Year : 2017

SOCIAL IMMUNITY IN ANTS: ANTIPATHOGEN DEFENSES IN INCIPIENT COLONIES AND USE OF ANTIMICROBIAL RESIN

BRÜTSCH Timothée

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département d'Écologie et Évolution

SOCIAL IMMUNITY IN ANTS:

ANTIPATHOGEN DEFENSES IN INCIPIENT COLONIES AND USE OF ANTIMICROBIAL RESIN

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Timothée BRÜTSCH

Master en Biologie (BEC) de l'Université de Lausanne

Jury

Prof. Chin Bin Eap, Président
Prof. Michel Chapuisat, Directeur de thèse
Prof. Laurent Keller, expert interne
Prof. William Hughes, expert externe

Lausanne 2017

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département d'Écologie et Évolution

SOCIAL IMMUNITY IN ANTS:

ANTIPATHOGEN DEFENSES IN INCIPIENT COLONIES AND USE OF ANTIMICROBIAL RESIN

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Timothée BRÜTSCH

Master en Biologie (BEC) de l'Université de Lausanne

Jury

Prof. Chin Bin Eap, Président
Prof. Michel Chapuisat, Directeur de thèse
Prof. Laurent Keller, expert interne
Prof. William Hughes, expert externe

Lausanne 2017

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président·e	Monsieur	Prof. Chin-Bin **Eap**
Directeur·rice de thèse	Monsieur	Prof. Michel **Chapuisat**
Experts·es	Monsieur	Prof. Laurent **Keller**
	Monsieur	Prof. William **Hughes**

le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Timothée Brütsch

Master of Science de l' Université de Lausanne

intitulée

SOCIAL IMMUNITY IN ANTS :
ANTIPATHOGEN DEFENSES IN INCIPIENT COLONIES AND USE OF ANTIMICROBIAL RESIN

Lausanne, le 28 avril 2017

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Chin-Bin Eap

Remerciements

Je tiens avant tout à remercier Michel pour ces années passées dans son labo, pour son enseignement, pour ses précieux conseils et pour sa confiance.

Merci à Laurent Keller, Bill Hughes et Chin Bin Eap, pour avoir accepté de faire partie de mon jury de thèse, et pour le temps qu'ils m'ont accordé.

Merci à Jessica Purcell, Antoine Felden, Anabelle Reber, Geoffrey Jaffuel, Armelle Vallat-Michel, Ted Turlings et Amaury Avril pour nos collaborations scientifiques.

Merci à l'Union Internationale pour l'Étude des Insectes Sociaux ainsi que la Société Suisse de Zoologie, pour les bourses et prix accordés.

Merci à mes collègues devenus amis, en particulier Romain et Thibault pour les délires dans et hors du bureau, Jessica Purcell avec qui j'ai pu co-signer mon premier article, Joël et Anabelle qui m'ont passé le flambeau, Amaury le divin enfant, Antoine et Geoff qui m'a fait l'affront de finir sa thèse avant moi. Merci également à Sophie, Charlène, Christine, Zoé, Alex, Auré, Gordon, Fred, Rob, Caro, ptit Romain, Ivan, Laura, Albertine, Marie, Céline Si et bien d'autres qui ont égayé ces années de thèse.

Merci aux personnes qui font le DEE, au groupe fourmis et au groupe Chapuisat, en particulier : Amaury Avril, Dumas Gálvez, Christine Clavien, Nayuta Brandt, Amaranta Fontcuberta, Sacha Zahnd, Nathalie Stroeymeyt, Raphaël Braunschweig et Eric Lucas.

Merci aux personnes avec qui j'ai eu le plaisir de faire de l'assistanat : Daniel Cherix qui a probablement initié mon amour pour les fourmis étant enfant à travers un de ses livres, Tanja Schwander, Philippe Christe, Franck Chalard, Frédéric Schütz, Pierre Bize, Marco Zini ainsi que les étudiants.

Merci aux personnes qui m'ont aidé pour l'administratif, l'entretien des fourmis ou leur espace de vie ainsi que le labo: France Pham, Lena Kolecek, Caroline Betto-Colliard, Jason Buser, Laélia Maumary, Jezaëlle Rufener, Yvan Marendaz, Catherine Berney.

Merci à Roland Maurer, un biologiste parmi les psychologues, sans qui je n'aurai peut-être jamais étudié la biologie à l'Université de Lausanne.

Merci aux anciens de psycho, Jecker, Mytam et Oli.

Merci aux étudiants que j'ai eu le plaisir d'encadrer: Geoffrey Jaffuel, Morgan Gueuning, Patrick Joye, Bahram « Moody » Kheradmand, Zoé Daeppen, Lea Megali Aude Rogivue et Théodora Steiner.

Merci à l'Interface Sciences-Société et L'éprouvette, qui me permet de donner un sens à mon parcours de psychologue-biologiste, et pour leur patience pendant ces derniers mois de thèse, en particulier: Alain Kaufmann, Patricia Auberson, Séverine Trouilloud, Mathilde Ythier, Delphine Ducoulombier et Virginie Népoux, et merci à l'équipe de choc des Mystères de l'UNIL: Diego Salvadore et Nicolas Schaffter sans oublier Edy Ceppi et le reste de l'équipe.

Merci à l'équipe d'Ammonite de mettre en valeur les fourmis du Jura à travers un magnifique documentaire à venir avec le grand David Attenborough, et pour cette expérience unique. Merci à Arnaud de partager cette expérience avec moi.

Merci à Cédric, mon plus vieil ami (même si il fait jeune), mon frère, que j'ai connu bien avant d'être psychologue ou biologiste. Merci pour nos 17 ans de musique en commun, parce qu'il n'y a pas que la science dans la vie.

Merci à ma famille et en particulier mon père, intéressé par toutes choses, ma mère, passionnée par un nombre précis de choses, qui ont fait de moi quelqu'un de passionné pour toutes choses. Merci à ma sœur Alex-Pipou-Head-of-Death, pour qui j'ai toujours eu la plus grande admiration.

Merci à Delphine, pour les années passées et pour le fils magnifique qu'elle m'a donné.

Merci à Ethan, un petit garçon incroyable qui a changé ma vie et qui me pousse à être à la hauteur.

Merci à Céline, une femme exceptionnelle avec qui j'ai la chance de partager ma vie, pour sa patience, sa constante positivité, son intelligence et pour son soutien. Merci de partager ta vie avec mon petit bonhomme et moi. Que notre vie à deux et en famille soit aussi belle à l'avenir qu'elle l'a été ces dernières années.

Enfin, merci et pardon aux fourmis que j'ai dû sacrifier pour mieux les comprendre. J'espère que leur mort n'aura pas servi à rien.

Pour Ethan, parce que ta curiosité, ton intelligence et tes questionnements me font réaliser qu'être un scientifique, c'est peut-être rester un peu un enfant.

Table of contents

SUMMARY

Social insects, and particularly ants, are extraordinarily ecologically successful organisms. Social life confers many advantages, such as co-operation for brood-rearing or foraging. However, social insects may be particularly susceptible to disease transmission, because their colonies consist of often closely related individuals living in densely populated nests. To combat pathogens, social insects have access to individual immune defenses, and have also evolved collective defenses known as "social immunity". In the first part of this thesis, I studied how ants defend themselves against pathogens during particularly sensitive life stages: colony founding and brood rearing. I first tested the ability of queens to detect and avoid pathogenic fungi when establishing incipient colonies. Unexpectedly, *Formica selysi* queens were attracted rather than repelled by pathogenic fungi. I then tested the hypothesis that under pathogen threat, queens associate during colony founding in order to benefit from social immunity. In *F. selysi*, the presence of pathogens in a nesting site did not induce queen associations. Moreover, in incipient colonies of *Lasius niger*, the queens and their workers did not benefit from social immunity, and rather invested in individual immunity. Finally, in *F. selysi*, the ability of young workers to resist a fungal infection did not depend on their mother queen, nor on the number of workers that reared them. In the second part of this thesis, I studied the use of antibiotic resin by the wood ant *Formica paralugubris*. I found that wood ants collect more resin when brood is present in their nest, and that they place resin near the brood. I also discovered that ants produce a potent antibiotic by depositing self-produced formic acid on the resin. This thesis illustrates some unexpected aspects of the host-pathogen relationship during colony founding and provides important insights into how ants use antibiotic substances such as resin.

RÉSUMÉ

Les insectes sociaux, et particulièrement les fourmis, sont des organismes au succès écologique extraordinaire. La vie sociale confère de nombreux avantages, comme la coopération pour l'élevage du couvain ou la recherche de nourriture. En revanche, les insectes sociaux peuvent être particulièrement susceptibles à la transmission de maladies, car leurs colonies se composent d'individus souvent très apparentés vivant dans des nids densément peuplés. Pour lutter contre les pathogènes, les insectes sociaux possèdent des défenses immunitaires individuelles, et ont également évolué des défenses collectives ou « immunité sociale ». Dans la première partie de cette thèse, j'ai étudié comment les fourmis se défendent contre les pathogènes pendant des stades de vie particulièrement sensibles chez les fourmis: lors de la fondation d'une colonie et pendant l'élevage de couvain. J'ai d'abord testé la capacité des reines à détecter et éviter des champignons pathogènes lors de l'établissement de nouvelles colonies. De façon inattendue, les reines de *Formica selysi* étaient attirées plutôt que repoussées par des champignons pathogènes. J'ai ensuite testé l'hypothèse selon laquelle en présence de pathogènes, les reines s'associent pour fonder une colonie afin de bénéficier d'une immunité sociale. Chez *F. selysi*, la présence de pathogènes dans un site de nidification n'incitait pas les reines à s'associer. De plus, dans les nouvelles colonies de *Lasius niger*, les reines et leurs ouvrières ne bénéficiaient pas de l'immunité sociale, et investissaient plutôt dans l'immunité individuelle. Finalement, chez *F. selysi*, la capacité de jeunes ouvrières à résister à un champignon pathogène ne dépendait ni de leur mère, ni du nombre d'ouvrières les ayant élevées. Dans la deuxième partie de cette thèse, j'ai étudié l'utilisation de résine antibiotique par les fourmis des bois *Formica paralugubris*. J'ai constaté que les fourmis des bois recueillaient davantage de résine quand le couvain était présent dans leur nid, et qu'elles plaçaient la résine près du couvain. J'ai également découvert que les fourmis produisaient un puissant antibiotique en déposant de

l'acide formique sur la résine. Cette thèse illustre certains aspects inattendus de la relation hôte-pathogène lors de la fondation de colonies, et apporte des informations importantes sur la façon dont les fourmis utilisent des substances antibiotiques telles que la résine.

General introduction

Social insects

Social insects, and particularly ants, are extremely ecologically successful organisms (Wilson 1987). Ants have been present on earth since the Cretaceous, surviving a mass extinction (Schultz 2000). About 13 000 ant species are known today, with probably the double of this number still to discover (Ward 2014). They represent on average 15-20% of the terrestrial animal biomass and up to 25% in certain areas (Schultz 2000). Ants are distributed widely on the planet, being absent only in Antarctica, Greenland, Iceland, and a few remote islands (Wilson 1987).

