in this thesis, is a clear example of behavioural resistance which serves to physically reduce parasite loads, and to alter with exposure to *Metarhizium*. Internal measures of immunity may be more related to how well ants can withstand infection levels, and may represent parasite tolerance (Siva-Jothy et al. 2005). Some entomopathogenic fungi, however, can show surprisingly few effects on hosts. Many Laboulbeniales fungi, for example, are effective at penetrating the insect cuticle and can reproduce to reach very high abundances inside the haemocoel without immediately killing the host, so in these species tolerance effects may be more important and more easily studied (Csata et al. 2013, 2014; Báthori et al. 2015). Because these components of defence can potentially result in very different outcomes for host-parasite dynamics, acknowledging the relative

importance of these types of defence is highly important, and future studies may be able to measure fitness costs given changing infection intensities in order to explore both these effects (Miller et al. 2005; de Roode and Lefèvre 2012).

Where species suffer from similar parasite pressures, organisms may show alternative strategies to defend themselves (Minchella 1985; Restif and Koella 2004; Boomsma et al. 2005; Schmid-Hempel 2011). Work from this thesis has shown this can be the case in ants. For example *O. smaragdina* weaver ants appears to rely primarily on its venom while *Polyrhachis* weaver ants use higher rates of selfgrooming, but both fill similar ecological niches within the same habitats, and likely encounter similar parasite pressures (Chapter 4). This demonstrates the considerable complexity of disease resistance traits and highlights the value of targeted, fine-scale comparative studies to identify and understand how disease resistance mechanisms are employed in different species. Where host life-history has led to potentially reduced parasite pressures, hosts are predicted to invest less into defence mechanisms (Bowers et al. 1994; Boomsma et al. 2005; Watson 2013). I provide evidence of this in Chapter 7, showing that socially parasitic species, which rely on their hosts for defensive duties, have significantly reduced behavioural, chemical and physiological immunity. Similarly the variation in investment in metapleural gland defence in attines in Chapter 5, and the use of alternative defences in weaver ant species in Chapter 4, suggest that life-history may have significant effects on investment into disease resistance.

The interspecific differences in disease resistance not only suggest significant costs from the different disease defences, but also demonstrate considerable plasticity in these traits, which can be up-regulated or reduced on both evolutionary and generational timescales, and in response to short-term threat. In slavemaker social parasites, it appears that behavioural defences were first to be lost, but there was no evidence of explicit trade-offs between mechanisms, as was the case in the weaver ant system (Chapters 4 and 5). Rather social parasites showed reduced immunity in all measures I examined. Demonstrations of trade-off may be not only important in understanding the specific mechanisms of host defence, but may also be able to explain the maintenance of genetic diversity in hosts (Bowers et al. 1994; Michod 2006). The costs of immunity have the potential to reduce the survival or fecundity of resistant hosts, and if parasite pressures are low, the cost of immunity may even outweigh the cost from parasitism (Gwynn et al. 2005). Thus the relative cost and benefit for possession of disease resistance traits is expected to fluctuate with parasite pressure, and natural selection will maintain variation in susceptibility alleles (Morand et al. 2015). Theoretical and empirical work suggests that host genetic diversity may promote disease resistance, however in order to fully demonstrate this link we need to better understand the genetic basis for resistance mechanisms themselves (Hughes and Boomsma 2004b, 2006; Calleri et al. 2006; Reber et al. 2008; King and Lively 2012). In the future we may be able to identify the genetic components that underlie important defence mechanisms, and then show that polymorphic systems are more resistant to diseases (Viljakainen 2015).

Most studies of host-parasite dynamics stem from classical models of two-way interactions, but abiotic and biotic external factors can have dramatic effects on

these systems (Poulin and Morand 2000; Miller et al. 2005; Watson 2013; Morand et al. 2015). With multiple interacting partners it makes measuring host fitness and parasite virulence challenging in the field, but it is vital to fully understanding the coevolution of host parasite interactions. In this thesis we predominantly used the parasite *Metarhizium* as an experimental parasite. This entomopathogenic fungi is a useful model parasite which can infect many insect species and can have significant fitness costs to hosts (Milner et al. 1998; Leger et al. 1999; Keller et al. 2003; Santos et al. 2007; Santi et al. 2010; Rännbäck et al. 2015). We found responses to this entomopathogen were generally stronger than to those of an opportunistic, facultative parasite, such as *Aspergillus* (Chapters 2 and 3), which is normally saprophytic, but can opportunistically infect insects, including ants, when the exposure level is very high or when the ants are immunocompromised (Glare et al. 1996; Scully and Bidochka 2005; Graystock and Hughes 2011). However, there may often be interaction between different parasites during exposure from multiple threats. Ants can show an increased fitness cost from exposure to an opportunistic parasite when they are additionally infected with *Metarhizium* (Hughes and Boomsma 2004a; Graystock and Hughes 2011). This can even result in the opportunistic facultative pathogen outcompeting the entomopathogenic parasite once the host defences have been weakened. Similarly, in Chapter 3, I found that the larvae of ants, that possess reduced immunity to disease are highly vulnerable to the opportunistic parasite *Aspergillus flavus* (Tragust, Ugelvig, et al. 2013). The defence of brood against opportunistic, facultative parasites, may therefore be just as important as against obligate, entomopathogenic parasites (Graystock and Hughes 2011). However, it is important to note that the parasite strains used in this thesis were all novel to the hosts used, and *Metarhizium* is also not host-specific, so the parasites are

unlikely to have undergone species-specific co-evolution that could have affected the independency and interpretation of the results. Nonetheless future studies explicitly investigating the effect of direct coevolution between host and more highly specialised parasites such as *Escovopsis* or *Ophiocordyceps* will be valuable for determining how specific defences may be employed against specialised parasites, and how this may differ from more general parasites (Frank 1993; Gerardo et al. 2006; Agosta et al. 2010; Evans et al. 2011a; Kobmoo and Mongkolsamrit 2012; Ruiz-González and Bryden 2012). The fact that organisms are generally exposed to a multitude of different parasites means that further studies looking at threats from other parasitic organisms such as bacterial and viruses, including mixed infections, will be vital for building a complete picture of the defence capabilities of social insects (Schmid-Hempel 1998; Boomsma et al. 2005). This will also allow us to compare how species may invest differently based on the varied and multiple parasitic threats they are exposed to, and to identify which defence mechanisms are specific and which are general. Thus we may be able to identify the extent to which natural selection may favour specificity in resistance, and how this may affect, or be affected by, the genetic diversity of hosts.

Additionally host environmental conditions will vary geographically and it is likely that individuals of the same species will experience changes in parasite pressure depending on the precise conditions they inhabit at the time (Morand et al. 2015). Host populations may then be viewed as inhabiting an environmental mosaic with varying communities and conditions (Hoberg and Brooks 2008; Wolinska and King 2009). This heterogeneity will affect the maintenance of genetic variation in host resistance and the spread of parasites between

populations, and will be increasingly important to understand in the context of climate change (Lively 1999; Brooks and Hoberg 2007; Hoberg and Brooks 2008; King and Lively 2012).

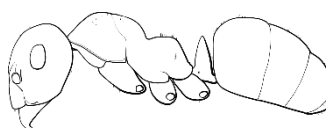
This work may prove useful for future disease and pest management strategies, both helping to control social insect species themselves, but also harnessing their own defenses. Many social insect species, especially ant and termites are economically significant pest, can be highly invasive, and consequently there is heavy focus on management schemes (Pimentel et al. 2001, 2005; Hill 2012; Rust and Su 2012; Evans et al. 2013; Lewis et al. 2014). There has been much interest in the use of pathogenic fungi to control insect populations (Butt et al. 2001; Lacey et al. 2001; Shah and Pell 2003; Chouvenc et al. 2012; Wang and Feng 2014). My work indicates that pathogenic fungi, especially specialized entomopathogens such as *Metarhizium*, represent a ubiquitous and potentially virulent threat to social insects. However, this threat is often not realised under normal circumstances because they have evolved highly effective defences and the generalist nature of *Metarhizium* means that it hasn't coevolved with specific hosts in order to overcome those defences (Frank 1996). These key defences include behavioural grooming and the production of antimicrobial secretions. This suggests that in order for biological control with fungal parasites to be most effective, it could be used in conjunction with methods which disrupt the natural behaviours of ants, perhaps through pheromones or insecticides, or reduce the antiseptic power of antimicrobial secretions (Shorey et al. 1992; Suckling et al. 2014). Conversely, the recent decline in honeybee populations, which is thought to be in part mediated by parasites, means that studies of social insect disease defence mechanisms is vitally important for understanding how we can best



conserve beneficial species (Allen-Wardell et al. 1998; Potts et al. 2010; Nazzi et al. 2012). For example, identification of the importance of the insects own defences has resulted in the selective breeding of genetic lines of ‘hygienic colonies’ which are more resistant to disease (Spivak and Reuter 1998; Harpur et al. 2014). Additionally, there is a wealth of unexplored avenues for therapeutic and commercial harnessing of social insect antimicrobial defences. For example, many novel antimicrobial peptides have been recently identified and isolated from social insects, and such substances are increasingly being recognized having powerful therapeutic value for human health (Bulet et al. 1999; Zasloff 2002; Reddy et al. 2004; Bulmer et al. 2009; Romanelli et al. 2011). On a wider scale, understanding how organisms are affected by, and respond to, different parasite pressures will be highly useful in predicting and mitigating the spread populations (Prenter et al. 2004).

It has often been suggested that the threat of parasite may in fact be an important driver of the evolution of sociality, because of the benefits that group living can confer in terms of resistance (Møller et al. 1993). However, whether the defence mechanisms we see in social insects are drivers of sociality, or necessary results of group living remains unclear. The comparative work in this thesis provides evidence that there may be a positive correlation between sociality and the degree of investment into disease resistance. Fungus growing ant species with larger and more complex colonies appear to invest more heavily into disease defence than the more basal species with smaller colonies (Chapter 5), and a derived leaf-cutting species also shows significantly higher levels of social immunity and hygienic personalities compared to dinosaur ants with simple societies (Chapter 6). This

may be because larger, more complex societies have more energy in which to invest into parasite defence, or because larger colonies are fundamentally more at risk from parasites and a greater investment in resistance is thus necessary (Schmid-Hempel 1998; Rifkin et al. 2012). Tracing the evolutionary history of these traits may be valuable in order to disentangle these hypotheses. For example, in socially parasitic species, these defences appear to have been secondarily reduced compared to closely related hosts and outgroup species (Sumner et al. 2003; Chapter 7). This shows that investment into disease resistance may also be reversed if selection pressures are relaxed, and implies there is a considerable cost to these defences. By using investment into disease resistance as a measure of parasite pressure, it will be possible to broadly explore how the threat of disease can shape the life history of species, including both spatial and evolutionary distributions. Thus future work demonstrating the explicit costs of individual components of disease resistance will be important for developing our understanding of how changes in parasite pressures can influence host biology and how organisms can survive in a world abundant with parasites.



*Formica rufa*

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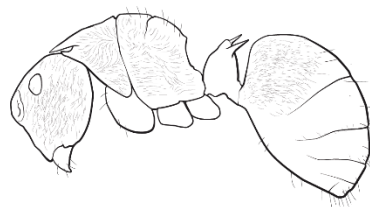
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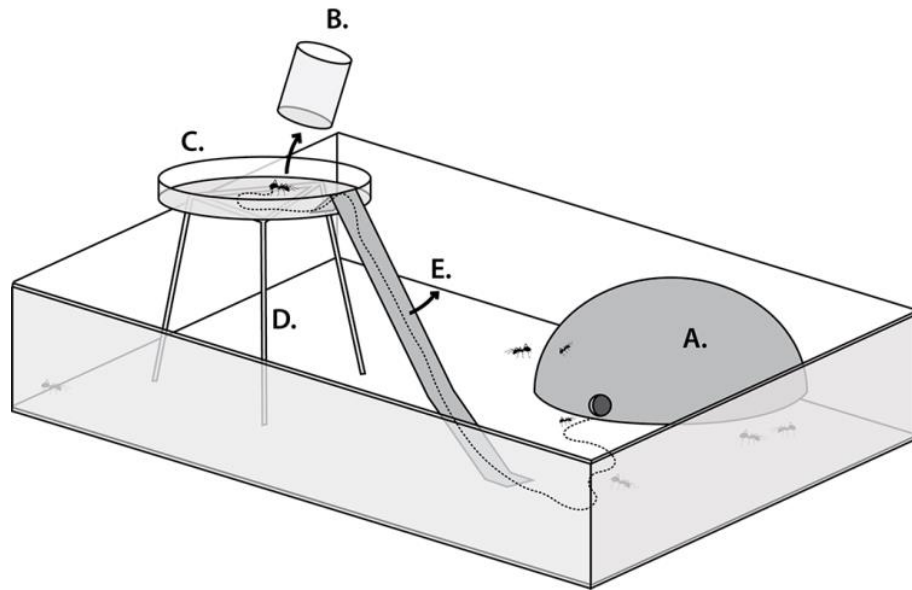
*Crematogaster scutellaris*

# Supplementary Figures

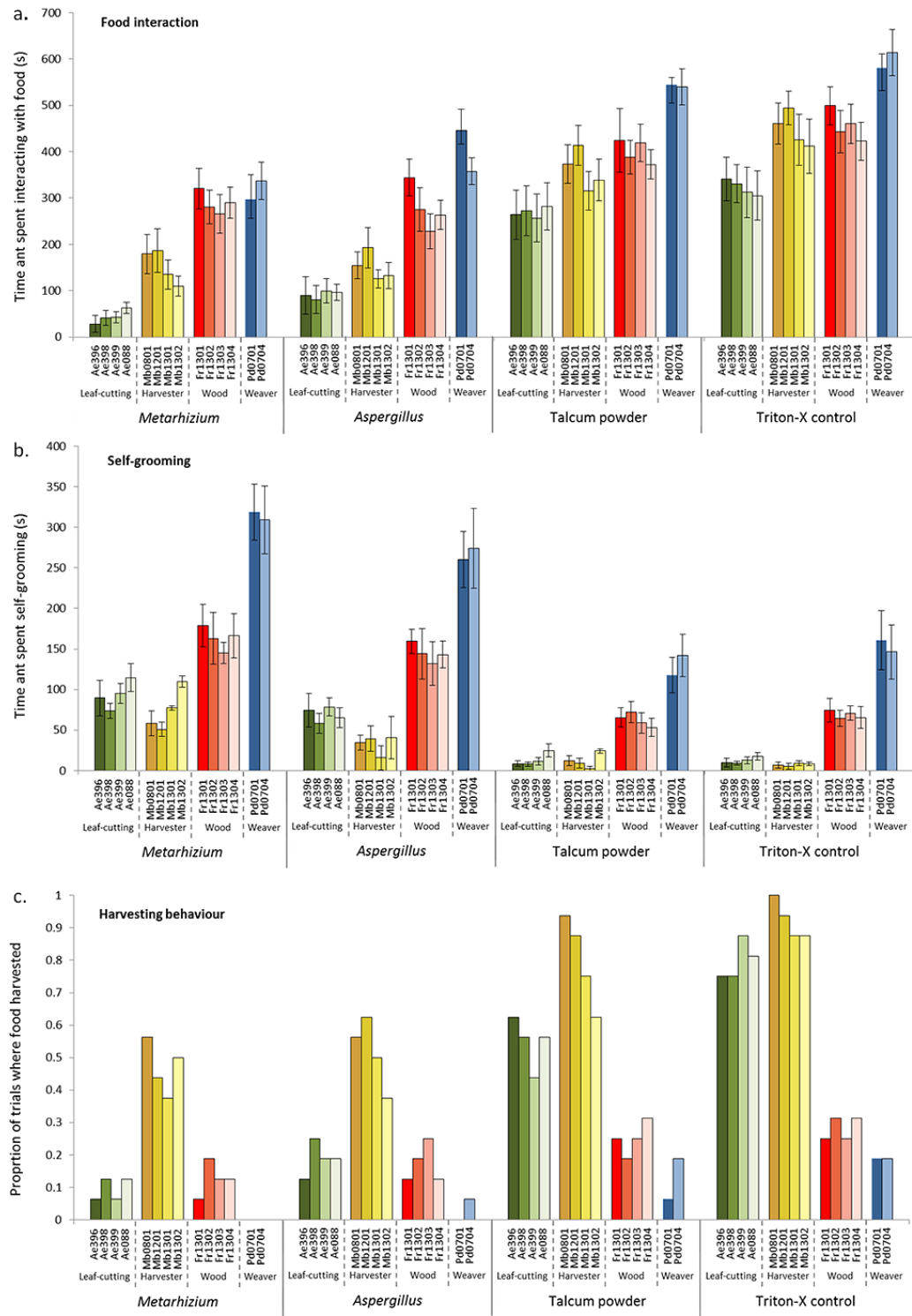
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*Polyrhachis foreli*