Because of their ecological success, ants have an important impact on ecosystems. In their natural range, they play key roles as predators, herbivores, or as ecosystem engineers when building their nests (Sanders & van Veen 2011). When introduced outside their native range, they can have devastating effects. Of the 17 land invertebrates included in the world's 100 worst invasive alien species, 7 are social insects, with 5 ants, one wasp, and one termite species (Lowe et al. 2000)

One of the main reasons behind the ecological success of social insects is their social system, called eusociality. Eusociality, considered as one of the major transitions in evolution (Szathmáry & Smith 1995), implies an overlap of

generations, collective care of the young, and reproductive division of labor. Reproductive division of labor means that only queens and males reproduce, while workers perform other tasks in the colony. Among the workers, there is another division of labor often based on age or morphology. For instance, young ants typically begin their life as nurses inside the colony and will as they age switch to tasks like nest cleaning and will finish their lives as foragers (Mersch, Crespi & Keller 2013).

Defenses against pathogens in ants and other social insects

Almost all organisms are subject to parasites, and according to some estimations, almost half of animals on the planet are themselves parasites (Poulin & Morand 2000). Hosts and their pathogens engage in a co-evolutionary arms race, with hosts continuously evolving new defenses to escape pathogen innovations (Ebert & Hamilton 1996).

Social insects offer an interesting system to study pathogen-host interactions, as they can be studied both at the individual level and at the collective level (Cremer, Armitage & Schmid-Hempel 2007; Cotter & Kilner 2010). Social insects and other group living animals, because they live in dense populations, potentially suffer from higher risks of disease transmission (Myers & Rothman 1995). Social insects may be even more at risk than gregarious animals: They usually interact closely in temporally and spatially stable nests where temperature and humidity

is controlled, conditions that are ideal for the proliferation of pathogens (Cremer *et al.* 2007). Additionally, because workers in a colony are often sisters, they are closely genetically related, and may thus be vulnerable to the same pathogens (Shykoff & Schmid-Hempel 1991).

To defend themselves against parasites such as fungi, bacteria or viruses, social insects generally benefit from the same individual immune defenses as non-social insects (Siva-Jothy, Moret & Rolff 2005). In addition, sociality has allowed the evolution of collective defenses, known as "social immunity" (Cremer *et al.* 2007; Cotter & Kilner 2010). I will briefly present here some of the antipathogen defenses relevant for the understanding of this thesis.

A first line of defense is the ability to detect and avoid pathogens. This basic measure may be cost effective, as it allows to avoid infection damage and the mounting of a costly immune response (Schulenburg et al. 2009). Arthropods vary in their ability to detect and avoid pathogens (Baverstock, Roy & Pell 2010). Ants are generally able to detect pathogens but respond to it by grooming and sanitary behavior, rather than by avoidance (Ugelvig & Cremer 2007; Reber *et al.* 2011; Tragust *et al.* 2013a). Pathogen avoidance may be a particularly efficient strategy for founding ant queens searching for nest sites, as pathogens present in the soil are responsible for a high proportion of colony failures (Baer, Armitage & Boomsma 2006). In **chapter one**, we tested this ability in young *Formica selysi* queens.

If contact with the pathogen is inevitable, the host may remove infective particles from its cuticle by self-grooming. While self-grooming can be performed by both social and non-social insects, mutual grooming (or allogrooming) is restricted to social insects (Reber *et al.* 2011; Tranter & Hughes 2015) and sub-social insects with parental care (Boos et al. 2014). In some cases, grooming may be used prophylactically, for example to prevent potential contamination by individuals returning from a foraging trip (Morelos-Juárez *et al.* 2010; Reber *et al.* 2011).

Grooming may be used in combination with a variety of self-produced antimicrobial substances. When exposed to pathogens, many ant species groom their metapleural glands (Fernández-Marín et al. 2006), which are antimicrobial producing structures found exclusively in ants (Yek & Mueller 2011). Workers may then spread the antimicrobial substance on themselves, nestmates, brood, or even their nest (Fernández-Marín *et al.* 2006; Tranter *et al.* 2013). Similarly, some ants use acid produced by their venom gland to groom brood (Tragust et al. 2013a) or their nest (Tranter *et al.* 2013) as a protection against fungal infections. Other antimicrobials may be found in the trophallaxis regurgitates. For example, immune challenged workers have been shown to increase trophallactic behavior, and to produce antimicrobial droplets that increase the survival of immune challenged nestmates (de Souza *et al.* 2008; Hamilton, Lejeune & Rosengaus 2011).

Some ant species also retrieve antimicrobials from their environment. The wood ant *Formica paralugubris* introduces resin with antimicrobial properties into their nests (Christe et al. 2003). Resin decreases the microbial load in the nests and protects workers or larvae against fungal and bacterial pathogens (Christe et al. 2003; Chapuisat et al. 2007). Little is known about the mechanisms governing resin use. Previous studies demonstrated that resin is used prophylactically, and that in the field, preference for resin over other types of nest material is higher in spring and summer than in autumn (Castella, Christe & Chapuisat 2008b). In **chapter 4**, we tested if *F. paralugubris* workers increase resin collection when brood is in the nest, and if they place it strategically near the brood. In **chapter 5**, we investigated if workers process the resin they collect to increase its antimicrobial effect.

If the pathogens manage to pass the cuticle barrier, insects can mount individual internal immune responses. Insects lack antibodies (Söderhäll & Cerenius 1998) supposedly required to mount an adaptive immune response, as found in vertebrates. However, studies on various species have shown that a type of immune memory, or "priming" (Konrad et al. 2012) may persist during the life of an individual (Little & Kraaijeveld 2004) or even be passed to its offspring, the latter being often referred to as "trans-generational immune priming" (Roth et al. 2010; López et al. 2014). In ants, immune memory has been observed in workers, after contact with a contaminated nestmate (Ugelvig & Cremer 2007;

Konrad *et al.* 2012), in larvae (Rosengaus, Malak & MacKintosh 2013) and in queens under certain conditions in some species (Gálvez & Chapuisat 2014). Trans-generational immune priming has however so far never been observed in ants. Although a growing number of studies have documented immune memory in invertebrates, insights into the mechanisms responsible remain rare (Konrad et al. 2012) .

Insect innate immune responses, on the other hand, are well documented. Hemocytes (insect blood cells), which are part of the cellular immune response, are involved in the phagocytosis of small pathogens or encapsulation of larger parasites (Gillespie, Kanost & Trenczek 1997). An important part of the humoral immune response is the activation from prophenoloxidase into phenoloxidase, an enzyme involved in the melanization of damaged tissues or encapsulation of particles of microbial origin (Söderhäll & Cerenius 1998). Melanization is usually accompanied by the production of toxic compounds which may help the antibiotic process (Cerenius & Söderhäll 2004). Other humoral immune defenses include antimicrobial peptides and proteins present in the hemolymph (Gillespie *et al.* 1997).

An increased immune activity may also be prophylactic, to counter an infection risk, rather than an actual infection. For example, insects that temporarily live in groups, like locusts, upregulate their physiological immune response to counter a higher transmission risk (Wilson *et al.* 2002; Wilson & Cotter 2008). This may

be more complex in social insects, as being in group might not constitute a risk, but rather a protection, as they can take advantage of social immunity. Indeed, workers exposed to a fungal pathogen survived better when they were kept with nestmates than when they were isolated (Hughes, Eilenberg & Boomsma 2002). In **chapter 2**, we tested if the immune response of *Lasius niger* queens depended on immune challenges and the number of queens founding the nest.

At the colony level, genetic diversity in a colony may prevent the propagation of pathogens (Shykoff & Schmid-Hempel 1991). Genetic diversity among workers may be achieved by polyandry, where the females mate with multiple males, or by polygyny, when more than one queen is present in the nest. In the socially polymorphic ant *Formica selysi*, for instance, workers in experimentally diverse groups survived exposure to a fungal pathogen better than ants originating from a single colony (Reber et al. 2008). However, workers from monogynous colonies survived better to a fungal challenge than workers from polygynous colonies, suggesting that something else than genetic diversity affected the resistance of polygynous colonies. In **chapter 3**, we tested if the number of workers caring for the brood might be one of the contributing factors affecting disease resistance. Genetic diversity may allow a better resistance to pathogens through different mechanisms. For example, genetic diversity may allow faster antipathogen behavioral response (Ugelvig et al. 2010). Alternatively, herd immunity may prevent the spread of pathogens in the nest if a sufficiently high number of

workers are resistant (Anderson & May 1985). A genetically diverse colony may also have access to a more varied arsenal of self-produced antibiotics (Fernández-Marín *et al.* 2006; de Souza *et al.* 2008; Hamilton *et al.* 2011; Tragust *et al.* 2013a; Tranter *et al.* 2013), which may even be mixed to create more potent antimicrobial cocktails (Mason & Singer 2015).

Finally, a trade-off between individual and social immunity may occur (Cotter & Kilner 2010). For instance, in wood ants, the presence of antimicrobial resin in the nest allowed the workers to decrease some components of their individual immune system (Castella et al. 2008a).

Fungal parasites

Social insects can be infected by a variety of pathogens, such as fungi, bacteria or viruses. In this thesis, I focused on entomopathogenic fungi. I used *Metarhizium brunneum*, previously known as *Metarhizium anisopliae* (Bischoff, Rehner & Humber 2009) and *Beauveria bassiana*, two pathogens that are frequently used in experimental studies of disease resistance in social insects (Tragust *et al.* 2013a; Yek, Boomsma & Schiøtt 2013; Loreto & Hughes 2016). *Metarhizium* and *Beauveria* are generalist entomopathogens; widespread on the globe, they can infect a variety of insect species and are widely used as biological control agents (Meyling & Eilenberg 2007). They are obligate killers: to propagate their conidia (asexually produced spores), they have to kill the insect

so that the cadaver can sporulate (Ebert & Weisser 1997). Sporulation of the cadavers gives a clear diagnosis of the cause of mortality, which is convenient to study pathogen impact and host defenses.

Conidia, upon contact with an insect, attach to its cuticle with the secretion of an adhesive mucus. The conidia then germinate and penetrate the cuticle with a germ tube and appresorium. This occurs with the combination of mechanical pressure, and the help of enzymes. The fungus then grows inside the insect body cavity, or haemocoel (Hajek & St Leger 1994; Thomas & Read 2007).

Aims of the PhD

This PhD has two main goals. The first one is to better understand antipathogen defenses in incipient colonies and the second one is to study the use of antimicrobial resin in wood ants.

In the first part of this thesis, I investigated the defenses against pathogens at particularly sensitive life stages: during colony founding and brood rearing. In **chapter 1**, we examined if young ant queens founding a colony were able detect and avoid fungal entomopathogens potentially present in nest sites. We used queens of *Formica selysi* (the Alpine silver ant), a pioneer species that nests in the soil, usually in sandy banks of rivers. Several species of fungal entomopathogens, including *M. brunneum* and *B. bassiana* have been found in the soil, near the Rhône river, where our study population is located (Reber &

Chapuisat 2012a). We then tested the hypothesis that young queens associate to benefit from social immunity. As *F. selysi* is socially polymorphic, with monogynous (one queen per nest) and polygynous (several queens per nest) colonies in the same area (Purcell *et al.* 2014), we tested if a contaminated nest site was a factor enticing queens to associate when founding a colony. Following the same idea, we tested in **chapter 2** if *Lasius niger* (the black garden ant) queens founding nests in temporary associations survived exposure to a fungal pathogen better than queens founding alone. We also examined if their investment in individual immunity depended on the presence of the pathogen or of other queens in their nest. *Lasius niger* is strictly monogynous, but queens sometimes associate temporarily to initiate colonies (Sommer & Hölldobler 1995). As soon as the workers emerge, queens engage in deadly fights, with only one queen remaining (Sommer & Hölldobler 1995). In **chapter 3**, we tested if the survival of young *F. selysi* workers exposed to the fungal pathogen *B. bassiana* depended on the number of workers that reared them (worker brood ratio) and their mother queen.

In the second part of this thesis, I investigated how the wood ant *Formica paralugubris* uses antimicrobial resin. *Formica paralugubris* live in coniferous forests, where they build large nests with conifer needles and small twigs. This species is highly polygynous, with each nest containing up to a thousand queens (Chapuisat & Keller 1999). In the Jura mountain, they form a supercolony of

about 1200 interconnected nests showing no aggression between them (Chapuisat, Goudet & Keller 1997). *F. paralugubris* workers collect pieces of conifer resin which they place into their nests. These pieces of resin have antibiotic properties, and protect workers or larvae from bacteria and fungi (Chapuisat *et al.* 2007). Little is known about how the ants use resin, aside from its prophylactic use (Castella et al. 2008b). In **chapter 4**, we asked if wood ants strategically place resin in the nest. Specifically, we tested if wood ants increased the amount of resin in the nest when brood is present, and if they place pieces of resin near the brood. In **chapter 5**, we tested if wood ants enhance the antibiotic activity of resin by using self-produced chemicals like formic acid.

Chapter 1: Ant queens (Hymenoptera: Formicidae) are attracted to fungal pathogens during the initial stage of colony founding

Timothée Brütsch, Antoine Felden, Anabelle Reber & Michel Chapuisat

Brütsch, T., Felden, A., Reber, A., & Chapuisat, M. (2014). Ant queens (Hymenoptera: Formicidae) are attracted to fungal pathogens during the initial stage of colony founding. *Myrmecological News*, *20*, 71–76.

ABSTRACT

Ant queens that attempt to disperse and found new colonies independently face high mortality risks. The exposure of queens to soil entomopathogens during claustral colony founding may be particularly harmful, as founding queens lack the protection conferred by mature colonies. Here, we tested the hypotheses that founding queens (i) detect and avoid nest sites that are contaminated by fungal pathogens, and (ii) tend to associate with other queens to benefit from social immunity when nest sites are contaminated. Surprisingly, in nest choice assays young *Formica selysi* (Bondroit, 1918) queens had an initial preference for nest sites contaminated by two common soil entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium brunneum*. Founding queens showed a similar preference for the related but non-entomopathogenic fungus *Fusarium graminearum*. In contrast, founding queens had no significant preference for the more distantly related non-entomopathogenic fungus *Petromyces alliaceus*, nor for heat-killed spores of *B. bassiana.* Finally, founding queens did not increase the rate of queen association in presence of *B. bassiana*. The surprising preference of founding queens for nest sites contaminated by live entomopathogenic fungi suggests that parasites manipulate their hosts or that the presence of specific fungi is a cue associated with suitable nesting sites.

INTRODUCTION

Hosts have several lines of defense to resist parasites and pathogens. First, they may avoid contact with the pathogens. If this behavioral defense fails, they may prevent pathogens from entering into their body, and finally stop pathogens from multiplying in their organs, generally by activating the immune system (Schmid-Hempel & Ebert 2003; Siva-Jothy *et al.* 2005). Pathogen avoidance may be particularly cost effective, as it minimizes pathogen-induced damage and avoids the costs of mounting an immune response (Schulenburg et al. 2009).

In arthropods, the ability to detect and avoid pathogens varies among species (Baverstock et al. 2010). For example, mole crickets tunneling through soil avoid generalist fungal entomopathogens (Villani *et al.* 2002; Thompson & Brandenburg 2005) such as *Beauveria bassiana* and *Metarhizium anisopliae*. In contrast, parasitoid wasps and potato beetles appear unable to detect *B. bassiana*, or do not perceive it as a threat (Lord 2001; Klinger, Groden & Drummond 2006). The host reaction may vary with conditions and life stage. For example, common flowerbugs avoid *B. bassiana* on leaves but not in soil (Meyling & Pell 2006), and Japanese beetle larvae keep away from soil contaminated by *M. anisopliae*, whereas adults increase oviposition in presence of the parasite (Villani et al. 1994). In a few cases, arthropods were attracted to the fungal pathogens. Specifically, collembolans showed a preference for substrate containing *B. brongnartii*, *B. bassiana* and *M. anisopliae* conidia (Dromph & Vestergaard

2002), and mosquitoes were attracted to spores of *B. bassiana* and *M. anisopliae*, as well as to *B. bassiana* infected caterpillars (George et al. 2013).

Social insects live in dense groups and often nest in soil, which exposes them to fungal entomopathogens. The ability of termites to detect and avoid *B. bassiana* and *M. anisopliae* has been well documented (Mburu et al. 2009; Rath 2010; Yanagawa et al. 2012). Ants are also generally able to detect these fungal pathogens. For example, *Formica selysi* (Bondroit, 1918) workers increased the rate of self-grooming (Reber et al. 2011) when exposed to *M. brunneum*, and *Lasius neglectus* (Van Loon, Boomsma & Andrásfalvy 1990) workers increased brood care and sanitary behavior in presence of contaminated workers or brood (Ugelvig & Cremer 2007; Tragust *et al.* 2013a). However, in contrast to termites, ants did not seem to avoid the pathogens. Indeed, contaminated individuals were not avoided, and were intensely groomed by nestmates (Reber et al. 2011; Konrad et al. 2012). Whether ants avoid direct contact with fungal pathogens in other contexts deserves to be further investigated.

Avoiding pathogens may be particularly important for young ant queens attempting to found a new colony independently, without the help of workers. Indeed, in soil-nesting species, generalist fungal entomopathogens appear to be responsible for a considerable rate of failures during colony founding (Baer et al. 2006). Lone founding queens lack the protection conferred by mature colonies, which may be mediated by allo-grooming (Walker & Hughes 2009; Reber *et al.*

2011), group diversity (Hughes & Boomsma 2004; Reber *et al.* 2008), sharing of antibiotic substances (Fernández-Marín et al. 2006; Chapuisat et al. 2007; Hamilton et al. 2011; Tragust et al. 2013a) or other forms of social immunity (Traniello, Rosengaus & Savoie 2002; Ugelvig & Cremer 2007; Konrad *et al.* 2012). Additionally, founding queens may be more susceptible to pathogens if they found claustrally (without foraging), as deprivation of food has been shown to affect immunity in insects (Siva-Jothy & Thompson 2002).

Another interesting hypothesis is that ant queens founding new colonies in nest sites that are contaminated by fungal parasites might increase their chances of success by associating with other queens. In line with this hypothesis, workers are more resistant to fungal parasites when they are in groups than when they are alone (Hughes et al. 2002; Johnson 2004). The influence of the presence of parasites on the propensity of queens to associate with other queens during colony founding has not been investigated so far.

Here, we studied the impact of the presence of fungi on the founding behavior of ant queens. The study species, *Formica selysi*, nests in the soil, where it is naturally exposed to fungal entomopathogens (Reber & Chapuisat 2012a) such as *Metarhizium brunneum* (formerly *M. anisopliae* Bischoff *et al.* 2009) and *Beauveria bassiana*. The frequency of pleometrosis in the field is unknown, but laboratory studies have shown that queens are able to found colonies

independently as well as in association with other queens (Reber, Meunier & Chapuisat 2010).

In a series of experiments, we tested whether founding queens detected and showed behavioral resistance to fungal entomopathogens during this crucial and exposed stage of their life-cycle. We first tested if young *F. selysi* queens avoided nest sites contaminated by *M. brunneum* and *B. bassiana*. We further examined if queens discriminated nest sites containing non-pathogenic fungi, and whether they distinguished between live and heat-killed *B. bassiana*. Finally, we tested if queens tended to associate with other queens when they had to found colonies in sites contaminated by *B. bassiana.*

MATERIAL AND METHODS

Ants sampling and experimental mating

We collected *F. selysi* ants from a well-studied population along the Rhône river between Sierre and Susten in Valais, Switzerland (Chapuisat, Bocherens & Rosset 2004). In summer 2009 and 2010, prior to the nuptial flight, we collected young males, young virgin queens, sexual pupae and workers. We transferred them to the laboratory, where we let the pupae hatch into queens and males. We kept queens and males in separate laboratory colonies, to prevent uncontrolled

mating. We supplied the ants with *ad libitum* water and jelly made of eggs, honey and agar, and maintained them at 25°C with a 12/12 h dark/night cycle.

Experimental mating took place outside, in the morning, under direct sunlight. We placed two virgin queens from the same colony with 5-10 males from several other colonies in a mating box (Reber et al. 2010). After mating, we kept each young queen in a glass tube with humid cotton wool until the beginning of the experiment.

Experimental nests

The young, freshly mated queens had to found colonies in experimental nests made of glass test tubes (10 cm long and 1.5 cm in diameter). The bottom of each tube was filled with water retained by small cotton wool plugs, and the tubes were wrapped in black paper. Such dark humid tubes constitute good nest sites, mimicking natural holes that are powerful attractants for founding queens (Tschinkel 1998). Each experimental nest contained a piece of filter paper (6 x 2 cm) on which we deposited either 500 µl of a solution of fungal spores diluted in 0.05% Tween 20 (fungal treatment), or 500 µl of spore-free 0.05% Tween 20 (control; e.g. Chapuisat et al. 2007; Reber et al. 2008). We placed the tubes in arenas (plastic boxes 13.5 cm long x 15 cm wide x 5 cm high) lined with Fluon to prevent queens from escaping.

Impact of pathogens on nest choice

We staged nest choice assays to investigate (i) if queens avoided founding colonies in sites contaminated by fungal entomopathogens (ii) if the presence of non-entomopathogenic fungi also affected their nest choice. In these tests, the queens had to choose between a nest containing fungal spores and an identical but spore-free control nest. We tested the choice of queens with respect to the presence of *B. bassiana* and *M. brunneum*, two fungal entomopathogens that are common in the site where we sampled the ants (Reber & Chapuisat 2012a), *Fusarium graminearum*, a pathogen of plants belonging to the same order as *B. bassiana* and *M. brunneum* (the Hypocreales), and *Petromyces alliaceus*, a non-entomopathogenic, phylogenetically more distant fungus belonging to another class (the Eurotiomycetes). We did not detect *F. graminearum* in the site where we sampled the ants, whereas *P. alliaceus* was very common (Reber & Chapuisat 2012a). These four species of fungi belong to the subdivision Pezizomycotina in the Ascomycota.

We tested 80, 62, 40 and 40 queens for *B. bassiana, M. brunneum*, *F. graminearum* and *P. alliaceus*, respectively, depending on the number of queens available at the time of the experiment. We used fungal solutions at 8.9×10^7, 3.5×10^7, 7.8×10^5 and 1.5×10^7 spores/ml, respectively. We selected these concentrations to account for the marked size differences between the spores of the four fungal species, in particular the larger spores of *F. graminearum*. These

concentrations also tend to compensate for the marginally higher lethality of *M. brunneum*, as compared to *B. bassiana* (Reber & Chapuisat 2012a).