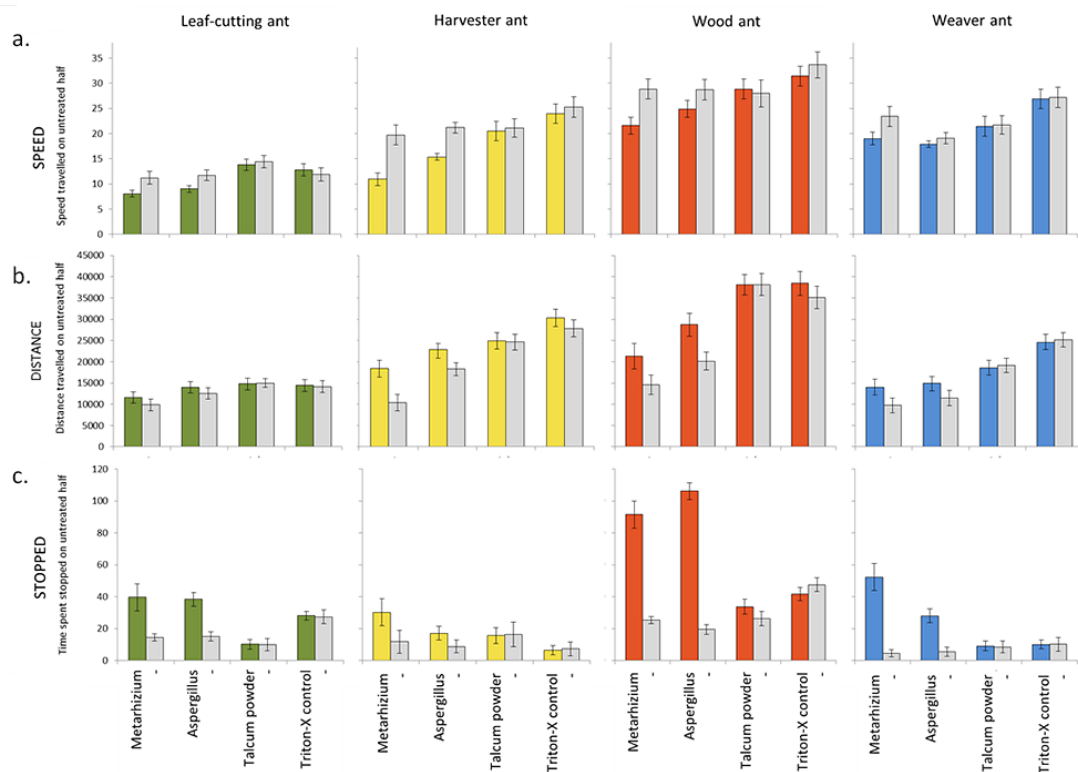


**Figure S2.1.** The experiment was performed on individual ants located, but isolated, within their own normal foraging arena to try and ensure most natural behaviours. Ants from the main nest (A) were able to access the Fluon-lined Petri dish (C) via a removable bridge (E). The Petri dish was kept isolated by being suspended on a Fluon coated tripod (D). When an ant entered the petri dish and the trial was to begin it was constrained initially using a plastic pot (B) to remove bias and help further acclimatise the ant to the trial.

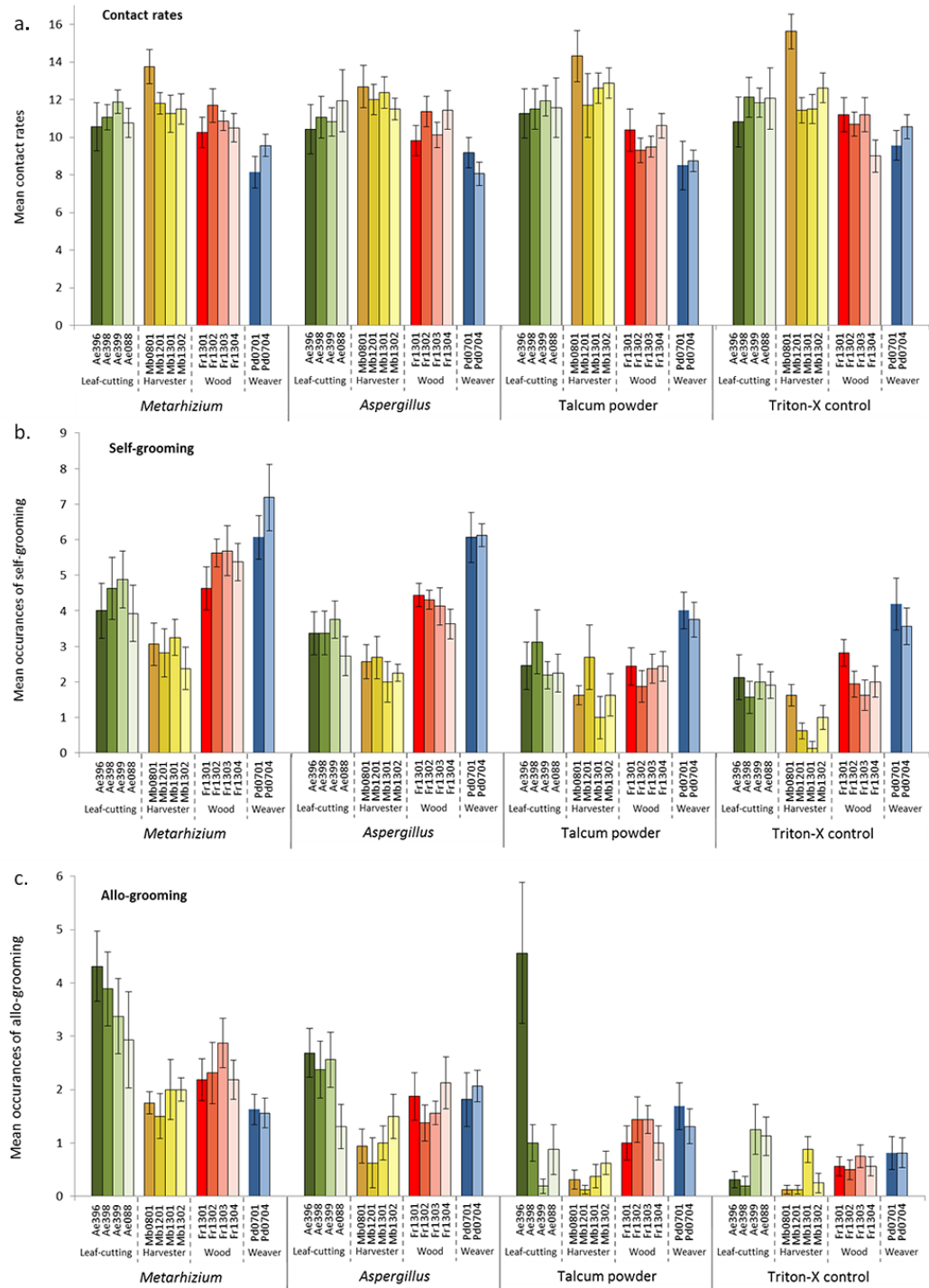


**Figure S2.2.** The (a) mean  $\pm$  s.e. time spent interacting with food, (b) mean  $\pm$  s.e. time spent self-grooming, (c) and proportion of food harvested in Experiment 1, by colonies of leaf-cutting ants, harvester ants, wood ants, and weaver ants when food had been treated with either the *Metarhizium* (Met.) or *Aspergillus* (Asp.) fungal pathogens, talcum powder control (Talc.) or control solution (Ctrl.).

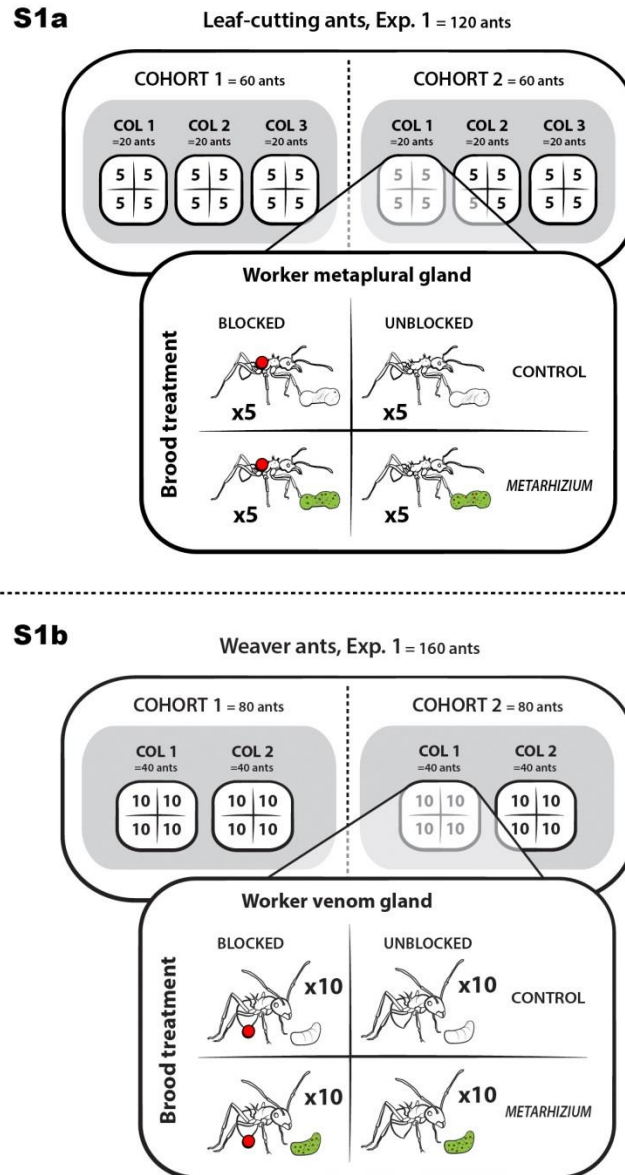




**Figure S2.3** The (a) proportion of time, (b) mean  $\pm$  s.e. speed travelled, (c) mean  $\pm$  s.e. distance travelled and (d) mean  $\pm$  s.e. time spent inactive in Experiment 2, on the *Metarhizium*, *Aspergillus*, talcum powder and Triton-X control treated side (coloured bars) compared to the Triton-X treated alternative treated side (grey bars).



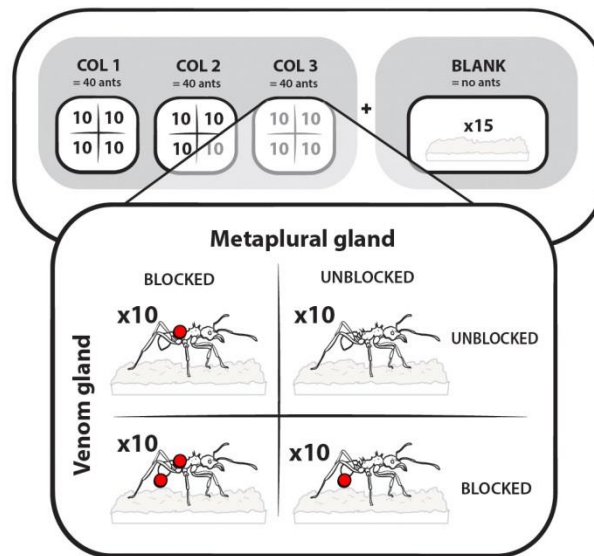
**Figure S2.4** The (a) mean  $\pm$  s.e. occurrence of contact and (b) self-grooming by the test ant, and of (c) allogrooming between treated and test ant (bottom) of leaf-cutting ants, for colonies of harvester ants, wood ants, and weaver ants in Experiment 3, to nestmates that had been treated with either the *Metarhizium* (*Met.*) or *Aspergillus* (*Asp.*) fungal pathogens, talcum powder control (Talc.) or control solution (Ctrl.).



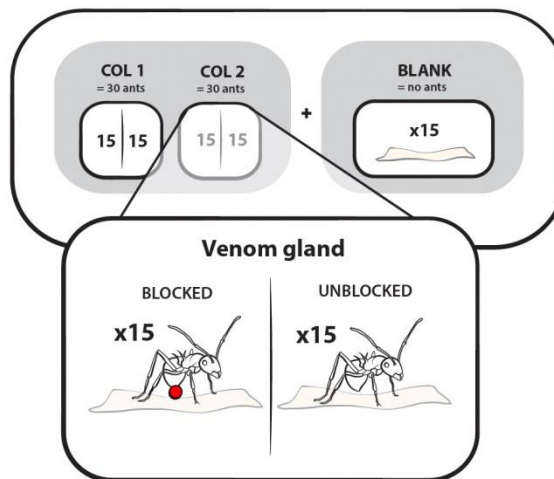
**Figure S3.1.** Details of treatment structure and subject assignment for **Experiment 1**. 120 leaf-cutting ants (a) were used in total, split between two equal cohorts. Each of the cohorts consisted of 20 ants from each of the three colonies, giving a total of 60 ants for each cohort. Within each cohort, ants from individual colonies were divided evenly into four treatment groups, consisting of five ants. This gave a total of 30 replicate ants per treatment across all colonies and cohorts. 160 weaver ants (b) were used in total, split between two equal cohorts. Each of the cohorts consisted of 40 ants from each of the two colonies, giving a total of 80 ants for each cohort. Within each cohort, ants from individual colonies were split evenly into four treatment groups, consisting of 10 ants. This gave a total of 40 replicate ants per treatment across all colonies and cohorts.