We introduced one queen in the middle of each arena. We recorded the position of each queen first shortly after introduction and then on a daily basis over a period of 13 days (nine days for *B. bassiana*). We analyzed the initial choice of queens, which was given by the first nest in which we found them. We also examined in which nests the live queens were on the last day. When a queen died, we surface sterilized its corpse with 14% bleach and kept it in an Eppendorf tube with wet cotton wool to check if it died from infection and produced fungal spores (Reber et al. 2011).

To get insight into temporal variation in the position of queens, we analyzed (i) the number of days spent in the initial nest, (ii) the proportion of queens that visited another nest and (iii) the total number of nest switches made by the queens, relative to the duration of the experiment. We examined if these measures of queen movements depended on the fungus used in the assay, on whether the first nest chosen was inoculated by a fungus or not, or on an interaction between these two factors. We only report significant results for these analyses.

In a follow-up experiment, we examined whether founding queens discriminated between live and dead spores of *B. bassiana*. We tested 120 queens, which had to choose between three nests sites, one inoculated with live spores (9.2×10^7 spores/ml), one with heat-killed spores (9.3×10^7 spores/ml) and one with control

solution. We monitored the position of the queens over a period of 13 days. For the heat-killed treatment, we autoclaved the fungus solution at 121°C for 20 minutes. With a microscope, we checked that the sterilization process had not affected the external structure of the spores. We also confirmed that the heat-killed spores did not grow on malt extract agar nutritive medium.

Impact of pathogen on queen association

To test if the presence of *B. bassiana* spores influenced the propensity of queens to associate with other queens during colony founding, we placed two queens in an arena containing a single nest site, which was either contaminated by spores of *Beauveria bassiana* (6.9×10^7 spores/ml) or contained the usual control solution. We tested 152 pairs of queens that we associated at random. Half of the pairs had access to a contaminated nest site, and the other half to a control nest site.

We monitored the position of the queens over a period of six days, recording if they were in or out of the nests. We estimated the initial frequency of queen association as the proportion of nests that contained two live queens on the second day of the experiment. At this time, 96% of the nests contained at least one queen. We analyzed the data with a general linear model including the type of nest (contaminated *vs.* uncontaminated) and whether the queens in a pair originated from the same or from different field colonies as explanatory variables. All

statistical analyses were performed in R 3.0.2 (R Core Team 2016). In all tests where an initial and final measure were analyzed, we adjusted the *p*-values with a Bonferroni correction to account for multiple comparisons.

RESULTS

Impact of pathogens on nest choice

Surprisingly, founding queens showed a strong initial preference for nests containing fungal pathogens. When given the choice between a nest contaminated with *B. bassiana* and a spore-free control nest, 75% of the 72 queens that entered a nest were first found in the contaminated one (Fig 1a; exact binomial test: $p < 0.0001$; all p-values are two-tailed; eight queens died without entering any nest). In the tests with *M. brunneum*, 67.7% of the 62 queens were first found in the nest containing the fungal pathogen (Fig 1a; exact binomial test: $p = 0.014$).

Founding queens also showed a strong initial preference for nests containing the non-entomopathogenic but phylogenetically close fungus *F. graminearum*: 80% of the 40 queens chose the nest containing the fungus (Fig 1; exact binomial test: $p = 0.0004$). The preference was weaker and not statistically significant when we tested the non-entomopathogenic but phylogenetically more distant fungus *P. alliaceus*: 65% of the 40 queens chose the nest containing the fungus (Fig 1a; exact binomial test: $p = 0.16$).

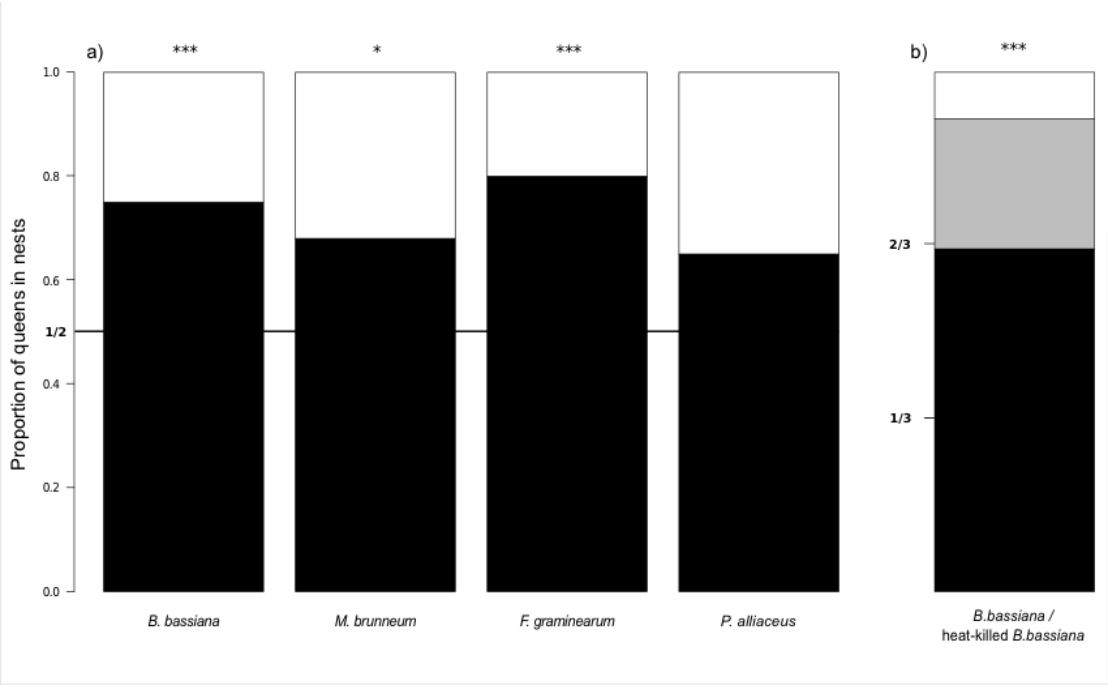


Figure 1: a) Proportion of queens that were first found in nests containing the fungi *B. bassiana*, *M. brunneum, F. graminearum* and *P. alliaceus* (black bars), as compared to control nests (white bars). b) Proportion of queens that were first found in nests containing live *B. bassiana* (black bar), heat-killed *B. bassiana* (grey bar) and control (white bar). Horizontal bars indicate "no choice", at 50% for a) and 1/3 and 2/3 for b). Asterisks signal significant deviations from 1/2 for a) and 1/3 for b) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The number of days spent by the queens in the initial nest depended on the fungus ($\chi^2 = 10$, $df = 3$, $p = 0.018$; it was higher in assays involving *F. graminearum*). On average and across all fungi tested, the queens that had initially chosen a

fungus-inoculated nest stayed for longer in it than queens that had initially chosen a spore-free nest (fungus *vs*. spore-free: 3.7 ± 2.6 days *vs.* 2.4 ± 1.7 days, respectively; $\chi^2 = 9.3$, $df = 1$, $p = 0.002$), and made fewer relative nest switches thereafter (fungus *vs*. spore-free: 0.18 ± 0.12 *vs.* 0.22 ± 0.13; $\chi^2 = 5.1$, $df = 1$, $p = 0.024$). For the proportion of queens that switched nests, there was a significant interaction between the fungus species and the type of nest initially chosen ($\chi^2 = 12.5$, $df = 3$, $p = 0.006$). This is because, in contrast to the pattern observed with the other three fungi, queens that had initially chosen a nest inoculated with *P. alliaceus* were more likely to visit another nest than the queens that had initially chosen a spore-free nest.

Overall, the mortality of queens depended on the fungi they were exposed to (binomial test, entomopathogens vs non-entomopathogens: $\chi^2 = 12.2$, $df = 1$, $p = 0.0005$). A larger proportion of queens died in treatments where one of the two nest sites was contaminated by an entomopathogenic fungus than by a non-entomopathogenic fungus (27.5% and 11.3% for *B. bassiana* and *M. brunneum,* respectively, versus 2.5% and 0% for *F. graminearum* and *P. alliaceus*, respectively). Eight out of the 22 queens that died during the test involving *B. bassiana* produced the typical white spores of this pathogen. None of the queens that died during the test involving *M. brunneum* sporulated with this pathogen. However, two queens that died after the end of the experiment produced *M. brunneum* spores, which confirms that this fungus was infectious.

By the end of the experiment, fewer queens remained in the nests, and the initial preference for contaminated nests was much reduced or non-existent. In the test with *B. bassiana*, 63.5% of the 52 live queens that were still in a nest at the end of the experiment were in the contaminated one (exact binomial test: $p = 0.14$), while in the test with *M. brunneum* this was the case of 50.9% of the 55 queens (exact binomial test: $p = 1$). In the test with *F. graminearum*, 55.3% of the 38 queens in a nest occupied the one containing spores (exact binomial test: $p = 1$), while in the test with *P. alliaceus* significantly more queens were found in the control nest (71.9% of the 39 queens found in a nest; exact binomial test: $p = 0.019$).

This change in the position of queens over time was not entirely explained by queen mortality: at the end of the experiment, we detected no significant preference for contaminated nests when we included dead queens in the analysis (exact binomial tests: *B. bassiana*: $p = 0.098$, *M. anisopliae*: $p = 0.79$, *F. graminearum*: $p = 0.63$, *P. alliaceus*: $p = 0.009$).

When given a choice between nest sites inoculated with live *B. bassiana*, heat-killed *B. bassiana* or a control, the queens significantly preferred the nest with the live entomopathogen (66%, 25%, 9% were found in each type of nest, respectively: Fig. 1b; $\chi^2 = 58.3$, $df = 2$, $p < 0.0001$; $n = 116$ queens, as four queens died without entering any nest site). By the end of the experiment, a marginally higher proportion of the queens remained in the nest with the live fungus (out of

the 101 queens that were still in a nest at the end of the experiment, 44%, 27% and 29% were in a nest with live *B. bassiana*, heat-killed *B. bassiana* and control, respectively; $\chi^2 = 5.8$, $df = 2$, $p = 0.11$).

Impact of pathogen on queen association

When two queens had access to a single nest during colony founding, the presence of the fungal pathogen *B. bassiana* in the nest had no significant influence on the initial propensity of queens to associate. On the second day of the experiment, 57.9% of the 76 contaminated nests contained two live queens, as opposed to 50% of the 76 control nests ($\chi^2 = 0.95$, $df = 1$; $p = 0.33$). Whether the queens originated from the same or from different field colonies had no significant influence on their initial propensity to associate ($\chi^2 = 0.005$, $df = 1$; $p = 0.95$). On the last day of the experiment, the presence of *B. bassiana* still had no significant impact on the frequency of queen association: 22.4% of the contaminated nests contained two live queens, as opposed to 25% of the control nests ($\chi^2 = 0.15$, $df = 1$, $p = 1$).