**S2a**

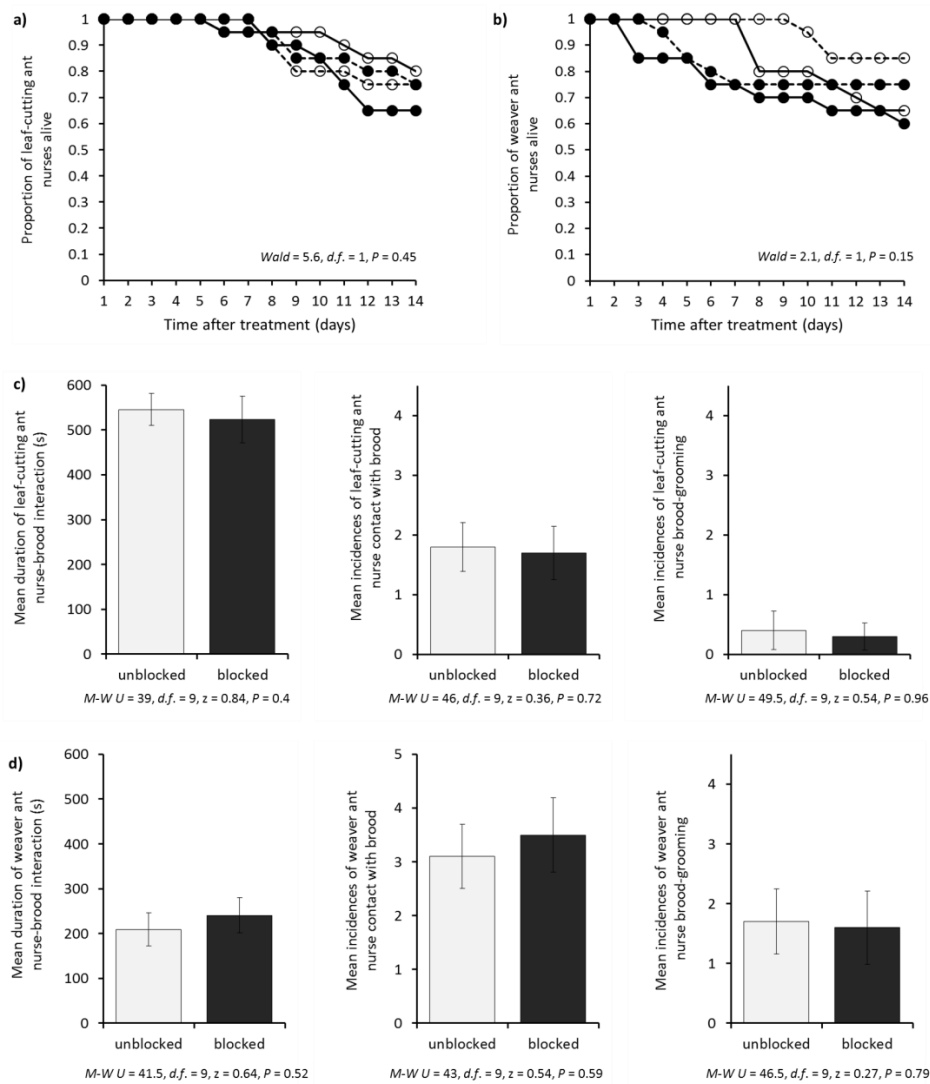
Leaf-cutting ants, Exp. 2 = 120 ants

**S2b**

Weaver ants, Exp. 2 = 60 ants



**Figure S3.2. Details of treatment structure and subject assignment for Experiment 2.** 120 leaf-cutting ants (a) were used in total, consisting of 40 ants from each of the three colonies. Ants from individual colonies were divided evenly into four treatment groups, consisting of 10 ants. This gave a total of 30 replicate ants per treatment across all colonies. Fifteen additional blank trials were conducted in the absence of any ant. 60 weaver ants (b) were used in total, consisting of 30 ants from each of the two colonies. Ants from individual colonies were divided evenly into two treatment groups, consisting of 15 ants each. This gave a total of 30 replicate ants per treatment across all colonies. Fifteen additional blank trials were conducted in the absence of any ant.



**Figure S3.3. Results of experiment comparing brood-care behaviour and survival of nurse ants with blocked and unblocked glands.** Both leaf-cutting ants (a;  $Wald=5.6, d.f.=1, p=0.45$ ) and weaver ants (b;  $Wald=2.1, d.f.=1, p=0.15$ ) showed no difference in survival of nurses with (open circles) or without (black circles) functional antimicrobial glands, whilst caring for brood treated with either *Metarhizium* parasite (solid lines) or control solution (dashed lines), over the course of the experiment. Additionally neither leaf-cutting ants (c) or weaver ants (d) showed any differences in the duration of time spent interacting with brood ( $U=39, d.f.=9, z=0.84, p=0.4$ , and  $U=41.5, d.f.=9, z=0.64, p=0.52$ , respectively), the incidences of contact with brood ( $U=46, d.f.=9, z=0.36, p=0.72$ , and  $U=43, d.f.=9, z=0.54, p=0.59$ , respectively), or incidences of brood-grooming ( $U=49.5, d.f.=9, z=0.54, p=0.96$ , and  $U=46.5, d.f.=9, z=0.27, p=0.79$ , respectively), between nurse ants with blocked (dark bars) and unblocked glands (light bars).

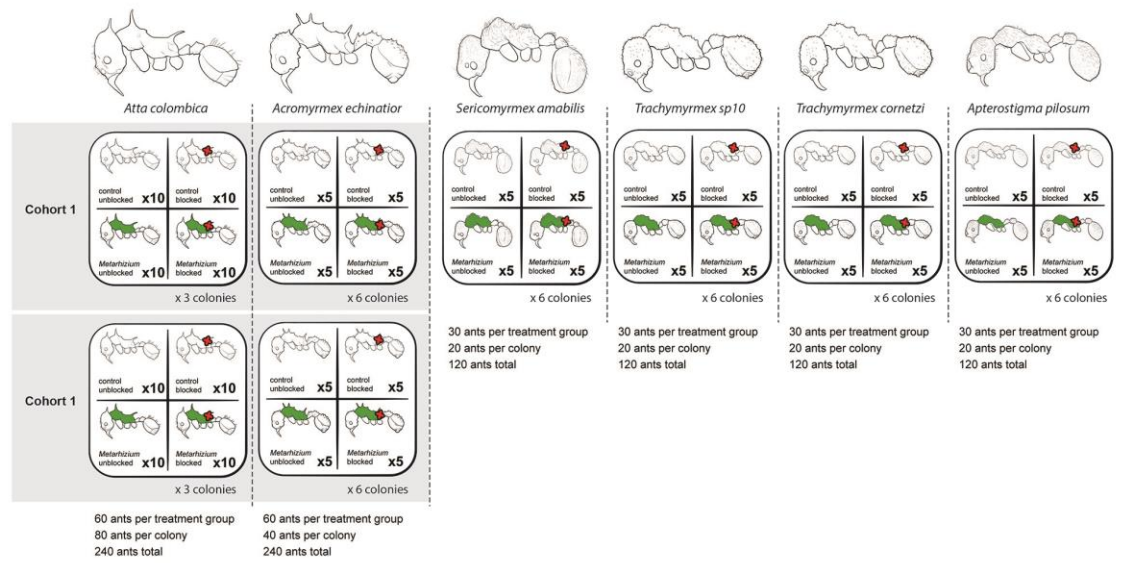
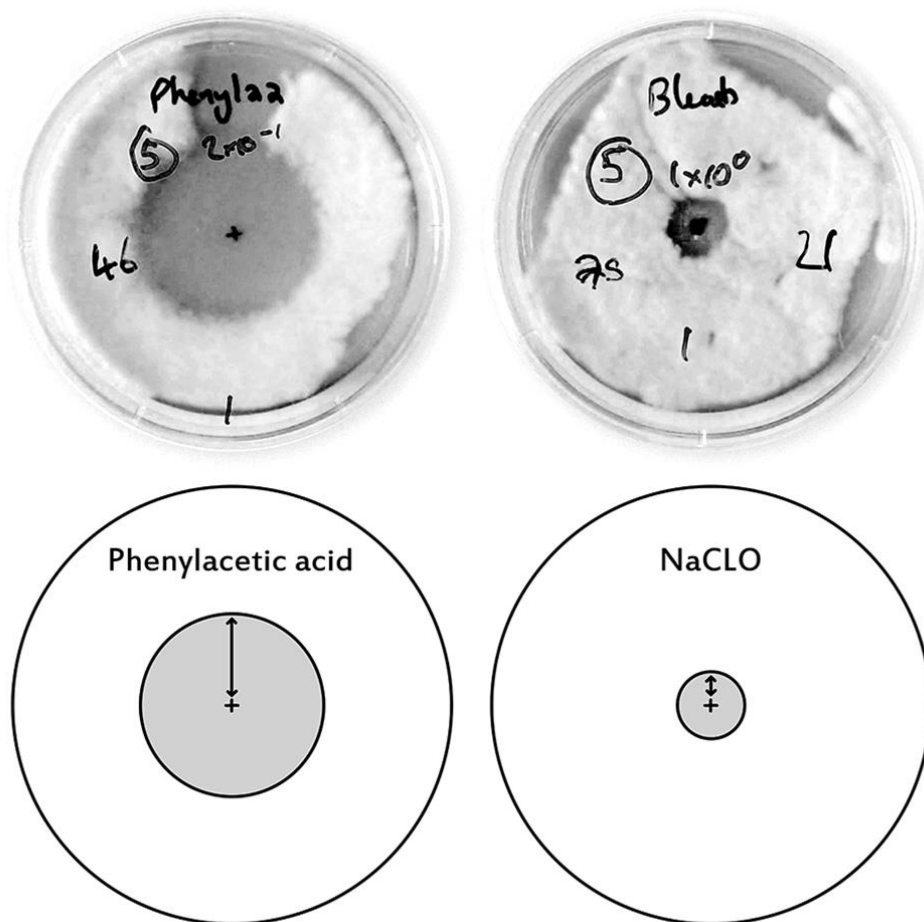
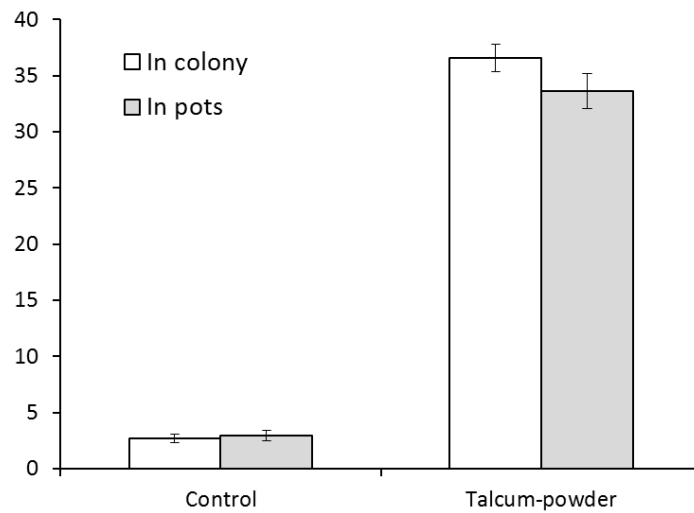


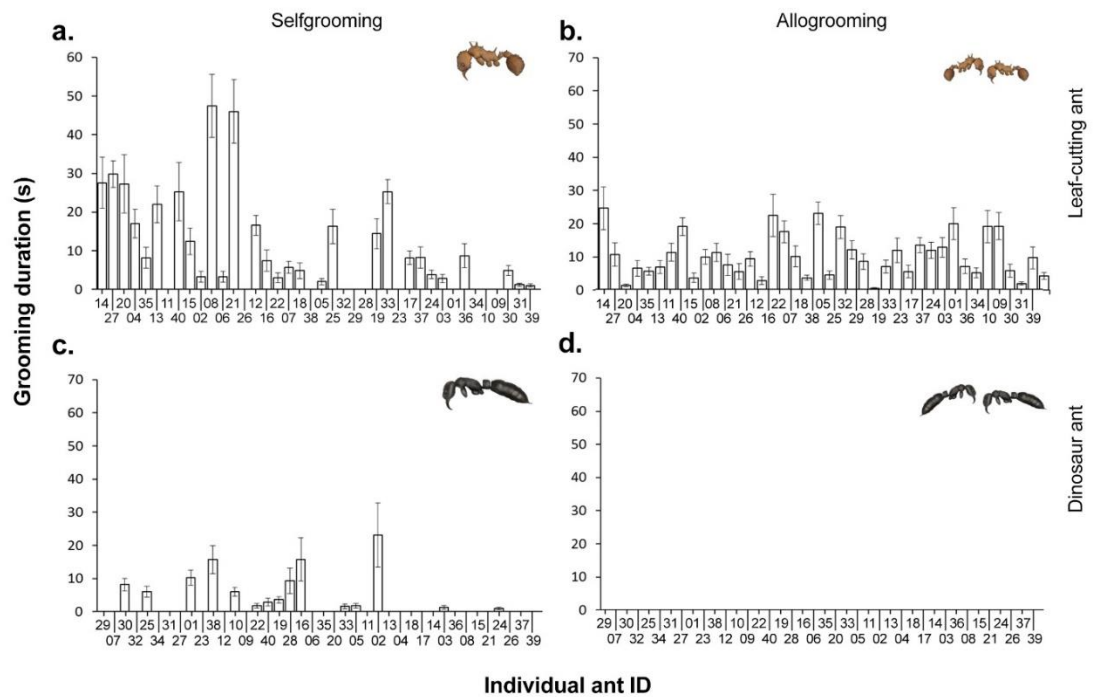
Figure S5.1. Experimental groups and species cohort information for Exp 1



**Figure S5.2.** Photos (top) of two plates (left: phenylacetic acid, right: bleach) showing zones around central application point in which growth of the *Metarhizium* fungal parasite was inhibited. Illustrations (bottom) show a representation of how zones were measured for analysis.



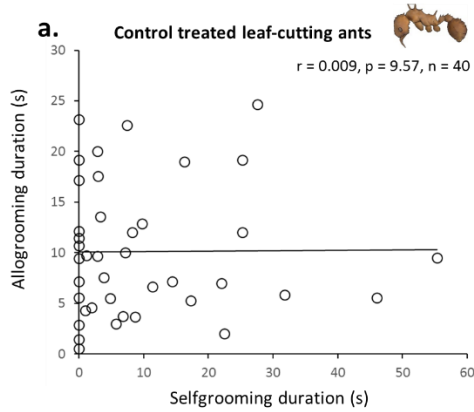
**Figure S6.1.** Mean  $\pm$  s.e. selfgrooming durations for ants maintained either in their colony (white bars) or in individual pots (grey bars) and treated with either control solution or talcum powder suspension.



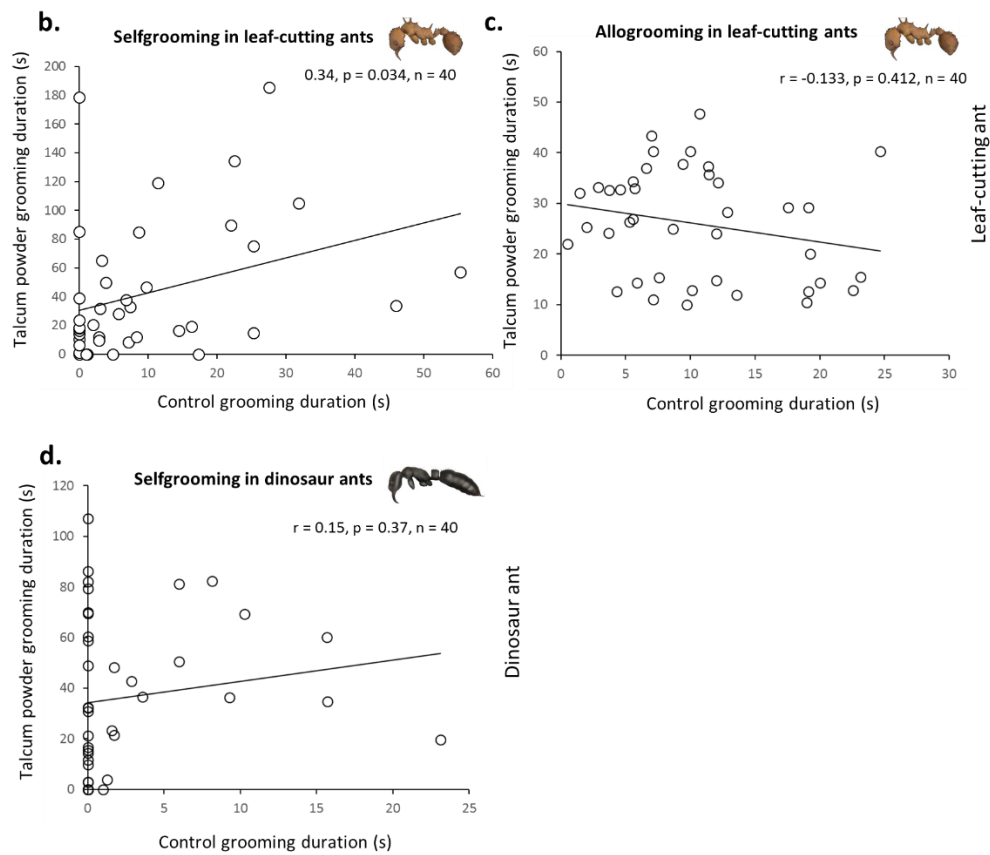
**Figure S6.2.** The mean  $\pm$  s.e. selfgrooming (a,b) and allogrooming (b,d) durations for individual leaf-cutting ants, (a-b) and dinosaur ants (c-d), treated with a control solution.