DISCUSSION

Ant queens founding new colonies independently are under high risk of dying from exhaustion (Camargo et al. 2011), desiccation (Mankowski & Morrell 2011), competition (Adams & Tschinkel 1995), predation (Nickerson et al. 1975) or

infection by parasites (Baer et al. 2006). As the presence of fungal pathogens in the soil jeopardizes the survival of the queen and her first brood, we expected that queens would avoid settling in nests contaminated by entomopathogenic fungi. In sharp contrast to this expectation, in nest choice assays *F. selysi* queens showed a strong and significant initial preference for nests contaminated by the common generalist fungal entomopathogens *B. bassiana* and *M. brunneum,* as opposed to spore free control nests. This preference of ant queens for entering contaminated nest sites is surprising, because *B. bassiana* caused significant mortality to the queens in this experiment, and both *B. bassiana* and *M. brunneum* killed workers in other experiments (Chapuisat *et al.* 2007; Reber *et al.* 2008; Purcell, Brütsch & Chapuisat 2012). Moreover, the two pathogens are common in the natural habitat of this ant species (Reber & Chapuisat 2012a).

Founding queens showed a similar preference for *Fusarium graminearum*, a plant pathogen belonging to the order Hypocreales, which also contains *B. bassiana* and *M. brunneum*. In contrast, founding queens had no significant initial preference for the more distantly related non-entomopathogenic fungus *Petromyces alliaceus*. There was a trend, however, and the sample size was lower, so that a general initial preference for fungi can't be excluded. Finally, the queens showed a much stronger preference for live than for heat-killed spores of *B. bassiana*. Together, these results indicate that queens are attracted to live fungi

belonging to the order Hypocreales, which are parasites of plants, invertebrates, or even other fungi (Spatafora et al. 2007).

The unexpected preference of founding queens for nest sites containing live entomopathogenic fungi may be explained in several ways. First, the fungal pathogens may manipulate their hosts, luring them with odor cues in order to increase infection probability. *Beauveria* and *Metarhizium* attract collembolans (Dromph & Vestergaard 2002), as well as mosquitoes (George et al. 2013). The hypothesis that *Beauveria* and *Metarhizium* manipulate uninfected insect hosts deserves further investigation (George et al. 2013). Records of pathogens attracting their hosts are indeed rare, as hosts are under strong selection to resist manipulation and avoid virulent pathogens (Poulin, Brodeur & Moore 1994).

Second, the presence of fungi may be a cue associated with suitable nesting sites, or may provide some direct or indirect benefits to the queens. For example, the presence of fungi may indicate favorable ecological conditions, for example humid, humus-rich soil. The queens might theoretically feed on fungi, but this seems unlikely given the lack of records of such behavior (Sanders 1964; Ayre 1967; cited by Cannon & Fell 2002).

Finally, contact of the queen with a pathogen might improve the defense of her offspring against the same pathogen. This process, known as "trans-generational immune priming", has been observed in diverse invertebrate taxa, such as bumble-bees (Moret & Schmid-Hempel 2001; Sadd *et al.* 2005), *Daphnia* (Little

et al. 2003), moths (Tidbury, Pedersen & Boots 2011) and beetles (Roth et al. 2010). The potential occurrence of trans-generational immune priming deserves to be investigated. It would however be surprising, given that trans-generational immune priming has not been reported in ants so far, and that we found no evidence of individual immune priming in workers (Reber & Chapuisat 2012b) or queens (Gàlvez & Chapuisat, unpublished results) of *F. selysi*.

Some queens died during the course of the experiment, and some moved between nests, so that in the assays involving pathogenic fungi the queens tended to be equally present in inoculated and spore-free nests by the end of the experiment. Queen movement did not appear to be prompted by the perception of queens that they were, or could be, infected. We found no significant difference between queens that initially entered contaminated nests and queens that initially entered spore-free nests for the number of days spent in the initial nest or the probability to abandon it. Only queens that initially entered *P. alliaceus*-inoculated nests were more likely to leave, suggesting that they were repelled by this non-entomopathogenic fungus. Indeed, in the assay involving *P. alliaceus*, most of the queens were in spore-free nests at the end of the experiment. A recording of queen movements in real time over a longer period, until the queens begin to lay eggs, would be useful to examine how initial nest choice, nest switching and mortality jointly determine the final settlement and success of the queens.

We also tested if the propensity of queens to associate with other queens during colony founding increased when nest sites were contaminated by *B. bassiana.* Indeed, by joining others, queens might benefit from allo-grooming or from other forms of social immunity (Cremer et al. 2007; Reber et al. 2011). However, founding queens did not increase the rate of queen association in presence of *B. bassiana*, which indicates that pleometrosis is not a conditional response to benefit from social immunity when the risk of infection is high.

Overall, our results indicate unexpected patterns in the colony founding behavior of ant queens in presence of fungi, including entomopathogens. Indeed, the queens did not avoid the fungal pathogens and one of the non-entomopathogens tested – to the contrary, they showed an initial preference for spore-inoculated nest sites. This surprising and potentially fatal attraction might result from parasite manipulation, or may be associated with correlated factors that are normally beneficial to the queens.

Acknowledgements

We thank Pierre-Henri Dubuis for providing us with the *F. graminearum* strain, Alain Reymond for his help in the lab, Jessica Purcell, Line Ugelvig and an anonymous reviewer for helpful comments on the manuscript. The study was supported by grant 31003A_125306 from the Swiss National Science Foundation to MC.

Chapter 2: No evidence for social immunity in co-founding queen associations

Timothée Brütsch, Amaury Avril & Michel Chapuisat

ABSTRACT

Colony founding by ant queens is a risky enterprise. In many species, queens establish incipient colonies either alone or in transient associations that last until the first workers emerge. Queens that associate during colony founding may benefit from improved disease resistance due to mutual grooming, sharing of antimicrobials, or higher genetic diversity among their workers. To test if queens in groups profit from such social immunity, we manipulated the number of queens in founding associations of the ant *Lasius niger* and measured their resistance to a common soil entomopathogen, *Metarhizium brunneum*. We also examined variation in queens' individual immunity. If queens in groups benefit from social immunity, we predict that they will invest less in individual immunity. On the contrary, in absence of social immunity, queens in groups are likely to increase individual immunity in order to respond to the higher risk of disease transmission. We found no evidence for social immunity in associations of founding queens. First, co-founding queens engaged in self-grooming, but performed very little allo-grooming or trophallaxis. Second, queens in associations did not show higher resistance to the fungal pathogen than solitary queens, and their workers were not more resistant either. Third, queens in groups had higher phenoloxidase activity, suggesting that they invest more in individual immunity than solitary queens and do not benefit from social immunity. Overall, our results provide no evidence that joint colony founding by *L. niger* queens increases their ability to resist fungal pathogens.

INTRODUCTION

In social insects, founding novel colonies is a precarious enterprise. Many ant species produce massive numbers of queens that fly away from their natal nest, mate, and seek to establish colonies independently (Keller 1991; Sommer & Hölldobler 1995). Incipient colonies are vulnerable to predation, competition and disease – their mortality has been estimated to be as high as 95% (Baer *et al.* 2006). Ant queens that are able to found colonies independently often associate with other queens, a mode of foundation called pleometrosis (Sommer & Hölldobler 1995; Bernasconi & Strassmann 1999). These associations are transient: after the first workers emerge, the queens fight to death, until only one remains (Sommer & Hölldobler 1995). Hence, joint colony founding is a gamble for queens, with maximal costs to losers and large benefits to the winner (Bernasconi & Strassmann 1999).

A major potential benefit of joint colony founding by ant queens is increased disease resistance, which might stem from various mechanisms conferring social immunity (Pull, Hughes & Brown 2013; Brütsch *et al.* 2014). Queens in group may benefit from mutual grooming (Reber *et al.* 2011). They may also share antimicrobial substances from the metapleural glands (Fernández-Marín *et al.* 2006), the venom gland (Tragust *et al.* 2013a), or in trophallactic fluids (Hamilton *et al.* 2011). Finally, queens in associations will produce groups of workers that are genetically more diverse (Sommer & Hölldobler 1995; Aron, Steinhauer &

Fournier 2009), and which might therefore better resist pathogens (Reber *et al.* 2008; Ugelvig *et al.* 2010).

The benefits from social immunity might be crucial during claustral colony founding, when the queens rely on their body reserves and are under strong energetic stress (Baer *et al.* 2006). Indeed, queens in groups may decrease their investment in energetically costly individual immunity, if they are protected by social immunity (Cotter & Kilner 2010). Such a trade-off has been documented in wood ants, who showed lower activation of their immune system when antimicrobial resin was present in their nests (Castella *et al.* 2008a). Conversely, in absence of social immunity, queens in groups are likely to increase individual immunity in order to respond to the higher risk of disease transmission (Elliot & Hart 2010; Godfrey 2013; Meunier 2015). Such density-dependent prophylaxis has been documented in desert locusts and thrips (Wilson & Reeson 1998; Wilson *et al.* 2002; Turnbull *et al.* 2011).

So far, evidence for social immunity in associations of co-founding ant queens have remained elusive. In *Lasius niger*, queens founding in pairs did not engage in allo-grooming and did not show higher survival than solitary queens when exposed to the fungal pathogen *Metarhizium pingshaense* (Pull *et al.* 2013). In *Formica selysi*, the presence of a fungal pathogen in the nest did not incite queens to associate, as would be expected if joint colony founding would increase their ability to resist the pathogen (Brütsch *et al.* 2014).

Here, we investigated if ant queens that found colonies in associations benefit from social immunity and modulate their individual immunity. We established experimental incipient colonies of *L. niger* with one, two or four queens, respectively, and tested their resistance to a common soil entomopathogen, *Metarhizium brunneum.* We also recorded the grooming behavior of queens, monitored some components of their individual immune system, and tested the fungal resistance of their workers. If ant queens in associations profit from social immunity, we predict that co-founding queens will (i) better resist to the fungal pathogen than solitary queens, (ii) engage in allo-grooming, (iii) decrease their investment in individual immunity, and (iv) produce more resistant workers.

MATERIAL AND METHODS

Queen sampling and experimental colony founding

The black garden ant *L. niger* is a common European species that nests in the soil. The species is strictly monogynous, with only one queen per mature colony (Sommer & Hölldobler 1995). After the nuptial flight, queens shed their wings and are found by hundreds roaming on the ground, searching for a nest site. The queens initiate new colonies without assistance from workers (independent colony founding). Pleometrosis is facultative, with 18% of incipient colonies having multiple queens in a field population (Sommer & Hölldobler 1995). Queens in associations are unrelated. They do not forage and entirely rely on their

energetic reserves to rear their first brood (Fjerdingstad & Keller 2004). As soon as the first workers emerge, the queens engage in deadly fights, leaving only one queen alive (Sommer & Hölldobler 1995; Bernasconi & Strassmann 1999).

On July 12th, 2013 we collected young mated queens that were walking on the campus of the University of Lausanne after the nuptial flight. The next day, we placed the queens in experimental nests either alone, in pairs, or in groups of four (N = 45 replicates for each queen number category). Experimental nests consisted of test tubes (17.5 cm long, 1.5 cm diameter) with water blocked by cotton wool at the bottom.