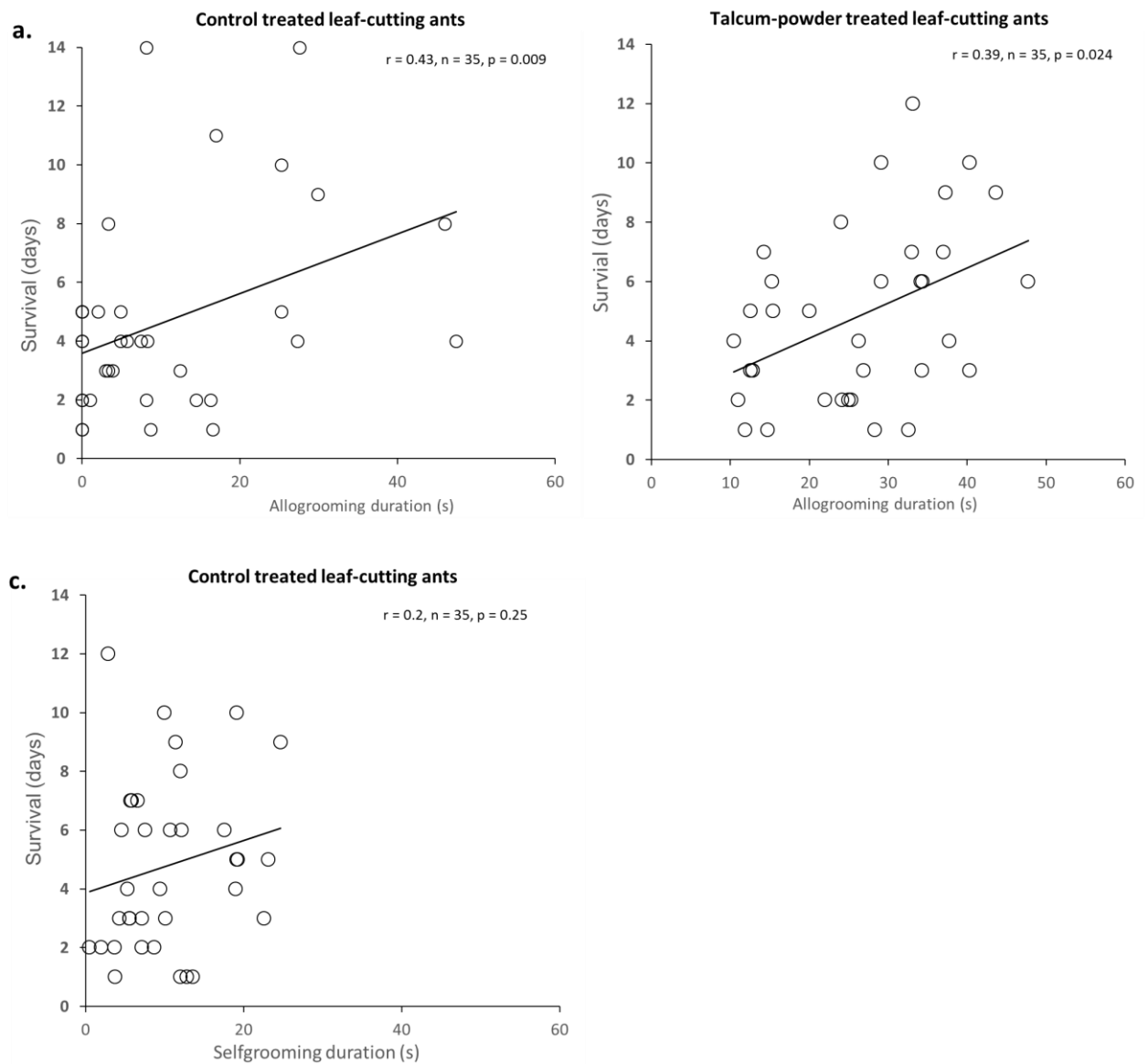
### Correlation between grooming types



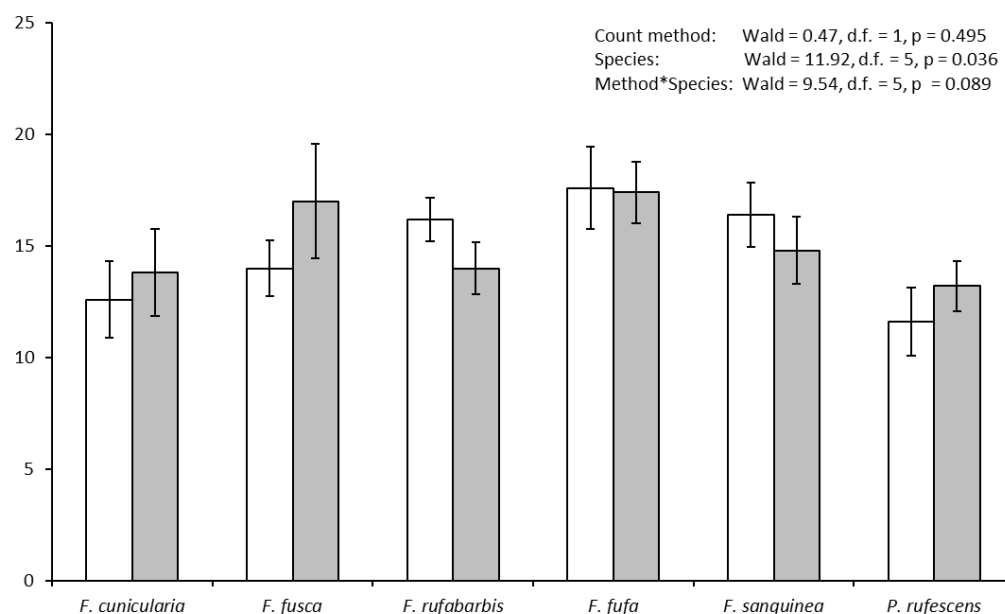
### Correlation between treatments



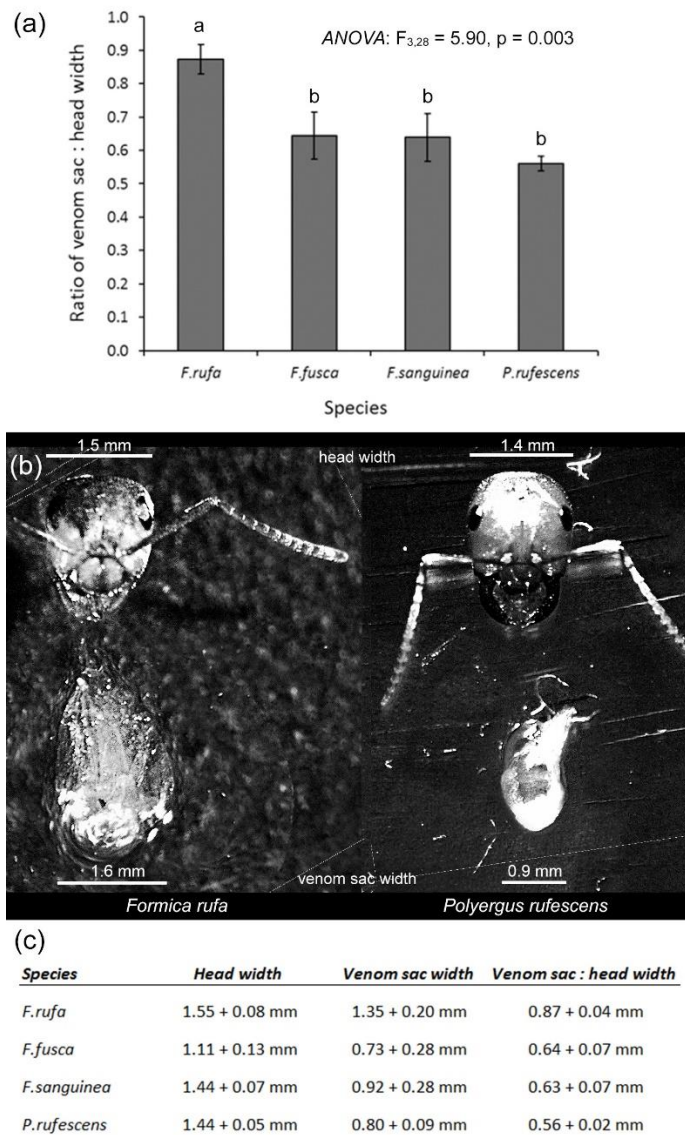
**Figure S6.3.** The relationships between allogrooming and selfgrooming durations after control treatment in leaf-cutting ants (a). The correlation between control and talcum-powder treatment in selfgrooming (b) or allogrooming durations (c) in leaf-cutting ants, and (d) in selfgrooming durations in dinosaur ants.



**Figure S6.4.** The correlation between fungal-treated ant survival and allogrooming duration, in (a) control treated, or (b) talcum-powder treated, leaf-cutting ants, and the correlation between fungal-treated ant survival and selfgrooming duration in control treated leaf-cutting ants.



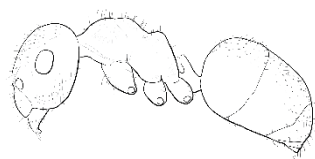
**Figure S7.1.** A comparison of cell count methods using fresh samples (white bars) and stored samples (grey bars) as per Baer et al. (2006). Although using the more destructive long-term storage method resulted in some degradation of cell structures it was still possible to accurately count the number present, and there was overall no difference between methods but a significant difference in cell counts between species. Haemolymph from each species were prepared for counting as live cells. 0.1  $\mu$ l of haemolymph was mixed with cell growth media (Schneider's Media with 10% foetal calf sera) onto a cover slip that had been previously coated with 100 $\mu$ l of 0.2% gelatin in PBS and incubated for 1h at 28°C. The slip was then washed twice with PBS before applying 80 $\mu$ l of Hoechst stain to the slide, left for 5mins, and finally washed in 100 $\mu$ l of PBS. The coverslip was then placed onto a sterile microscope slide, hydrated with 50% glycerol in pbs and sealed with Vaseline before counted as above.



**Figure S7.2.** Size of venom gland. a) The size of the venom gland relative to body size (head width) differed significantly between the free-living *Formica rufa* and *F. fusca*, the facultative social parasite *F. sanguinea* and the obligate social parasite *Polyergus rufescens*, with *F. rufa* having substantially larger venom glands for their body size than the other species. Different letters above columns indicate species which differed significantly from one another in pairwise comparisons as  $P < 0.05$ . b) Photographs showing the dissected gland reservoirs of the *F. rufa* and *P. rufescens*, with the heads of their respective ants to show the considerable size differences in venom reservoir size between these species. c) Mean  $\pm$  s.e. size (head width), venom sac width, and size of venom gland relative to body size for *Formica rufa*, *F. fusca*, *F. sanguinea* and *P. rufescens*, showing the considerable variation in the size of the venom gland, presumably due to emptying of the venom gland in some cases which made accurate measurements difficult. Note however that this variation was much smaller in *P. rufescens* where in all cases the venom reservoir appeared full on removal.

# Supplementary Tables

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*Lasius niger*

**Table S2.1.** Five items of each of the three different food-types were dosed with a conidia solution ( $1.5 \times 10^8$ ) and left to dry for 10 minutes. They were then placed in 500 $\mu$ l of ethanol and vortexed for 5 minutes. The number of conidia in the resulting liquid were quantified using a blank haemocytometer. This washing was repeated sequentially three further times for each food item, to achieve a total number of conidia retrieved, and this as a percentage of the total applied. These results are consistent with conidia recovery rates reported in previous studies. There was no difference in the number of conidia retrieved between the three food-types (Kruskal-Wallis:  $H = 0.62$ , d.f. = 2,  $p = 0.733$ )

Foodstuff	Conidia applied	Wash 1	Wash 2	Wash 3	Wash 4	Total retrieved	% of applied
Leaf	$1.5 \times 10^8$	$1.12 \times 10^8 \pm$	$1.84 \times 10^7 \pm$	$1.02 \times 10^5 \pm$	$1.60 \times 10^2 \pm$	$1.31 \times 10^8 \pm$	$87.30 \pm$
		$1.53 \times 10^7$	$1.80 \times 10^7$	$2.22 \times 10^5$	$2.61 \times 10^2$	$8.62 \times 10^6$	5.75
Rice	$1.5 \times 10^8$	$9.70 \times 10^7 \pm$	$3.32 \times 10^7 \pm$	$3.87 \times 10^4 \pm$	$1.60 \times 10^2 \pm$	$1.30 \times 10^8 \pm$	$86.83 \pm$
		$1.34 \times 10^7$	$1.97 \times 10^7$	$4.73 \times 10^4$	$1.82 \times 10^2$	$1.54 \times 10^7$	10.24
Mealworm	$1.5 \times 10^8$	$1.04 \times 10^8 \pm$	$1.97 \times 10^7 \pm$	$2.31 \times 10^5 \pm$	$1.00 \times 10^2 \pm$	$1.24 \times 10^8 \pm$	$82.83 \pm$
		$1.24 \times 10^7$	$1.84 \times 10^7$	$4.73 \times 10^4$	$1.27 \times 10^7$	$1.27 \times 10^7$	8.49

**Table S2.2. Fidelity of ‘AntTrak’ software**

Video	Frame-by-frame track analysis <sup>1</sup>			Overall analysis <sup>2</sup>
	Sensitivity	1 - Specificity	Accuracy	% Error
49	0.913	0.800	0.783	1.3
395	0.839	0.083	0.864	0.8
399	0.999	0.173	0.962	0.9
401	0.747	0.092	0.848	0.4
403	0.831	0.714	0.812	1.5
404	0.867	0.628	0.993	1.8
4452	0.883	0.178	0.879	0.7
4453	0.899	0.528	0.870	0.3

<sup>1</sup> Data computed from each frame of the video giving measures of fidelity for tracking for eight randomly selected videos based manual frame-by-frame tracking for comparison. <sup>2</sup> Percentage error calculated based on differences in overall values for ‘Time ant spent on treated side’ calculated from the videos by the software and those recorded by human observation

**Table S2.3.** Overall statistical results of experiment 1: ‘Food’

Behaviour analysed	Factor(s)	Test score	d.f.	P
<b>Time spent interacting with food</b> <i>GLMM gamma log link</i>	Species	82.14	3,816	< 0.001
	fungal treatment	100.96	3,816	< 0.001
	species*treatment	17.04	9,816	< 0.001
	colony	0.87		= 0.387
<b>Proportion of trials with food harvested</b> <i>GLMM binomial probit link</i>	species	48.87	3,816	< 0.001
	fungal treatment	20.25	3,816	< 0.001
	species*treatment	3.45	9,816	= 0.002
	colony	0.39		= 0.68
<b>Time spent self-grooming</b> <i>GLMM gamma log link</i>	species	118.76	3,816	< 0.001
	fungal treatment	80.82	3,816	< 0.001
	species*treatment	4.49	9,816	< 0.001
	colony	0.46		= 0.65

**Table S2.4.** Statistical results of experiment 1: ‘Food’ for each species

Species	Behaviour analysed	Factor(s)	Test score	d.f.	P
<b>Leaf-cutting ant</b> <i>Acromyrmex echinator</i>	<b>Time interacting with food</b> <i>GLMM gamma log link</i>	treatment	69.15	3,252	< 0.001
		colony	0.48		= 0.64
	<b>Proportion with food harvested</b> <i>GLMM binomial probit link</i>	treatment	25.73	3,252	< 0.001
		colony	0.45		= 0.72
	<b>Time spent self-grooming</b> <i>GLMM gamma log link</i>	treatment	55.94	3,252	< 0.001
		colony	0.76		= 0.45
<b>Harvester ant</b> <i>Messor barbarus</i>	<b>Time interacting with food</b> <i>GLMM gamma log link</i>	treatment	39.46	3,188	< 0.001
		colony	0.51		= 0.29
	<b>Proportion with food harvested</b> <i>GLMM binomial probit link</i>	treatment	10.43	3,188	< 0.001
		colony	0.25		= 0.82
	<b>Time spent self-grooming</b> <i>GLMM gamma log link</i>	treatment	22.08	3,188	< 0.001
		colony	1.07		= 0.29
<b>Wood ant</b> <i>Formica rufa</i>	<b>Time interacting with food</b> <i>GLMM gamma log link</i>	treatment	11.98	3,252	< 0.001
		colony	0.130		= 0.892
	<b>Proportion with food harvested</b> <i>GLMM binomial probit link</i>	treatment	10.43	3,252	= 0.110
		colony	0.33		= 0.742
	<b>Time spent self-grooming</b> <i>GLMM gamma log link</i>	treatment	34.62	3,252	< 0.001
		colony	5.43		< 0.001
<b>Weaver ant</b> <i>Polyrhachis dives</i>	<b>Time interacting with food</b> <i>GLMM gamma log link</i>	treatment	22.29	3,124	< 0.001
		colony	0.48		= 0.640
	<b>Proportion with food harvested</b> <i>GLMM binomial probit link</i>	treatment	0.312	3,124	= 0.816
		colony	0.22		= 0.820
	<b>Time spent self-grooming</b> <i>GLMM gamma log link</i>	treatment	11.72	3,124	< 0.001
		colony	0.63		= 0.490



**Table S2.5.** Overall statistical results of experiment 2

Behaviour analysed	Factor(s)	Test score	d.f.	P
<b>Time spent on the treatment side</b> <i>GLMM gamma log link</i>	Species	8.712	3,816	= 0.003
	fungus treatment	50.98	3,816	< 0.001
	species*treatment	5.98	9,816	= 0.001
	colony	0.19		= 0.36
<b>Speed on the treatment side</b> <i>GLMM gamma log link</i>	species	6.02	3,816	= 0.024
	fungus treatment	29.15	3,816	< 0.001
	species*treatment	4.24	9,816	= 0.006
	colony	1.02		= 0.307
<b>Time inactive on the treatment side</b> <i>GLMM gamma log link</i>	species	8.33	3,816	= 0.007
	fungus treatment	6.09	3,816	= 0.041
	species*treatment	16.65	9,816	< 0.001
	colony	1.61		= 0.1

**Table S2.6.** Statistical results of experiment 2: ‘Environment’ for each species

Species	Behaviour analysed	Factor(s)	Test score	d.f.	P
<b>Leaf-cutting ant</b> <i>Acromyrmex echinator</i>	<b>Time spent on the treatment side</b> <i>GLMM gamma log link</i>	treatment	12.19	3,252	< 0.001
		colony	0.95		= 0.34
	<b>Speed on the treatment side</b> <i>GLMM gamma log link</i>	treatment	1.91	3,252	= 0.200
		colony	0.14		= 0.81
	<b>Time stopped</b> <i>GLMM neg-binomial log link</i>	treatment	3.29	3,252	= 0.037
		colony	0.22		= 0.71
<b>Harvester ant</b> <i>Messor barbarus</i>	<b>Time spent on the treatment side</b> <i>GLMM gamma log link</i>	treatment	15.05	3,188	< 0.001
		colony	1.697		= 0.09
	<b>Speed on the treatment side</b> <i>GLMM gamma log link</i>	treatment	11.11	3,188	< 0.001
		colony	1.00		= 0.08
	<b>Times stopped</b> <i>GLMM neg-binomial log link</i>	treatment	2.33	3,188	= 0.051
		colony	0.20		= 0.35
<b>Wood ant</b> <i>Formica rufa</i>	<b>Time spent on the treatment side</b> <i>GLMM gamma log link</i>	treatment	10.70	3,252	< 0.001
		colony	0.57		= 0.63
	<b>Speed on the treatment side</b> <i>GLMM gamma log link</i>	treatment	3.38	3,252	= 0.049
		colony	0.23		= 0.66
	<b>Times stopped</b> <i>GLMM neg-binomial log link</i>	treatment	9.69	3,252	< 0.001
		colony	0.25		= 0.82
<b>Weaver ant</b> <i>Polyrhachis dives</i>	<b>Time spent on the treatment side</b> <i>GLMM gamma log link</i>	treatment	9.06	3,124	< 0.001
		colony	0.28		= 0.77
	<b>Speed on the treatment side</b> <i>GLMM gamma log link</i>	treatment	0.94	3,124	= 0.081
		colony	1.72		= 0.21
	<b>Times stopped</b> <i>GLMM neg-binomial log link</i>	treatment	9.75	3,124	= 0.015
		colony	0.26		= 0.14

**Table S2.7.** Overall statistical results of experiment 3

Behaviour analysed	Factor(s)	Test score	d.f.	P
<b>Contact rates between nestmates</b> <i>GLMM gamma log link</i>	Species	10.82	3,816	< 0.001
	fungal treatment	2.21	3,816	= 0.085
	species*treatment	1.80	9,816	= 0.066
	colony	5.95		< 0.001
<b>Self-grooming rates</b> <i>Negative binomial log link</i>	species	61.47	3,816	< 0.001
	fungal treatment	56.57	3,816	< 0.001
	species*treatment	2.77	9,816	= 0.003
	colony	0.57		= 0.565
<b>Allogrooming rates</b> <i>GLMM gamma log link</i>	species	16.83	3,816	< 0.001
	fungal treatment	29.14	3,816	< 0.001
	species*treatment	2.03	9,816	= 0.030
	colony	1.92		= 0.08

**Table S2.8.** Statistical results of experiment 3 ‘Nestmates’ for each species

Species	Behaviour analysed	Factor(s)	Test score	d.f.	P
<b>Leaf-cutting ant</b> <i>Acromyrmex echinator</i>	<b>Number of contacts</b> <i>GLMM gamma log link</i>	treatment	0.60	3,252	= 0.620
		colony	0.95		= 0.340
	<b>Time allogrooming</b> <i>GLMM gamma log link</i>	treatment	17.4	3,252	< 0.001
		colony	4.71		< 0.001
	<b>Time self-grooming</b> <i>GLMM gamma log link</i>	treatment	4.99	3,252	= 0.002
		colony	0.65		= 0.520
<b>Harvester ant</b> <i>Messor barbarus</i>	<b>Number of contacts</b> <i>GLMM gamma log link</i>	treatment	1.553	3,188	= 0.202
		colony	1.697		= 0.090
	<b>Time allogrooming</b> <i>GLMM gamma log link</i>	treatment	14.05	3,188	< 0.001
		colony	0.80		= 0.423
	<b>Time self-grooming</b> <i>GLMM gamma log link</i>	treatment	10.67	3,188	< 0.001
		colony	0.16		= 0.781
<b>Wood ant</b> <i>Formica rufa</i>	<b>Number of contacts</b> <i>GLMM gamma log link</i>	treatment	1.78	3,252	= 0.149
		colony	0.12		= 0.802
	<b>Time allogrooming</b> <i>GLMM gamma log link</i>	treatment	16.58	3,252	< 0.001
		colony	0.41		= 0.621
	<b>Time self-grooming</b> <i>GLMM gamma log link</i>	treatment	48.91	3,252	< 0.001
		colony	0.32		= 0.749
<b>Weaver ant</b> <i>Polyrhachis dives</i>	<b>Number of contacts</b> <i>GLMM gamma log link</i>	treatment	16.06	3,124	< 0.001
		colony	0.33		= 0.742
	<b>Time allogrooming</b> <i>GLMM gamma log link</i>	treatment	4.3	3,124	= 0.006
		colony	0.72		= 0.117
	<b>Time self-grooming</b> <i>GLMM gamma log link</i>	treatment	1.77	3,124	= 0.160
		colony	0.38		= 0.650

**Table S5.1.** Fungus growing ant life-history traits

	Colony size	Life history summary	MG grooming rates	Actinomycete coverage
<i>Atta colombica</i>	Very large $10^6$	Polymorphic. Huge underground colonies. Cut leaves as fungus substrate  'Leafcutter group'	Very high	None
<i>Acromyrmex echinator</i>	Large $10^4$	Polymorphic. Cut leaves as fungal substrate  'Leafcutter group'	High	Low
<i>Sericomyrmex amabilis</i>	Medium $10^3$	Monomorphic. Multiple fungal chambers.  'Higher agriculture'	High	Low
<i>Trachymyrmex cornetzi</i>	Small ca. $10^2$	Monomorphic  'Higher agriculture'	Very low	High
<i>Trachymyrmex sp10</i>	Medium $10^3$	Monomorphic  'Higher agriculture'	Low	None
<i>Apterostigma pilosum</i>	Very small $10^1$	Monomorphic. Dead plant or arthropod material as substrate.  'Coral fungus group'	Very low	Low

References: (Weber 1972; Hölldobler and Wilson 1990; Murakami *et al.*, 2000; Mikheyev *et al.*, 2007; Pitts-Singer and Espelie 2007; Baer *et al.*, 2009; Fernández-Marín *et al.*, 2009, 2013; Mehdiabadi and Schultz 2010; Leal *et al.*, 2011; Mueller *et al.*, 2011; Bruner *et al.*, 2013)

**Table S5.2.** MG compounds tested as a percentage of total secretion volume for Attine genera.

Modified from Vieira *et al.*, 2006. Product source identifier in brackets (Sigma-Aldrich); n.d. = not detectable.

		% chemical constituent of MG secretion					
		Indole (I3408)	Skatole (M51458)	Methyl oleate (311111)	Methyl-3- indoleacetate (I9770)	2-Nonanone (W278505)	Phenylacetic acid (P16621)
Attine species	<i>Apterostigma</i>	1.4	95.5	n.d	2.3	n.d	n.d
	<i>pilosum</i>						
	<i>Mycetarotes</i>	13.1	55.9	15.5	n.d	n.d	n.d
	<i>parallelus</i>						
	<i>Trachymyrmex</i>	7.9	68.5	1.1	10.9	n.d	n.d
	<i>fuscus</i>						
	<i>Acromyrmex</i>	n.d	40.9	1	2.8	11.7	n.d
	<i>coronatus</i>						
	<i>Atta laevigata</i>	8.7	36.2	0.5	5.1	6.1	29.3

**Table S5.3.** Statistical results of survival analysis in six attine species with blocked or functional metapleural glands, and treated with *Metarhizium pinshaense* fungal parasite or control solution.

Ant species	Factor	Wald	Significance
<i>Atta colombica</i>	Gland blockage	22.04	<0.001*
	Fungal treatment	8.27	0.004*
	Blockage * Fungal	4.36	0.037*
	Colony	0.37	0.841
<i>Acromyrmex echinator</i>	Gland blockage	18.35	<0.001*
	Fungal treatment	6.96	0.008*
	Blockage * Fungal	3.92	0.048*
	Colony	0.58	0.989
<i>Sericomyrmex amabilis</i>	Gland blockage	28.60	<0.001*
	Fungal treatment	10.55	0.001*
	Blockage * Fungal	5.01	0.025*
	Colony	10.56	0.061
<i>Trachymyrmex sp10</i>	Gland blockage	2.94	0.087
	Fungal treatment	4.36	0.035*
	Blockage * Fungal	0.10	0.75
	Colony	1.73	0.885
<i>Trachymyrmex cornetzi</i>	Gland blockage	1.32	0.25
	Fungal treatment	9.80	0.002*
	Blockage * Fungal	0.07	0.79
	Colony	12.08	0.06
<i>Apterostigma pilosum</i>	Gland blockage	0.63	0.43
	Fungal treatment	5.85	0.016*
	Blockage * Fungal	0.01	0.92
	Colony	8.65	0.124

**Table S5.4.** Isolated MG secretions listed by their abundances as reported in GC-MS fractions (Do Nascimento and Schoeters 1996; Ortius-Lechner *et al.*, 2000; Vieira *et al.*, 2012b) and natural gland secretions from *Acromyrmex* ants. Total secretion volumes taken as an average of 4  $\mu$ L (Ortius-Lechner *et al.*, 2000; Yek *et al.*, 2012). Calculated dilution series based on natural concentrations and appropriate solvent are also listed. Concentration 3 in bold is representative of the calculated natural concentration thought to be found in ants. Compounds for each dilution were first dissolved in their appropriate solvent and then further diluted with ddH<sub>2</sub>O to reach the correct concentration of test compound and a concentration of solvent which matches the appropriate dilution of control solvent. E.g. 2.4g of indole were dissolved in 0.5 mL of acetone, which was then added to 0.5 mL of ddH<sub>2</sub>O.

MG secretion test compounds	Amount found in GC-MS analysis (ng)	Concentration in ant MG secretion (ng/ $\mu$ L)	Concentrations tested (g/mL)					Solvent used (see below)	Volume solution applied ( $\mu$ L)
			1 $1 \times 10^{-2}$	2 $1 \times 10^{-1}$	3 $1 \times 10^0$	4 $1 \times 10^1$	5 $1 \times 10^2$		
Indole	95	24	$2.4 \times 10^{-4}$	$2.4 \times 10^{-3}$	$2.4 \times 10^{-2}$	$2.4 \times 10^{-1}$	$2.4 \times 10^0$	Acetone	20
Skatole	430	108	$1.1 \times 10^{-3}$	$1.1 \times 10^{-2}$	$1.1 \times 10^{-1}$	$1.1 \times 10^0$	$1.1 \times 10^1$	Acetone	20
Methyl oleate	6	1.5	$1.5 \times 10^{-5}$	$1.5 \times 10^{-4}$	$1.5 \times 10^{-3}$	$1.5 \times 10^{-2}$	$1.5 \times 10^{-1}$	Acetone	20
2-Nonanone	30	7.5	$7.5 \times 10^{-5}$	$7.5 \times 10^{-4}$	$7.5 \times 10^{-3}$	$7.5 \times 10^{-2}$	$1.2 \times 10^{-1}$	Hexane	20
Phenylacetic acid	350	87.5	$8.8 \times 10^{-4}$	$8.8 \times 10^{-3}$	$8.8 \times 10^{-2}$	$8.8 \times 10^{-1}$	$8.8 \times 10^0$	ddH <sub>2</sub> O	20
Methyl-3-indoleacetate	40	10	$1.0 \times 10^{-4}$	$1.0 \times 10^{-3}$	$1.0 \times 10^{-2}$	$1.0 \times 10^{-1}$	$1.0 \times 10^0$	Acetone	20
			Dilutions tested						
Solvent control chemical	Control type		1	2	3	4	5	Diluted in	
Hexane	solvent control		$5 \times 10^{-5}$	$5 \times 10^{-4}$	$5 \times 10^{-3}$	$5 \times 10^{-2}$	$5 \times 10^{-1}$	ddH <sub>2</sub> O	
Acetone	solvent control		$5 \times 10^{-5}$	$5 \times 10^{-4}$	$5 \times 10^{-3}$	$5 \times 10^{-2}$	$5 \times 10^{-1}$	ddH <sub>2</sub> O	
Bleach	positive control		$5 \times 10^{-5}$	$5 \times 10^{-4}$	$5 \times 10^{-3}$	$5 \times 10^{-2}$	$5 \times 10^{-1}$	ddH <sub>2</sub> O	
ddH <sub>2</sub> O	negative control		1	1	1	1	1	n/a	

Dose volumes applied standardised for body size

*Atta*, *Acromyrmex*: 0.5  $\mu$ L, *Sericomyrmex*, *Trachymyrmex sp10*: 0.4  $\mu$ L, *Trachymyrmex cornetzi*, *Apterostigma*: 0.3  $\mu$ L