Queen behavior, immune challenges and survival

We monitored queen behavior by scanning each nest for five seconds, five times per day, over seven days. We recorded instances of self-grooming, allo-grooming and trophallaxis (oral exchange of liquid). After 10 days, all queens were subjected to a first immune challenge, which consisted in a small puncture of the thorax with a glass micro capillary and the extraction of 1 µl of hemolymph (that we will use to measure individual immunity, see below). At day 22, queens from half of the experimental nests were exposed to the generalist entomopathogen *Metarhizium brunneum* (Reber & Chapuisat 2012a). In exposed nests, 500 µl of spore solution ($1.75x10^8$ spores/ml in 0.05% Tween 20) were deposited on a filter paper (6.5 x 2 cm). In control nests, queens were exposed to 500 µl of 0.05%

Tween 20. After this fungal challenge, we monitored again the queen behavior over seven days, as described above. Two days after the fungal challenge, which is enough time for spores to germinate and elicit an immune response, we performed a second puncture to extract hemolymph. We monitored queen survival for a total of 87 days, and counted the number of workers produced in each nest with queens alive at day 81.

Worker resistance

We tested if the resistance of workers to *M. brunneum* depended of the number of queens that founded their nest. We used workers from control nests that had no previous exposure to the pathogen. From each control nest that produced at least 10 workers, we made as many groups of five workers as possible (2 to 10). These five-worker groups were kept in 9 cm diameter petri dishes with a filter paper disk at the bottom. Half of the groups from each nest were exposed to spores of *M. brunneum* (500 µl of 0.05% Tween 20 with 1.8 x 10^8 spores/ml deposited on the filter paper), while the other half of the groups were kept as control (500 µl of spore-free 0.05% Tween 20 deposited on the filter paper). We monitored worker survival over 14 days.

Immune measures

The individual immunity of queens was assessed by measuring the phenoloxidase and antifungal activities of their hemolymph. Phenoloxidase (PO) is an essential

component of the innate immune defense of insects. This enzyme is involved in the melanization of damaged tissues and pathogens, and prophenoloxidase is converted in active phenoloxidase by the presence of particles of microbial origin or by wounding (Söderhäll & Cerenius 1998; Cerenius & Söderhäll 2004). We measured both active PO and total PO (active PO + prophenoloxidase), following the methods described in Castella et al. (2009). Briefly, the sample of 1 µl of hemolymph was diluted in 10 µl of sodium cacodylate, and 3 µl of diluted hemolymph was used per measure of PO (Castella *et al.* 2009). The absorbance was measured at 492 nm every 10s for 800 reads at 30°C. We analyzed the active PO and total PO curves with the software PO-CALC (Kohlmeier, Dreyer & Meunier 2015).

The antifungal activity of the hemolymph was measured as described in Konrad et al. (2012). We used 96-well plates containing 2 µl of fungal spore solution (8 x 10^6 spores/ml in 0.05% Tween 20) diluted in 50 µl of Sabouraud Dextrose Broth (SDB). We added either 3µl of diluted hemolymph or 3 µl of sodium cacodylate, as control. Fungal growth was estimated by subtracting the absorbance in a spectrophotometer immediately after the set up from the absorbance after 24 hours (Konrad *et al.* 2012). The antifungal activity of hemolymph was standardized with respect to controls. Specifically, the fungal growth in wells with hemolymph was divided by the average fungal growth in controls.

Statistical analyses

We calculated the frequency of occurrence of self-grooming, allo-grooming and trophallaxis (number of observations divided by number of scans). We used a binomial generalized linear model to determine if the occurrence of self-grooming depended on the number of queens in founding associations and on the immune challenges.

Queen survival was analyzed with a Cox proportional hazards model. The proportion of queens alive was the response variable, while the explanatory variables were the number of queens in founding associations, the exposure to fungal spores, and the interaction between the two factors (we expect an interaction if queens in groups are more resistant to the pathogen). The experimental nest was included as a random factor. Worker survival was analyzed in a similar manner, with the group of workers nested in the nest of origin as random factors. We used Cox mixed-effects models, as implemented in the package "coxme" (Therneau 2015).

We analyzed if queen number, cuticle puncture and exposure to fungal spores influenced the level of active and total PO with mixed effects models. We constructed separate models, with the level of active or total PO as the response variable. We analyzed 1) the immune activity of queens before immune challenges (with queen number as explanatory variable and the nest as random factor); 2) the change in immune activity after cuticle puncture (with cuticle

puncture as explanatory variable, and the nest and queen identity as random factors, to compare immune measures from the same queens before and after puncture) and 3) the immune activity of queens after exposure to fungal spores (with queen number and exposure to fungal spores as explanatory variables and the nest as random factor). Active PO was squared-root transformed to satisfy the assumptions of normality of residuals and homogeneity of variances.

The effect of queen number, cuticle puncture and exposure to fungal spores on the antifungal activity of hemolymph was analyzed with mixed effects models. We constructed separate models, with relative fungal growth as response variable. We analyzed 1) the antifungal activity of hemolymph before immune challenges (with queen number as explanatory variable and the nest as random factor); 2) the change in antifungal activity of hemolymph after cuticle puncture (with cuticle puncture as explanatory variable, and the nest and queen identity as random factors) and 3) the antifungal activity of hemolymph after exposure to fungal spores (with queen number and exposure to fungal spores as explanatory variables and the nest as random factor). Antifungal activity was log transformed to satisfy the assumptions of normality of residuals and homogeneity of variances.

RESULTS

Behavior

Founding queens had very few social interactions and did not increase allo-grooming after being exposed to spores of a fungal pathogen. We recorded only 12 occurrences of allo-grooming, out of a total of 17004 five-second scans (0.07 %). Nine of these allo-grooming events occurred in the first week of observation, before any immune challenge. One occurred in the controls (cuticle puncture only), and two after the fungal challenge (cuticle puncture + exposure to *M. brunneum*). A single occurrence of trophallaxis was observed, after exposure to the pathogen.

In contrast to allo-grooming, self-grooming was frequent and increased after exposure to the pathogen. Overall, we recorded 998 occurrences of self-grooming, out of a total of 19769 scans (5 %). The frequency of self-grooming did not vary with the number of queens in the nest (Fig. 1; χ^2=0.72, df = 2, P = 0.7), but did depend on immune challenges (Fig. 1; χ^2=298.9, df = 2, P < 0.0001). Specifically, self-grooming increased after cuticle puncture (Fig. 1; Tukey post-hoc test, z = 11.3, P < 0.0001) and in response to exposure to fungal spores of *M. brunneum* (Fig. 1; Tukey post-hoc test: z = 4.72, P < 0.0001).

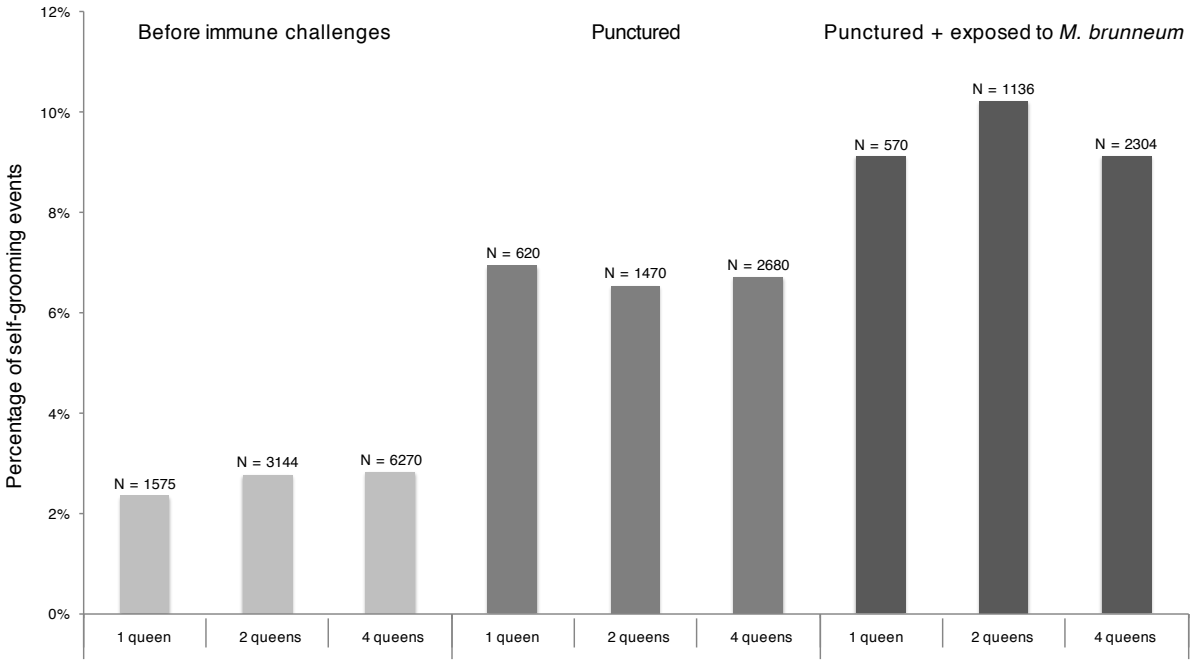


Figure 1. Frequency of self-grooming events, expressed as the number of occurrences of self-grooming divided by the total number of scans per queen (= N). The frequency of self-grooming did not vary with the number of queens in founding associations (1, 2 or 4 queens), but increased after both types of immune challenges (punctured and exposed to *M. brunneum*; see text for details).

Queen survival

The fungal pathogen caused a strong and significant mortality to the queens (Fig. 2; $\chi^2 = 117.9$, df = 1, P < 0.0001). The number of queens in founding associations did not influence queen survival (Fig. 2; $\chi^2 = 0.3$, df = 2, P = 0.87). Finally, queens in group did not show higher resistance to the pathogen (Fig. 2; there was no significant interaction between queen number and pathogen exposure, $\chi 2 = 0.8$, df = 1, P = 0.69).

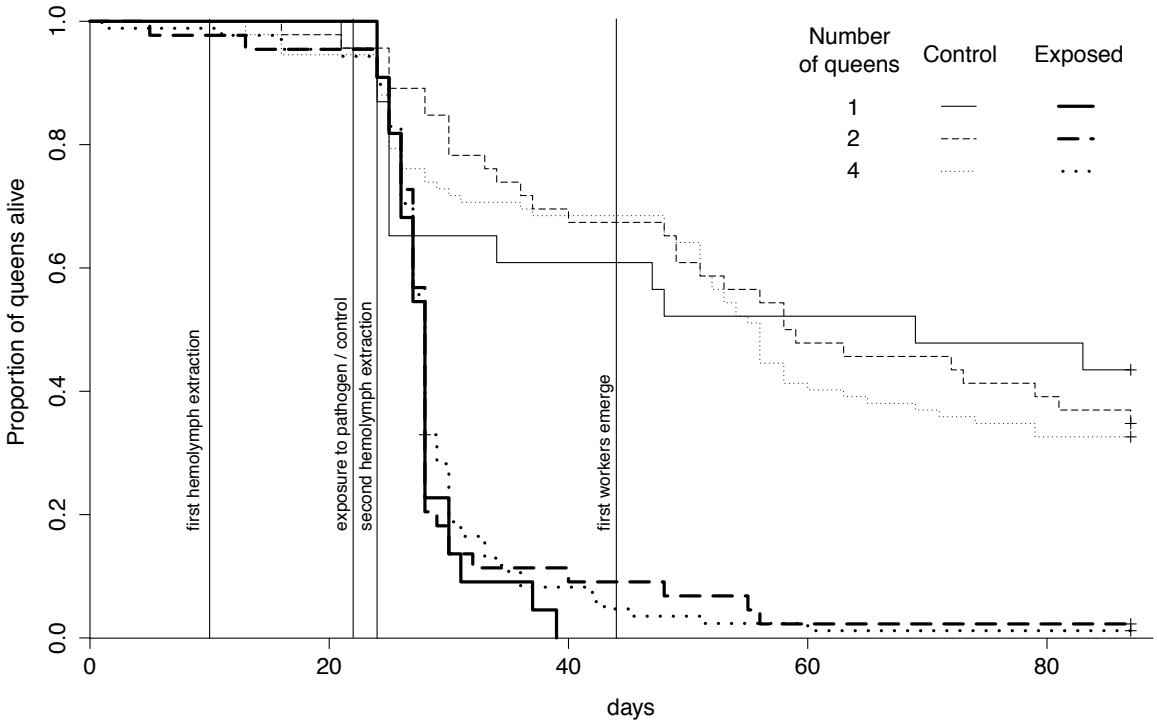


Figure 2. Survival of queens, in function of both the number of queens in founding associations (1, 2 or 4 queens) and the exposure to spores of *M. brunneum* (control versus exposed).

Worker survival

Most (85.5%) of the control nests (no fungal exposure) produced workers, which emerged between day 43 and 51 (average 45). In contrast, only one of the nests exposed to the fungal pathogen managed to produce a single worker. The average number of workers produced per control nest with queens alive at the end of the experiment increased with queen number (Mean ± SE: 14.6 ± 2.1, 18.6 ± 2.8 and 26.3 ± 3.1 workers per nest with 1, 2 and 4 founding queens, respectively; Kruskall-Wallis rank sum test: χ^2 = 7.2 , df = 2, P = 0.027). In contrast, the average number of workers per queen alive at the end of the experiment decreased with queen number (14.6 ± 2.1, 9.3 ± 1.4, 6.6 ± 0.79 workers per queen in nests with 1, 2 and 4 founding queens, respectively; Kruskall-Wallis rank sum test: χ2 = 12.02, df = 2, P = 0.002).

The fungal pathogen caused a significant mortality to the workers originating from control nests (Fig. 3; χ^2 = 116.5, df = 1, P < 0.0001). The number of queens that initiated the colony did not influence workers survival overall (Fig. 3; χ^2 = 1.6, df = 1, P = 0.2), nor the ability of workers to resist to the pathogen (Fig. 3; there was no significant interaction between queen number and pathogen exposure; χ^2 = 2.2, df = 1, P = 0.14).

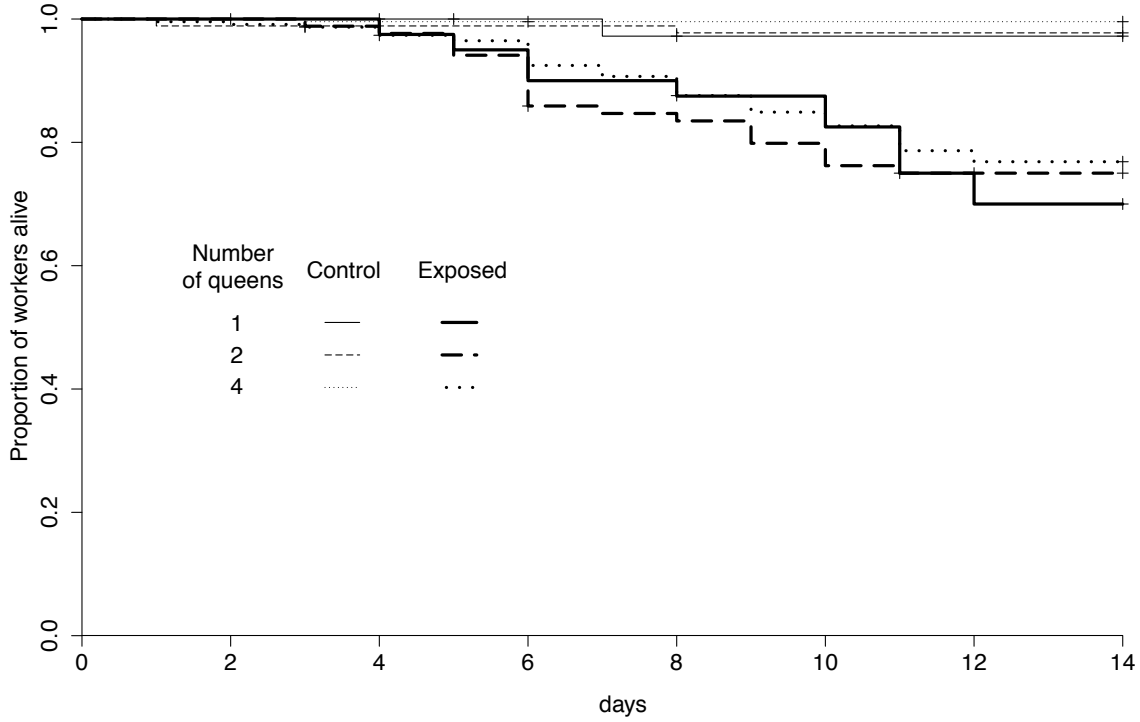


Figure 3. Survival of workers originating from control nests, in function of both the number of founding queens (1, 2 or 4 queens) and the exposure to spores of *M. brunneum* (control versus exposed).

Individual immune defenses of queens

Before any immune challenge, the level of active PO in the queens' hemolymph increased slightly but significantly with the number of founding queens (Fig. 4; ANOVA: $F = 6.9$, $df = 2$, $P = 0.001$). After cuticle puncture, the level of active PO increased greatly (Fig 4; $F = 445$, $df = 1$, $P < 0.0001$), and did not vary with queen number (Fig. 4; $F = 0.24$, $df = 2$, $P = 0.78$). The level of active PO did not further change in response to exposure to fungal spores of *M. brunneum* (Fig.

4; F = 1.15, df = 1, P = 0.29).

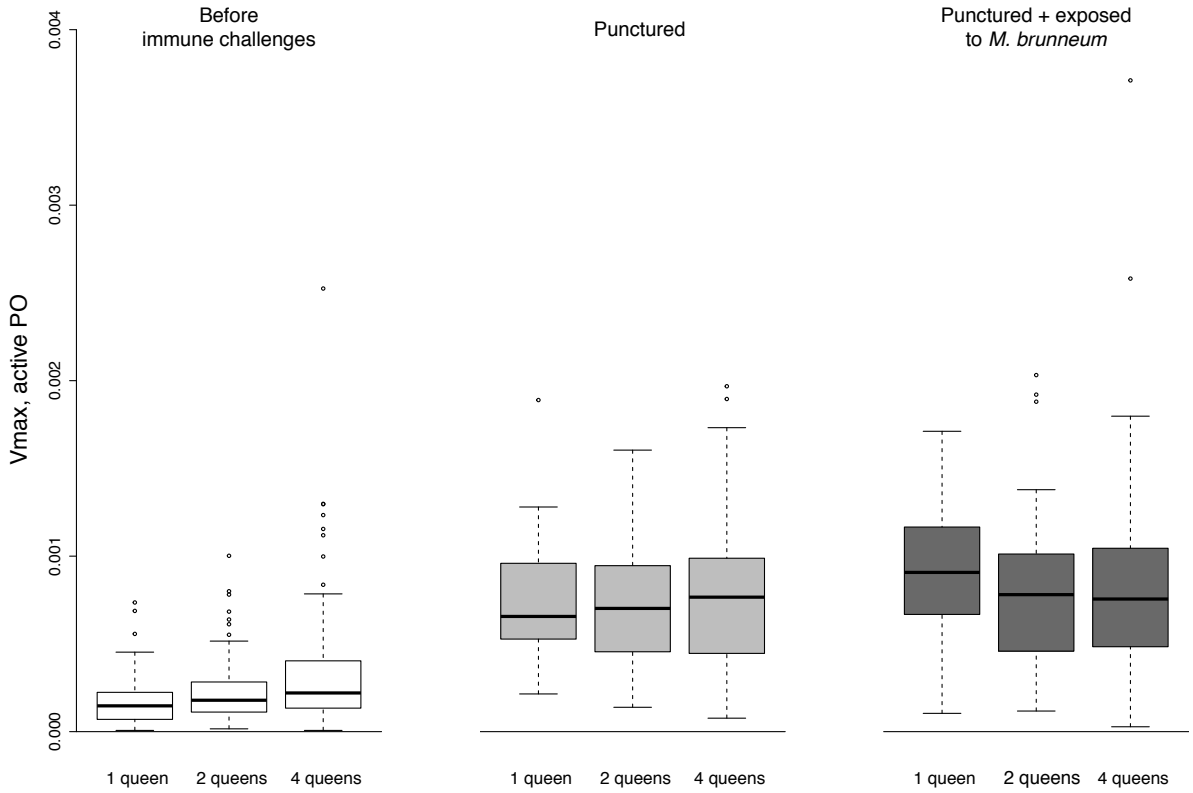


Figure 4. Level of active PO in queens, in function of both the number of queens in founding associations and the immune challenges.

Before any immune challenge, the level of total PO did not vary with queen number (Fig. 5; F = 0.47 , df = 2, P = 0.63). After cuticle puncture, the total PO decreased significantly (Fig. 5; F = 17, df = 1, P < 0.0001), and increased with queen number (Fig. 5; F = 15.8 , df = 2, P < 0.0001). The level of total PO did not further change in response to exposure to fungal spores of *M. brunneum* (Fig. 5; F = 0.79, df = 1, P = 0.38).

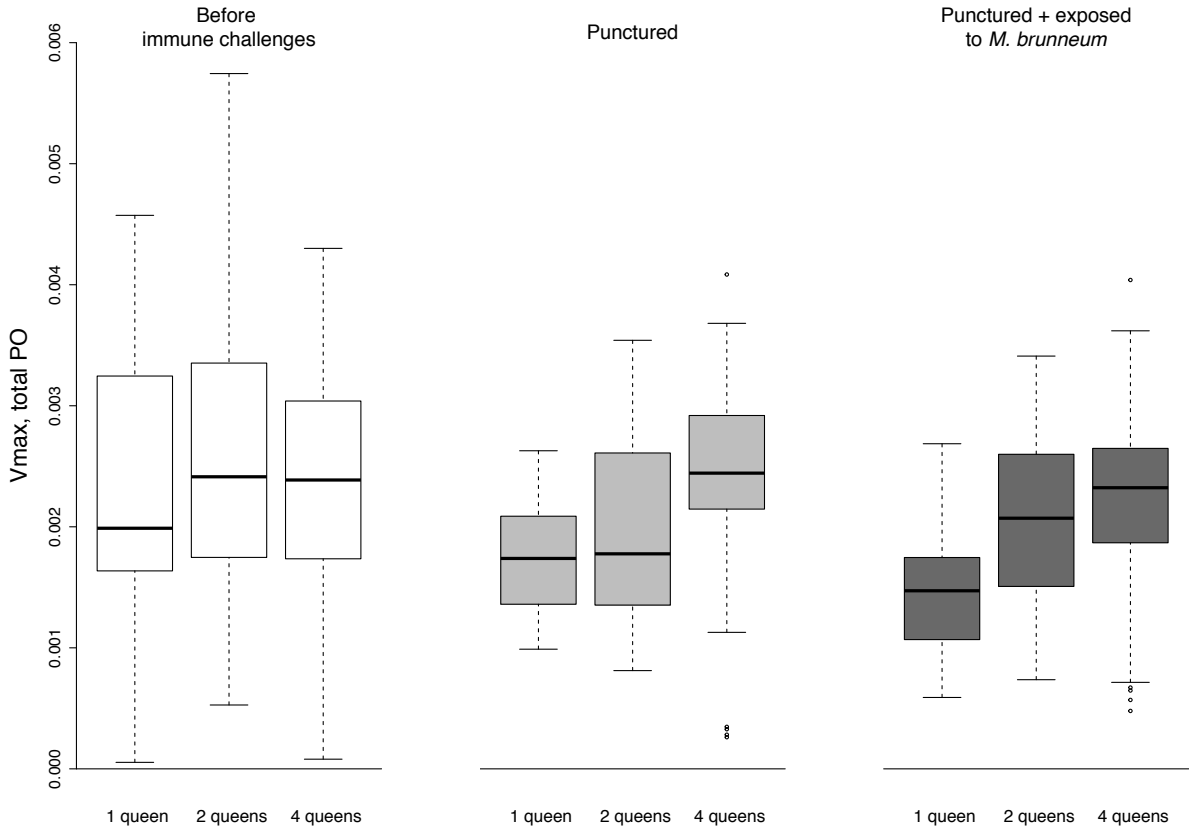


Figure 5. Level of total PO in queens, in function of both the number of queens in founding associations and the immune challenges.