**Table S5.5.****Natural abundances**

<i>Species</i>	<i>Indole</i>	<i>Skatole</i>	<i>Methyl oleate</i>	<i>Methyl-3-indol.</i>	<i>2-Nonanone</i>	<i>Phenylacetic acid</i>	<i>total</i>
<i>A.cephalotes</i>	8.70	36.20	0.50	5.10	6.10	29.30	85.90
<i>A.echinatior</i>	0.00	40.90	1.00	2.80	11.70	0.00	56.40
<i>S.amabilis</i>	2.45	50.71		6.61	14.00		73.77
<i>T.sp10</i>	7.90	68.50	1.10	10.90	1.10	0.00	89.50
<i>T.cornetzi</i>	3.90	84.11	0.80	10.90	0.00	0.00	99.71
<i>A.pilosum</i>	1.40	95.50	0.00	2.30	0.00	0.00	99.20

**Compound average antimicrobial activities (Fig 3a)**

<i>Indole</i>	<i>Skatole</i>	<i>Methyl oleate</i>	<i>Methyl-3-indol.</i>	<i>2-Nonanone</i>	<i>Phenylacetic acid</i>	<i>total</i>
10.45	11.88	15.70	10.45	27.87	41.29	

**Percentage composition**

<i>Species</i>	<i>Indole</i>	<i>Skatole</i>	<i>Methyl oleate</i>	<i>Methyl-3-indol.</i>	<i>2-Nonanone</i>	<i>Phenylacetic acid</i>	<i>total</i>
<i>A.cephalotes</i>	10.13	42.14	0.58	5.94	7.10	34.11	100.00
<i>A.echinatior</i>	0.00	72.52	1.77	4.96	20.74	0.00	100.00
<i>S.amabilis</i>	3.32	68.74	0.00	8.96	18.98	0.00	100.00
<i>T.sp10</i>	8.83	76.54	1.23	12.18	1.23	0.00	100.00
<i>T.cornetzi</i>	3.91	84.35	0.80	10.93	0.00	0.00	100.00
<i>A.pilosum</i>	1.41	96.27	0.00	2.32	0.00	0.00	100.00

**Antimicrobial activity x % composition**

<i>Species</i>	<i>Indole</i>	<i>Skatole</i>	<i>Methyl oleate</i>	<i>Methyl-3-indol.</i>	<i>2-Nonanone</i>	<i>Phenylacetic acid</i>	<i>total</i>
<i>A.cephalotes</i>	1.06	5.01	0.09	0.62	1.98	14.08	22.84
<i>A.echinatior</i>	0.00	8.61	0.28	0.52	5.78	0.00	15.19
<i>S.amabilis</i>	0.35	8.17	0.00	0.94	5.29	0.00	14.74
<i>T.sp10</i>	0.92	9.09	0.19	1.27	0.34	0.00	11.82
<i>T.cornetzi</i>	0.41	10.02	0.13	1.14	0.00	0.00	11.70
<i>A.pilosum</i>	0.15	11.44	0.00	0.24	0.00	0.00	11.83

**Table S7.1.** Details of experimental design indicating how many, and from which colonies, ants were used for each section of this study

Species	colony #	#uid	StudyID	Prelim	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Notes
				Trial use	Ant survival	Grooming rates	Slave ratios	fungal viability	venom pH	PO & Cell counts
<i>Formica cunicularia</i>	1	cuni01oct11	Fc1	20				30		
<i>Formica cunicularia</i>	2	cuni02oct11	Fc2		20			30		
<i>Formica cunicularia</i>	3	cuni03oct11	Fc3							5 used to supply brood to P3-4
<i>Formica cunicularia</i>	4	cuni04oct11	Fc4							5 used to supply brood to P3-4
<i>Formica cunicularia</i>	5	cuni05oct11	Fc5							5
<i>Formica cunicularia</i>	6	cuni06oct11	Fc6							5
<i>Formica cunicularia</i>	7	cuni07mar13	Fc7		20	30		30		
<i>Formica cunicularia</i>	8	cuni08mar13	Fc8		20					
<i>Formica cunicularia</i>	9	cuni09mar13	Fc9		20	30		30		
<i>Formica cunicularia</i>	10	cuni10mar13	Fc10			30				
<i>Formica cunicularia</i>	11	cuni11mar13	Fc11			30				
<i>Formica fusca</i>	1	fusc01oct11	Ff1	20				30		5
<i>Formica fusca</i>	2	fusc02oct11	Ff2		20	30		30		5
<i>Formica fusca</i>	3	fusc03oct11	Ff3					30		5
<i>Formica fusca</i>	4	fusc04oct11	Ff4							5
<i>Formica fusca</i>	5	fusc05oct11	Ff5		20	30		30		
<i>Formica fusca</i>	6	fusc06oct11	Ff6							
<i>Formica fusca</i>	7	fusc07oct11	Ff7							v. small colony size - not used
<i>Formica fusca</i>	8	fusc08dec11	Ff8		20	30				
<i>Formica fusca</i>	9	fusc09dec11	Ff9							used to supply brood to P1-2
<i>Formica fusca</i>	10	fusc10dec11	Ff10							
<i>Formica fusca</i>	11	fusc11mar13	Ff11		20	30				
<i>Formica fusca</i>	12	fusc12mar13	Ff12							used to supply brood to P1-2
<i>Formica rufibarbis</i>	1	rufb01oct11	Frb1		20	30				
<i>Formica rufibarbis</i>	2	rufb02oct11	Frb2	20	20	30		30		5
<i>Formica rufibarbis</i>	3	rufb03oct11	Frb3		20	30				
<i>Formica rufibarbis</i>	4	rufb04oct11	Frb4		20	30				
<i>Formica rufibarbis</i>	5	rufb05oct11	Frb5							5
<i>Formica rufibarbis</i>	6	rufb06oct11	Frb6							5
<i>Formica rufibarbis</i>	7	rufb07oct11	Frb7							5
<i>Formica rufibarbis</i>	8	rufb08mar13	Frb8					30		
<i>Formica rufibarbis</i>	9	rufb09mar13	Frb9					30		
<i>Formica rufibarbis</i>	10	rufb10mar13	Frb10					30		
<i>Formica rufa</i>	1	fru01jul13	Fru1	40	20	30		30		5
<i>Formica rufa</i>	2	fru02jul13	Fru2		20	30		30		
<i>Formica rufa</i>	3	fru03jul13	Fru3		20	30		30		5
<i>Formica rufa</i>	4	fru04jul13	Fru4		20	30		30		
<i>Formica rufa</i>	5	fru05jun14	Fru5						5	5
<i>Formica rufa</i>	6	fru05jun14	Fru6						5	5
<i>Formica sanguinea</i>	1	sang01oct11	Fs1	20			10			
<i>Formica sanguinea</i>	2	sang02oct11	Fs2		20	30		30		5
<i>Formica sanguinea</i>	3	sang03oct11	Fs3				10			
<i>Formica sanguinea</i>	4	sang04oct11	Fs4				10			
<i>Formica sanguinea</i>	5	sang05oct11	Fs5				10			
<i>Formica sanguinea</i>	6	sang06oct11	Fs6				10			
<i>Formica sanguinea</i>	7	sang07dec11	Fs7				30			
<i>Formica sanguinea</i>	8	sang08dec11	Fs8							5
<i>Formica sanguinea</i>	9	sang09oct12	Fs9		20	30	10	30		5
<i>Formica sanguinea</i>	10	sang10dec12	Fs10		20	30	30	30		
<i>Formica sanguinea</i>	11	sang11dec12	Fs11		20	30	30	30		5
<i>Polyergus rufescens</i>	1	plyg01oct11	P1					3		Kept with fusca slaves
<i>Polyergus rufescens</i>	2	plyg02oct11	P2					3		Kept with fusca slaves
<i>Polyergus rufescens</i>	3	plyg03oct11	P3					3		kept with cunicularia slaves
<i>Polyergus rufescens</i>	4	plyg04oct11	P4		8	10		5		5 kept with cunicularia slaves
<i>Polyergus rufescens</i>	5	plyg05oct11	P5		8	10		4		5 kept with fusca slaves
<i>Polyergus rufescens</i>	6	plyg06aug14	P6		8	10		30	5 5	5 kept with cunicularia slaves, slave
<i>Polyergus rufescens</i>	7	plyg06aug14	P7		8	10		30	5 5	5 kept with cunicularia slaves, slave

**Table S7.2.** Standardisation of volume and dose of *Metarhizium* and talcum powder applied to species of different body size in Experiments 1 & 2 (n=10). Stock concentrations of  $5 \times 10^7$  conidia/particles per ml.

Species	Weber's length	Volume applied	Conidia/mm <sup>2</sup>	Talc particles/mm <sup>2</sup>
<i>Formica cunicularia</i>	1.91±0.38	0.2µl	2610	2610
<i>Formica fusca</i>	2.02±0.37	0.2µl	2340	2340
<i>Formica rufibarbis</i>	1.96±0.34	0.2µl	2480	2480
<i>Formica rufa</i>	2.86±0.24	0.4µl	2330	2330
<i>Formica sanguinea</i>	3.27±0.32	0.5µl	2230	2230
<i>Polyergus rufescens</i>	2.16±0.20	0.2µl	2040	2040

**Table S7.3.** Survival of ants from six species over 14 days with or without exposure to *Metarhizium* and venom gland blockage

Species	Treatment	Wald	Sig.	Exp(B)
<i>Formica cunicularia</i>	Fungal treatment *	3.291	0.040	0.233
	Gland blockage *	5.103	0.024	2.909
	Block * Fungal	0.923	0.337	.346
<i>Formica fusca</i>	Fungal treatment *	4.596	0.032	0.103
	Gland blockage *	6.206	0.013	2.997
	Block * Fungal	0.000	0.991	1.013
<i>Formica rufibarbis</i>	Fungal treatment *	4.037	0.045	0.117
	Gland blockage *	4.258	0.039	2.728
	Block * Fungal	0.417	0.518	.381
<i>Formica rufa</i>	Fungal treatment	0.345	0.557	0.487
	Gland blockage	2.374	0.123	3.521
	Block * Fungal	0.574	0.449	.290
<i>Formica sanguinea</i>	Fungal treatment *	6.538	0.011	0.140
	Gland blockage	1.277	0.258	1.578
	Block * Fungal	0.086	0.770	1.323
<i>Polyergus rufescens</i>	Fungal treatment *	4.579	0.032	0.279
	Gland blockage	2.073	0.069	2.040
	Block * Fungal	1.457	0.227	0.353

**Table S8.1. Details of Primer and PCR conditions used for amplification of host ant (*COI*) and *Wolbachia* symbiont sequences.**

Gene	Primer	PCR Mix	PCR conditions
<b>COI</b>  (710-bp)	<b>LCO (f)</b> GGTCAACAAATCATAAAGATATTGG	8.2µl ddH <sub>2</sub> O 4.0µ FlexiBuffer 2.0µ MgCl <sub>2</sub> 1.6µl dNTPs	94°C for 2 min 37 cycles: 94°C for 30 s 59°C for 45 s 72°C for 1 min 30 s
	<b>HCO (r)</b> TAAACTTCAGGTGACCAAAAAATCA	1.0µl primer (x2) 0.2µl TAQ 2.0µl DNA	1 cycle: 72°C for 10 min 4°C hold
<b>wsp</b>  (603-bp)	<b>wsp_f</b> GTCCAATARSTGATGARGAAAC	9.6µl ddH <sub>2</sub> O 4.0µ FlexiBuffer 2.5µ MgCl <sub>2</sub> 1.0µl dNTPs	94°C for 2 min 37 cycles: 94°C for 30 s 59°C for 45 s 72°C for 1 min 30 s
	<b>wsp_r</b> CYGCACCAAYAGYRCTR TAAA	0.4µl primer (x2) 0.1µl TAQ 2.0µl DNA	1 cycle: 72°C for 10 min 4°C hold
<b>MLST</b> <b>gatB</b> (471-bp)	<b>gatB_f</b> GAK TTA AAY CGY GCA GGB GTT <b>gatB_r</b> TGG YAA YTC RGG YAA AGA TGA		$\Delta = 54^{\circ}\text{C}$
<b>MLST</b> <b>coxA</b> (487-bp)	<b>coxA_f</b> TTGGRGCRATYAACTTTATAG <b>coxA_r</b> CTAAAGACTTTKACRCCAGT	9.6µl ddH <sub>2</sub> O 4.0µ FlexiBuffer 2.5µ MgCl <sub>2</sub> 1.0µl dNTPs	95°C for 2 min 35 cycles: 95°C for 30 s $\Delta^{\circ}\text{C}$ for 30 s
<b>MLST</b> <b>hcpA</b> (515-bp)	<b>hcpA_f</b> GAAATARCAGTTGCTGCAAA <b>hcpA_r</b> GAAAGTYRAGCAAGYTCTG	0.4µl primer (x2) 0.1µl TAQ 2.0µl DNA	72°C for 1 min 1 cycle: 72°C for 7 min 4°C hold
<b>MLST</b> <b>ftsZ</b> (524-bp)	<b>ftsZ_f</b> ATYATGGARCATATAAARGATAG <b>ftsZ_r</b> TCRAGYAATGGATTTRGATAT		$\Delta = 52^{\circ}\text{C}$
<b>MLST</b> <b>fbpA</b> (509-bp)	<b>fbpA_f</b> GCTGCTCCRCTTGGYWTGAT <b>fbpA_r</b> CCRCCAGARAAAAYYACTATTC		$\Delta = 59^{\circ}\text{C}$

# Appendices

## A preliminary study of nest structure and composition of the weaver ant *Polyrhachis* (*Cyrtomyrma*) *delecta* (Hymenoptera: Formicidae)

### Abstract

*Polyrhachis* weaver ants build their nests from vegetation bound together using silk produced by their larvae. Here we provide a pilot study of the colony composition and the physical nest structure of the arboreal silk nests of three colonies of *Polyrhachis* (*Cyrtomyrma*) *delecta* based on examination of three colonies. We found broadly similar nest architecture and size of the nests with each containing six or seven identifiable chambers, and describe the distribution of ants of different castes and life stages between them. We also note the construction of silk ‘girder’ structures which spanned larger chambers and we hypothesise these provide additional strength to the internal nest structure. This study highlights the importance of more detailed investigation of the internal nest structure and composition in *Polyrhachis*, and other weaver ant species, which will help develop our understanding of this specialised form of nest construction and nesting habits in a diverse group of ants.

### Introduction

The ability of social insects to locate suitable nesting sites and, through manipulation of the environment, to construct often highly complex nests is key to the success of the colony and social insects in general (Hölldobler & Wilson 1990). In ants, nests can range in size and complexity from the vast underground networks of *Atta* leaf-cutting ants, to a whole colony of *Temnothorax* contained within a single acorn (Hölldobler & Wilson 1990). The nesting habits of organisms are an important factor in their life history and a powerful driver of their morphology and ecology (Jeanne 1975; Mikheyev & Tschinkel 2004;). The architecture of nests themselves is believed to be key in the evolution of division of labour which has contributed to the ecological success of ant societies (Hölldobler & Wilson 1990). The internal structure of a nest and the internal arrangement of ants and brood within it can potentially also have important implications for the spread of infectious diseases within colonies (Schmid-Hempel 1998; Naug & Camazine 2002). Social insects may be particularly vulnerable to parasites due to the very high population densities, homeostatic environmental conditions and low genetic diversity within colonies, which contribute to an increased risk of parasite transmission. In leaf-cutting ants, waste management tasks are partitioned spatially and between castes, which helps isolate the main colony from the increased risk of contamination (Bot et al. 2001; Waddington & Hughes 2010). Similarly, compartmentalisation between chambers within the nest may help prevent transmission of parasites to vulnerable aspects of the colony such as the queen or brood (Pie et al. 2004; Boomsma et al. 2005).

*Polyrhachis* Fr. Smith is a diverse genus of ants within the subfamily Formicinae with over 600 species widely distributed across Africa, Asia, and Australasia. Commonly termed ‘weaver ants’, many species form arboreal nests constructed from vegetation bound together with silk produced by their larvae, but may vary widely from subterranean nests formed from intertidal mangrove mud to lignicolous and lithocolous species (Robson & Kohout 2007). There is also an extreme range of colony sizes and compositions from very small colonies with just a few tens of individuals, through to

colonies with almost a million workers (Liefke et al. 1998; Dornhaus et al. 2010). Colonies tend to be polydomous, and in some species may also be polygynous with multiple dealate queens within a nest (Liefke et al. 1998; van Zweden et al. 2007). Like other formicines, weaver ants produce acidic venom from their venom gland which they can use to disinfect themselves and their brood (Graystock & Hughes 2011; Tragust et al. 2013; Tranter et al. 2014). Additionally, *Polyrhachis* and *Oecophylla* weaver ants use this venom to maintain acidic conditions of their nest silk (Tranter et al., 2014; CT and WOHH unpublished data). The general nesting habits of these ants have been well documented (Kohout 2000; 2012; Robson et al. 2015) and recently set into a phylogenetic framework, broadly describing patterns of weaving behaviour and basic nest composition (Robson & Kohout 2005, 2007). There has been a comprehensive study of the unusual nesting habits of the estuarine species *Polyrhachis* (*Chariomyrma*) *sokolova*, as well as brief details of mainly external colony architecture, and records of colony composition for a few other *Polyrhachis* species (Jinfu & Jue 1996; Nielsen 1997; Liefke et al. 1998; Downes 2015). However, detailed observation of the finer scale structure and colony composition of *Polyrhachis* nests is less well documented, especially considering the large number of species in the genus and the diversity of nesting habits. Here we provide some preliminary information on this from three nests of the arboreal and silk weaving species of Australian weaver ant *Polyrhachis* (*Cyrtomyrma*) *delecta*.

## Methods

This work represents a preliminary study of the nest structure and nest composition of *P. delecta* (Kohout 2006) which we hope can provide a basis for further exploration of this interesting and understudied species. Ants were identified using keys available in Kohout (2006). Three nests of *P. delecta* were collected from around Centenary Lakes (-



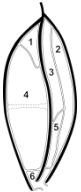
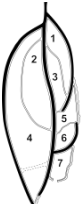

16.902°S, 145.749°E), Cairns, QLD, Australia in July 2014. The nests were externally formed from interwoven leaves hanging in vegetation approximately 1.5 m above ground and were suspended at a single point. At each site the nests collected were the only nests visible in the vicinity. Nests 2 and 3 were collected from approximately 20 m apart, whilst Nest 1 was located about 40 m from either of the other nests; none of the nests were therefore found on the same plant. Nests were measured externally about three axes to give a height, breadth, width measurement using a 30 cm ruler. Nests were all approximately ellipsoid in shape and estimated volumes were calculated using the formula  $V = 4/3\pi abc$ , where  $a = \frac{1}{2}\text{height}$ ,  $b = \frac{1}{2}\text{breadth}$ ,  $c = \frac{1}{2}\text{width}$ . The whole nest was collected by cutting the branch above the nest and gently releasing the nest into a plastic container. Returning worker ants were collected individually for a period of 15 min after collection of the nest and stored separately in 95% ethanol. This collection method resulted in minimal disturbance of the ants. The nest was then left for 6 h during which time the few ants which had emerged during collection returned to within the nest. The whole nest was then rapidly chilled by placing it in the freezer at -20°C for 3 h. The nest was then removed from the freezer, measured externally, and carefully dissected. The position and size of chambers within the nest were recorded, and their contents: queens, gynes (alate queens), males, workers, and brood, collected in ethanol for later counting under a stereomicroscope. Whilst every effort was made to minimise disturbance of the nest prior to dissection it was not possible to determine the extent to which ants within the nest may have repositioned during procedures. Therefore any positional data of castes within the colony should be treated cautiously. *P. delecta* larvae do not spin larval cocoons, the loss of which is thought to be restricted to the *Cyrtomyrma* and *Mymatopa* subgenera, and pupae are exposed within the nest (Robson & Kohout 2007; Robson et al. 2015). Whilst Downes (2015) reported that it is possible to discriminate between incipient workers and incipient sexuals in the closely related *P. australis* due to the present of wing buds on exposed pupae, we were not able to reliably do so here for *P.*

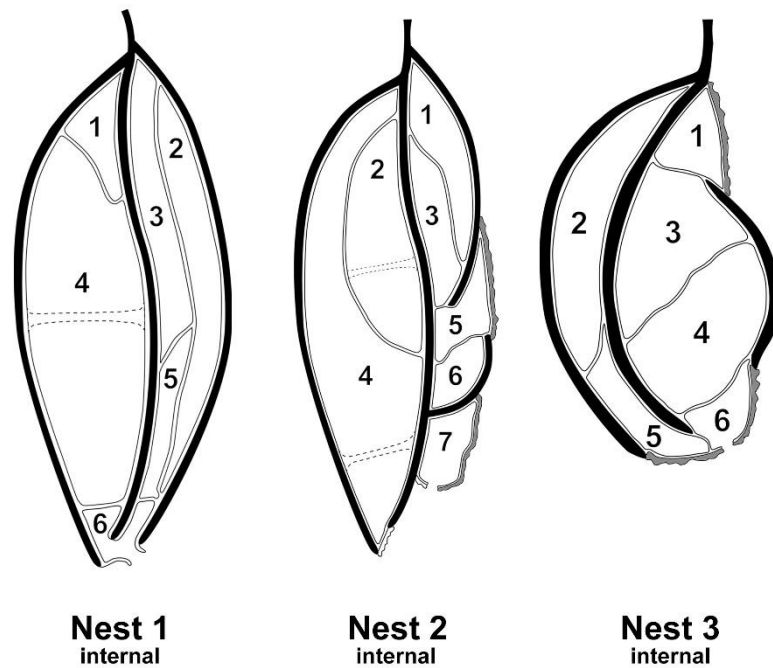
*delecta*. After removal with soft forceps of all ants and brood, the internal nest structure was recorded with sketches and photographs (Canon 7D and Canon MPE-65mm or 100mm f/2.8 macro) as the dissection progressed. Samples are stored at the University of Sussex, UK.

## Results

Nests 1 and 2 were similar in their external structure, dimensions and construction, whilst Nest 3 was larger and more spherical in shape (Table 1).

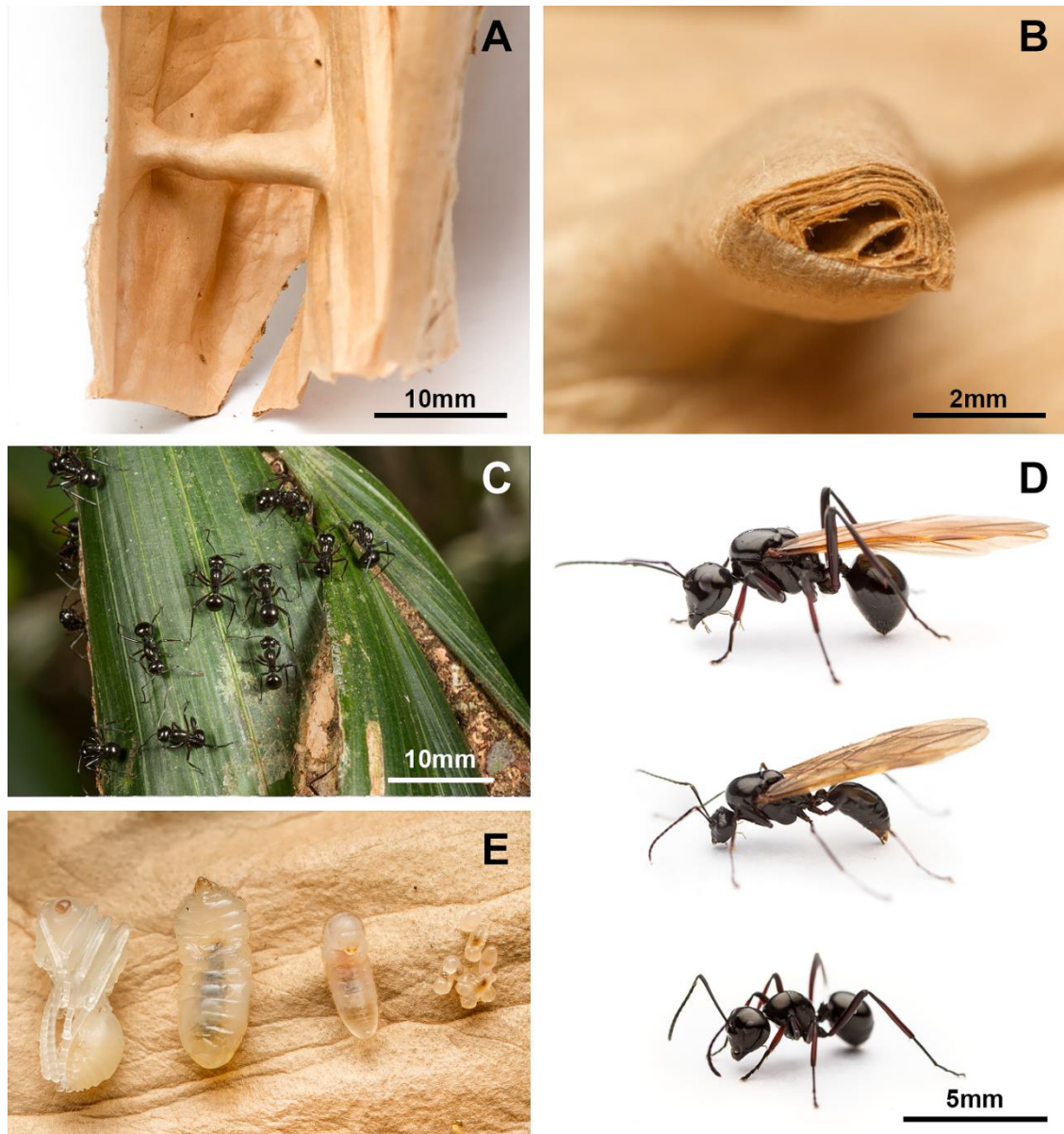
**Table 1.** Information on external and internal colony structure of three nests of *P. delecta*. Numbering detailed in Figure 1. Approximate volumes are calculated from dimensions.

Nest	External dimensions (height x width x depth )	Internal chamber details		
		Count (n)	Chamber	Volume (%)
1. 	Formed of 3 leaves  140 x 70 x 65 mm volume: 333.53cm <sup>3</sup>	6	1	10
			2	20
			3	10
			4	44
			5	10
			6	5
2. 	Formed of 5 leaves  135 x 75 x 60 mm volume: 318.09cm <sup>3</sup>	7	1	10
			2	20
			3	5
			4	35
			5	10
			6	10
			7	10
3. 	Formed of 3 leaves  110 x 85 x 80 mm volume: 39165 cm <sup>3</sup>	6	1	10
			2	20
			3	25
			4	25
			5	10
			6	10



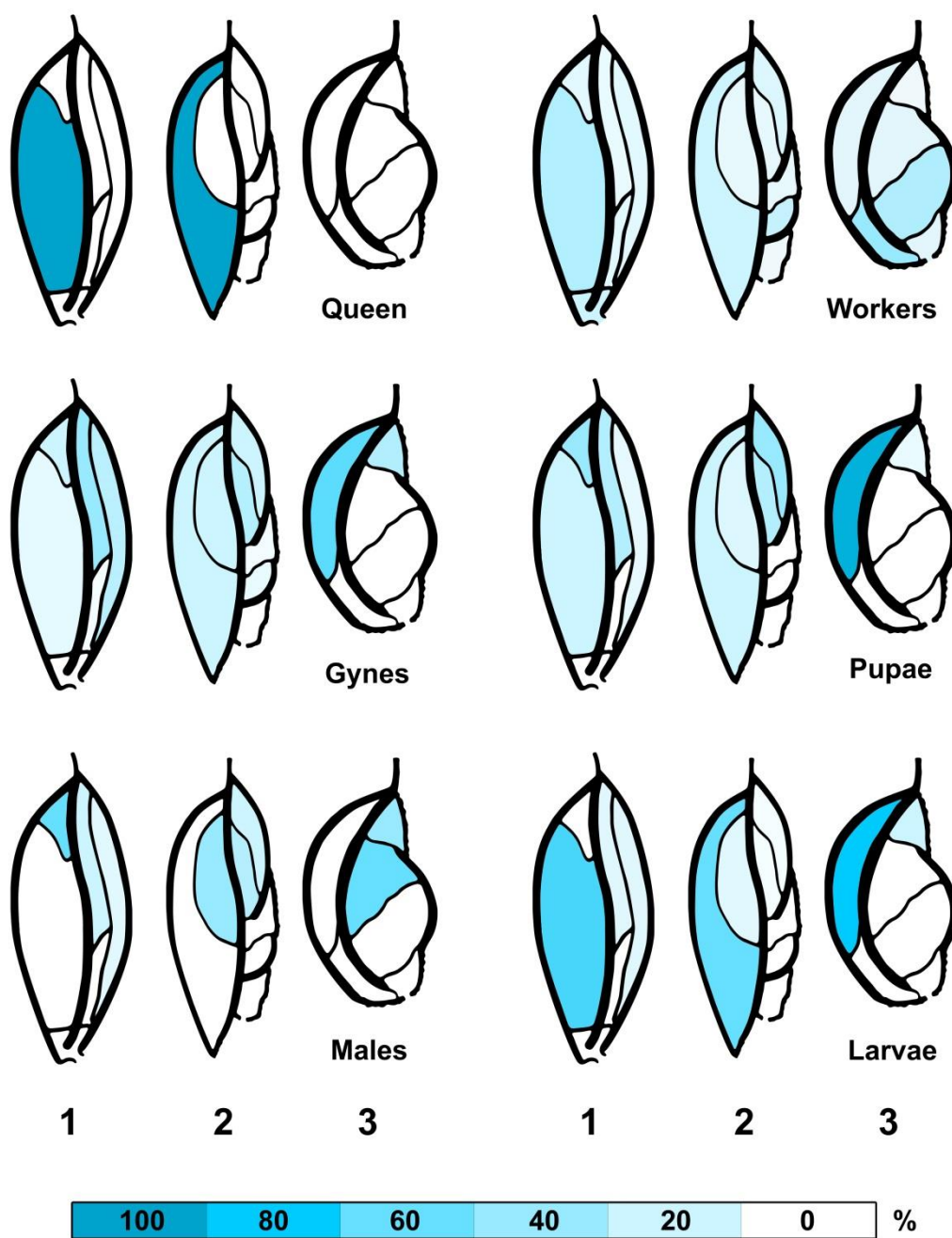
**Figure 1.** Lateral view schematic illustrations of the internal chamber arrangement of three nests of *Polyrhachis delecta* weaver ants. Chambers are labelled (1-7). Nests were suspended from vegetation at the top and thick black lines show the core arrangement of leaves which were divided into chambers through construction with larval silk (thin lines). Sections with wavy outlines in gray on the outer surface of the nest indicate areas constructed from carton. Internal structures with dashed lines indicate the position of internal 'girders'. Nest openings are portrayed oriented towards the lower right of each nest.

All three nests were formed from less than five leaves at the terminus of a hanging branch, with the leaves slightly folded and woven together with silk and carton material, and in some cases (ca. 20%) split along veins (Figure 2C). All nests had one entrance located at the bottom of the colony. Internally nests were composed of 1-2 large chambers, usually formed as one whole section between two leaves, and a number of smaller chambers towards the periphery (Figure 1).



**Figure 2.** Photographs detailing a cross section through the silk support structures ('girders') spanning some chambers within nests (A-B), a view of the outside of Nest 1 showing workers on sections of the folded leaf with an area of visible carton in the bottom right (C), examples of an alate queen (top), male (middle) and worker (bottom) of *Polyrhachis delecta* weaver ants (D), and examples of the various brood stages including a pupa (left) and variously sized larvae (middle to right) found within colonies (E).

These chambers were lined with silk sheets which varied in thickness from so thin as to be almost transparent to the approximate thickness of standard 75 gsm copy paper (100  $\mu\text{m}$ ). Three of the larger chambers possessed cylindrical protuberances, which we term here ‘girders’. These girders emerged from the inner wall and spanned the chamber, and were formed from tightly layered silk (Figure 2A-B). A single large girder was present in Chamber 4 of Nests 1 and 2 and a smaller girder was also observed spanning the walls of Chamber 2 in Nest 2. Nests 1 and 2 contained similar numbers of workers, alate queens and brood (Table 2; Figure 2D-E). Nest 3 however had much fewer workers and brood, no dealate queens and very few alate queens present, unlike the other two nests. There were some similarities between the three nests in the location in which the majority of each of the castes were found (Figure 1,3), most evident between Nests 1 and 2 which shared a more similar physical structure. The dealate queen in Nests 1 and 2 were both located in the largest chamber of the nest which also contained a large number of eggs and small larvae. The males tended to be located in chambers towards the nest’s middle. Workers were found throughout the nests in all chambers but with indication of some concentrated in chambers adjacent to nest entrances, and those chambers towards the top of the nest. These uppermost chambers also contained large numbers of alate queens and larger larvae.



**Figure 3.** Panel showing the percentage of each ant life stage within each chamber of each of three nests of *Polyrhachis delecta* weaver ants.

Table 2. Overall details of the composition of ants in three nests of *P. delecta*

Caste or life stage	Nest 1						Nest 2						Nest 3						
	Totals						Totals						Totals						
	By chamber (%)						By chamber (%)						By chamber (%)						
	1	2	3	4	5	6	1	2	3	4	5	6	7	1	2	3	4	5	6
Adult ants																			
Dealate queens	1	0	0	0	100	0	1	0	0	0	100	0	0	0	0	0	0	0	0
Alate queens	79	14	27	42	11	3	92	17	22	27	21	3	9	1	6	33	67	0	0
Males	38	63	16	21	0	0	37	22	41	32	3	0	3	0	15	40	0	60	0
Workers																			
Within colony	394	12	13	7	33	12	446	16	12	12	19	24	11	7	183	4	11	10	28
Outside colony	27						19								33				39
Total	421						465								216				9
Brood																			
Pupae	37	35	14	30	22	0	64	33	14	28	22	3	0	0	18	12	88	0	0
Large larvae	53	0	17	17	66	0	21	14	5	14	67	0	0	0	10	30	70	0	0
Small larvae	19	0	0	0	100	0	35	0	23	0	77	0	0	0	5	0	100	0	0
Eggs	yes						yes								no				

## Discussion

We present here the first observations and descriptive data on the structure and composition of three nests of the weaver ant *Polyrhachis delecta*. There was evidence of segregation of castes within the various chambers in the nest and similarities in their spatial position between nests. We also noted the inclusion of tightly wrapped sections of silk, that we term here ‘girders’, which horizontally spanned a number of the larger chambers. These girders were extremely rigid compared to the rest of the nest construction and we hypothesise that they function to provide internal support to stop lateral compression and collapse of inner chambers and the nest as a whole.

Many ant colonies undergo seasonal variation in their size and composition, which is often best seen in the different rates of caste production (i.e. workers vs. sexuals) or the season in which brood may tend to be produced. Downes (2015) demonstrated seasonal fluctuations in the colony composition of *P. australis*. In their study, alate queens and alate queen pupae were mostly present only from July to December. In our study, conducted in July, we also found the presence of alate queens in quite high numbers. In addition, we found generally similar colony sizes to Downes (2015), with the exception of males which were more numerous in the colonies in our study. Compared to other *Polyrhachis* species these nest populations are small overall for the genus, many species of which have thousands of workers, but are more representative of species within the *Cyrtomyrma* subgenus as a whole (Dorow et al. 1990; Dorow 1995; Glaser 1997; Liefke et al. 1998). In this study we estimated that the proportion of foragers constituted around 5-18% of the total worker force, although returning foragers were only collected for 15 min after colony collection. In *Odontomachus brunneus* the foraging population found outside the nest was estimated to be 77% of the total workforce (Hart & Tschinkel 2011), much higher than we estimated here, but in general it is unknown



quite how the proportions of foragers varies depending on ant species or overall colony size (Tschinkel 1999) .

As the size of nests and number of chambers increases, the chance of a complete mixing of colony members decreases and thus larger nests may promote colony complexity through task differentiation or protection against disease through compartmentalisation (Sendova-Franks & Franks 1995; Naug & Camazine 2002; Naug 2008; Konrad et al. 2012). In this study there were indications of some degree of compartmentalisation of colony components to different chambers within the nest, although the potential for relocation of ants between collection and dissection, and the fact that the study was limited to only three nests means that these data must be treated cautiously. As with other social insect nests (Kugler & Hincapie 1983; Longino 1991; Ito et al. 1994; Baracchi & Cini 2014), the brood, and especially the queen, tended to be located away from the periphery of the nest, and usually away from the nest entrance. Additionally nest architecture is important in producing correct internal nest microclimates and brood may be moved within colonies in order to raise them at the correct temperature which may be in part responsible for the location of brood observed in this study (Sendova-Franks & Franks 1995; Tschinkel 1999). Further investigation of more *P. delecta* nests is needed to confirm the descriptive data provided here.

Polydomy occurs in a number of *Polyrhachis* species, some of which have been described as supercolonial (Yamauchi et al. 1987; van Zweden et al. 2007), and it is possible that the presence of the queenless, brood-bearing and otherwise healthy nest in this study is evidence for this in this species. Thus it is hard to know if assemblages collected here represent distinct colonies or, as we term them here, just one of possibly many ‘nests’ which comprise the colony as a whole. As three nests were collected for this study and all were relatively small, collected from only where nests were readily discoverable and easily collected, and from a limited geographic locality, it is likely that

there is selection bias in these results. Larger colonies or those located higher in vegetation, or at a different location, may differ significantly in their structure and colony composition. It is likely that there will have been some internal relocation of ants within nests between collection and dissection, so the data on the intranidal location of ants needs to be treated cautiously. Nonetheless Nests 1 and 2, which were similar in size and structure, seemed to also share similarities in their spatial location of ants.

Although general nesting patterns are quite well studied across *Polyrhachis* species, this study provides the first descriptive data of the interesting structure of *P. delecta* nests. We hope that this work will stimulate future more detailed studies on nest structure and composition in order to further explore the intricacies of this specialised form of nest construction, which may help to elucidate evolutionary patterns of nest-building and habitat preference in this highly diverse genus of ants.

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## Life-history information of Formicine social parasites and their hosts

*Polyergus* is an obligately duoltic (obligate slave-maker) social parasite that is completely dependent on the ants within the *Formica* subgenus *Serviformica* in order to survive (Topoff et al. 1989; King and Trager 2007). Individuals possess characteristic scythe-shaped mandibles which assist in their periodic group-raids of other colonies, where they retrieve brood which may be reared to eclosion and integrated into the worker population (Topoff et al. 1989; Topoff 1990). Young *Polyergus* queens are unable to found a colony on their own and will invade an existing *Serviformica* nest, kill or usurp the queen and take over the colony.

*Polyergus* workers lack the ability to feed, even when presented with food, or clean themselves or other ants, and will not care for brood (Topoff, Inez-Pagani, et al. 1985; Topoff, LaMon, et al. 1985; Topoff et al. 1989; Hölldobler and Wilson 1990). The facultatively duoltic species *Formica sanguinea*, a medium sized species with colonies of several thousands of individuals, however, retains some ability for its workers to undertake necessary day-to-day tasks within a colony (Snelling and Buren 1985; Mori et al. 2001). Individuals will feed and groom themselves and nestmates, can survive without slaves, and queens can found new colonies either on their own or through parasitism as in *Polyergus* (Mori et al. 2001). *F. sanguinea* will however conduct raids of *Serviformica* colonies, retrieving and raising workers from host colonies. The hosts themselves: *F. fusca*, *F. cunicularia* and *F. rufibarbis* tend to be smaller ants, but with colonies of many thousands of individuals, which, when not being raided, live as isolated independent colonies – foraging for scavenged food and tending to start

new colonies as solitary foundresses or through colony fission (Bergström et al. 1968; Francoeur 1973; D’Ettorre et al. 2002). *Formica rufa* builds nests formed of sticks and conifer needles, with very large colonies often composed of several hundred thousand workers (Francoeur 1973; Laakso and Setälä 1997; Domisch et al. 2009). Workers forage for honeydew and non-selectively scavenge and predate invertebrates (Domisch et al. 2009).

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## Supplementary material for Chapter 6. Simple societies and sanitary syndromes

### Supplementary methods

#### Study species

We used ten colonies of *Dinoponera quadriceps*, a monomorphic, queenless, ponerine ant species with small, highly simple, societies collected from Bahia state, Brazil, in November 2014, and four colonies of *Acromyrmex echinator*, a myrmicine leaf-cutting ant with large, complex societies and highly polymorphic workers, collected in Gamboa, Panama, in 2011.

*Dinoponera* dinosaur ants are a monomorphic Neotropical Ponerine ant genus, considered the biggest ants of the world. They exhibit amongst the most primitive societies of all ants, with a simple linear dominance hierarchy headed by an inseminated worker or gamergate, instead of a distinct queen. *Dinoponera quadriceps* Santschi, 1921 forms colonies of 10s to a few 100s of individuals in subterranean nests. Workers are able to be categorised very simply into two groups: 1) low-rank workers that perform foraging activities outside the nest for arthropod prey, and guard and maintain the nest, and 2) higher-ranking workers that engage in helper activities, e.g. brood tending, mainly within the nest. (Choe and Crespi 1997; Monnin and Peeters 1997; Araújo and Rodrigues 2006; Nascimento et al. 2012; Sousa et al. 2012)

*Acromyrmex* leaf-cutting ants are highly polymorphic Neotropical Myrmicine genus that cuts leaves on which they grow a fungal crop as a food source. Unlike dinosaur ants, *Acromyrmex echinator* leaf-cutting ant colonies can reach 100,000s of individuals, headed by a very large distinct queen, and are organised into morphological and behaviourally distinct castes which vary by orders of magnitude in size. These

include enlarged soldiers which possess powerful mandibles for colony defence, through to miniscule worker ‘minims’ which live in the fungus. Leaf-cutting ants show much more distinct division of labour and task partitioning within colonies compared to dinosaur ants (Hölldobler and Wilson 1990; Schultz and Brady 2008; Waddington and Hughes 2010; Nygaard et al. 2011).

As a parasite we used the entomopathogenic fungus *Metarhizium pingshaense* (KVL02-73 isolated soil in Panama; (Pull et al. 2013)), which is a natural parasite of ants (Boomsma et al. 2005), but a generalist insect pathogen and thus unlikely to have coevolved with either ant species used here

### Colony care

All colonies were maintained in the laboratory at the University of Sussex at 23°C and 60% relative humidity for at least three months prior to use and were in good health at the time of the experiment. Dinosaur ant colonies had previously been extensively censused and the ages of all individuals were approximately known, and we used external foragers of similar size (mesosoma length:  $7.9 \pm 0.5$  mm; width:  $3.1 \pm 0.2$  mm) and age ( $> 8$  months) for the experiments. For the leaf-cutting ants, we also used external foragers of similar size (mesosoma length:  $3.2 \pm 0.2$  mm; width:  $0.78 \pm 0.02$  mm) and age (estimated from cuticular colouration; (Armitage and Boomsma 2010)). Foragers may be most likely to be exposed to fungal pathogens in the environment.

### Experimental set-up

Dinosaur ants were individually marked for identification with small numbered discs attached to the mesosoma, and individual ants were removed from the colony for observation and then replaced in their colony after each repeat. This was impractical in the much larger *A. echinator* colonies, so workers were housed in individual pots (70 x 35 mm, with sucrose solution and water supplied *ad libitum*) for the duration of the

experiment, and the experiment was done over a shorter time period to minimise the impact of segregation from the colony.

In order to check that this difference in methodology did not affect the results, an additional 20 dinosaur ants from different colonies were kept in individual pots and tested over 7 days to mimic the set-up for the leaf-cutting ants, and this confirmed that the difference in setup had minimal impact on the behaviour of the ants (See results section ‘housing comparison’ below).

### **Treatment protocol**

Metarhizium conidia, and talcum powder, suspensions were thoroughly vortexed to ensure homogeneity prior to use. Individual ants were gently held with soft forceps and the treatment was applied with a calibrated micropipette. Positive application of talcum powder treatment could be visually confirmed by the presence of a fine white coating to the ants cuticle after drying.

## **Supplementary results**

### **Housing comparisons**

We compared the effect on grooming behaviour of keeping dinosaur ants either a) in the colony or b) in separate pots during the grooming experiment, using a GLMM with gaussian distribution and log link function and colony included as a random factor. There was no significant difference in grooming durations between ants kept in their colonies or in pots for either control or talcum treated ants (respectively:  $F_{1,58} = 0.035$ ,  $p = 0.85$ ,  $F_{1,58} = 0.003$ ,  $p = 0.96$ ; Figure S6.1 p.267).

## Social immunity and personalities

There was a significant interaction between treatment and species on overall selfgrooming and allogrooming ( $F_{1,156} = 9.19$ ,  $p = 0.003$ ;  $F_{1,156} = 61.71$ ,  $p < 0.001$ ; respectively; Figure 6.1c-f; S6.2). There was no significant correlation between allogrooming and self-grooming in control-treated ants ( $r = 0.009$ ,  $n = 40$ ,  $p = 9.57$ ; Figure S6.3a). Leaf-cutting ants that had higher baseline levels of selfgrooming in the control treatment, showed a significant correlation with having higher selfgrooming rates after talc exposure ( $r = 0.34$ ,  $p = 0.034$ ,  $n = 40$ ; Figure S6.3b). There was no similar correlation between the treatments in leaf-cutting ant allogrooming durations ( $r = -0.133$ ,  $n = 40$ ,  $p = 0.41$ ; Figure S6.3c) or in the self-grooming of dinosaur ants or ( $r = 0.15$ ,  $n = 40$ ,  $p = 0.37$ ; Figure S6.3d).

## Personalities and disease resistance

In leaf-cutting ants treated with *Metarhizium* there was a significant positive correlation between allogrooming duration and survival in both control, and talcum-powder treated trials (respectively:  $r = 0.43$ ,  $n = 35$ ,  $p = 0.009$ ;  $r = 0.34$ ,  $n = 35$ ,  $p = 0.024$ ; Figure S6.4a-b), but no significant correlation between selfgrooming duration and survival in control trials ( $r = 0.2$ ,  $n = 35$ ,  $p = 0.25$  Figure S6.4c).

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## Attine life-history

Photo overleaf. The fungus growing ants form a monophyletic clade within the Myrmicinae which can be split based on their mode of agriculture. The leaf-cutting ants, which include *Acromyrmex* and *Atta*, are the most evolutionarily derived genera (c-d overleaf) and have the most sophisticated fungal agriculture where they cut fresh vegetation to cultivate their fungal crops. The higher agriculture genera of *Sericomyrmex* (a) and *Trachymyrmex* use found plant material on which to grow their fungus. The most basal genera belong to the lower agriculture group, including *Apterostigma* (b), which tend to use more diverse substrates for their fungus, including scavenged insect cadavers and other detritus. Within the lower agriculture groups there are also different fungal types which are grown, including ‘coral fungus’ and ‘yeast fungus’