The antifungal activity of the queens' hemolymph did not vary with queen number (Fig. 6; before challenges: $F = 0.37$, $df = 2$, $P = 0.69$; after puncture, with and without exposure to fungal spores: $F = 0.52$, $df = 2$, $P = 0.6$). Fungal growth in the queens' hemolymph overall increased significantly after the immune challenges (cuticle puncture with and without exposure to fungal spores: Fig. 6; $F = 55.5$, $df = 1$, $P < 0.0001$). The growth of the fungus in the queens' hemolymph tended to be reduced after exposure of the queens to the fungal pathogen (Fig. 6; $F = 3.7$, $df = 1$, $P = 0.058$).

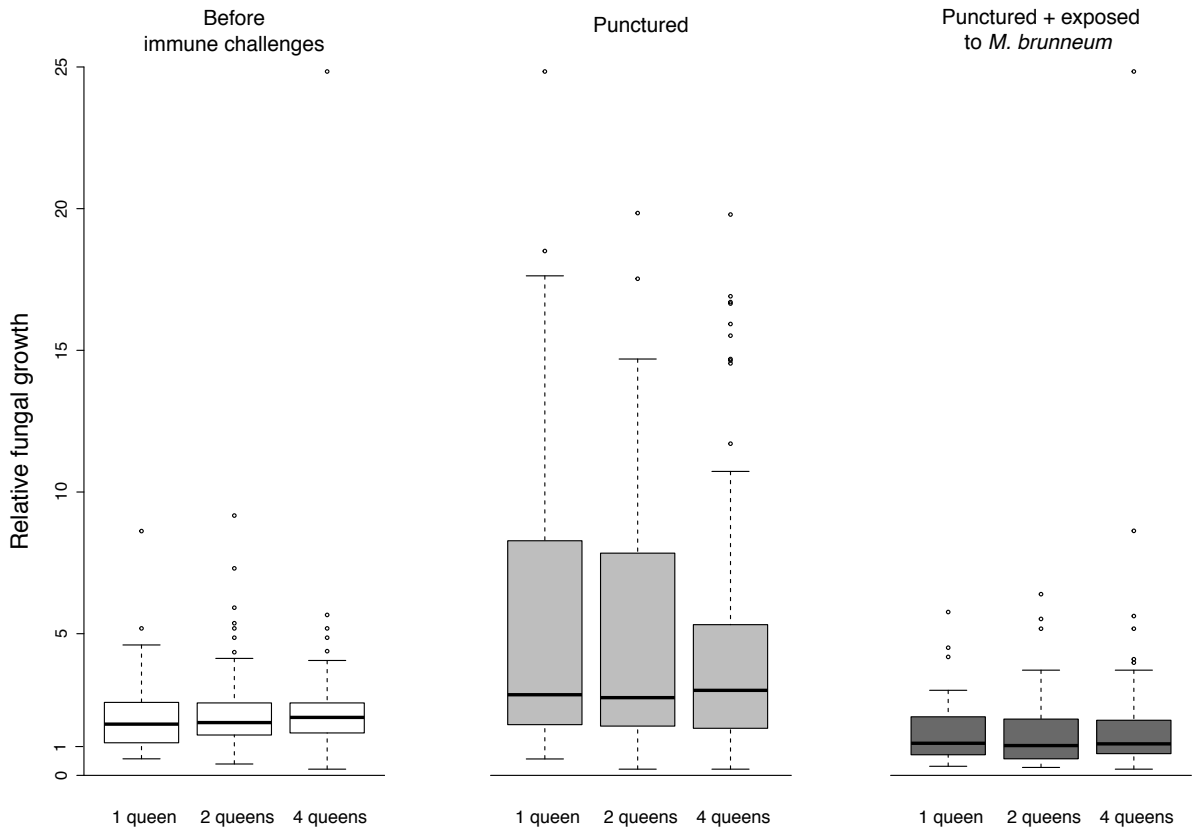


Figure 6. Relative fungal growth of *M. brunneum* in presence of the queens' hemolymph, relative to control, and in function of both the number of queens in founding associations and the immune challenges.

DISCUSSION

We found no evidence that *L. niger* queens jointly establishing incipient colonies benefit from social immunity. Compared to solitary queens, queens founding in associations did not engage in mutual grooming, did not show higher resistance to the common soil fungal pathogen *M. brunneum*, did not decrease their

investment in individual immunity, and their workers did not better resist to the fungal pathogen. Overall, joint colony founding by ant queens did not appear to improve their resistance to fungal pathogens.

The results of our experiments are fully in line with the findings of a previous study that documented an absence of social immunity among co-founding *L. niger* queens (Pull et al., 2013). In both experiments, allo-grooming was almost absent, even in presence of fungal spores. In contrast, self-grooming was common and occurred at higher frequency after exposure to fungal spores or other types of immune challenges. Indeed, in our experiment self-grooming rate increased after cuticle puncture, as well as after exposure to spores of *M. brunneum*. A similar pattern has been documented in *F. selysi,* with higher rates of self-grooming, but no change in allo-grooming, when workers were exposed to *Metarhizium* spores (Reber *et al.* 2011).

A key result of our experiment is that queens in founding associations did not benefit from improved disease resistance, which further confirms the findings of Pull et al (2013). Indeed, when exposed to spores of *M. brunneum,* the survival of queens in groups was not higher than the one of solitary queens. Compared to Pull et al (2013), we used more stringent immune challenges, with a combination of cuticle puncture and group exposure by walking on fungal spores. Hence, the mortality of queens exposed to the pathogen was higher, and we did not detect signs that queens tolerate the infection (Pull *et al.* 2013). We also used a different

pathogen species, and tested associations of four queens. The congruent findings, across various conditions, that co-founding queens do not show higher resistance to fungal pathogens than lone foundresses suggest that the absence of social immunity during joint colony founding by ant queens is a robust and general result.

In addition, we also tested the pathogen resistance of workers produced in incipient colonies. We found no evidence that groups of workers originating from co-founding queens were better able to resist to the fungal pathogen, compared to groups of workers originating from solitary founding queens. Groups of workers originating from co-founding queens are expected to be genetically more diverse, because all queens in associations contribute to brood production (Aron *et al.* 2009). Yet, the higher genetic diversity among incipient workers did not confer higher resistance to *M. brunneum* in the conditions tested, which contrasts with findings in other situations (Reber *et al.* 2008).

We measured some components of the queens' immune defenses, to examine if the benefits of social immunity resulted in a decreased investment in individual immunity (Cotter & Kilner 2010). We found the reverse pattern for active PO: before any immune challenge, queens in groups had higher PO activity than solitary queens. In a similar vein, bumble-bees kept in groups also increased active PO (Ruiz-González, Moret & Brown 2009). The level of total PO did not vary with queen number before the immune challenge, but increased with queen

number after cuticle puncture and fungal exposure. Overall, these results are consistent with an absence of social immunity, and suggests higher investment in individual immunity when in groups, possibly because of higher pathogen transmission risk (Wilson *et al.* 2002; Wilson & Cotter 2008).

The queens' hemolymph tended to favor the growth of *M. brunneum*. This is not fully unexpected, as the hyphae of this fungus penetrates the cuticle and grows inside the insect body, and may thus use hemolymph as food source (Gillespie *et al.* 2000). Yet, a previous study showed that the workers' hemolymph inhibited fungal growth (Konrad *et al.* 2012). The causes of these contrasted results remain to be investigated. It is conceivable that queens just after mating are under energetic stress and tend to be immunocompromised, possibly as a result of sperm storage (Baer *et al.* 2006). In our experiment, the growth of the fungus tended to be reduced when queens had been exposed to the fungus, suggesting that these queens activated some antifungal defenses.

Incipient colonies founded by multiple *L. niger* queens produced more workers than colonies with a lone foundress. Specifically, colonies founded by two and four queens produced 1.3 and 1.8 times more workers, respectively, than colonies founded by a single queen. A higher productivity of co-founding associations is expected, as queens rely on their limited energy reserves to produce the first generation of workers (Fjerdingstad & Keller 2004; Aron *et al.* 2009). The production of a larger worker force when multiple queens found nests together

has been documented in several ant species (Bernasconi & Strassmann 1999). This demographic benefit of co-founding is likely to confer a major advantage when the first workers open the nest and start to compete with other colonies for foraging and brood raiding (Bernasconi & Strassmann 1999).

In conclusion, our results indicate that joint colony founding provide no social immunity benefit to ant queens. Co-founding queens did not engage in allo-grooming, but performed extensive self-grooming. Queens in group did not better resist to the fungal pathogen than solitary queens, and did not produce more resistant workers. Finally, queens in groups tended to increase their investment in active PO, a component of their individual immune defenses. An absence of social immunity and a strong investment in individual immunity might reflect the competitive nature of co-founding queen associations. Indeed, queens fight to death after the first workers emerge (Bernasconi & Strassmann, 1999), and the presence of infectious cadavers can have devastating effects in small incipient colonies (Loreto & Hughes 2016).

Acknowledgements

We thank Aurélie Laurent for her help in the lab and the Swiss National Science Foundation for founding (grant 31003A-146641 to MC).

Chapter 3 : Effects of the social environment on the survival and fungal resistance of ant brood

Jessica Purcell, Timothée Brütsch & Michel Chapuisat

Purcell, J., Brütsch, T. & Chapuisat, M. (2012). Effects of the social environment on the survival and fungal resistance of ant brood. *Behavioral Ecology and Sociobiology*, 66, 467-474.

ABSTRACT

The phenotype of social animals can be influenced by genetic, maternal and environmental effects, which include social interactions during development. In social insects, the social environment and genetic origin of brood can each influence a whole suite of traits, from individual size to caste differentiation. Here, we investigate to which degree the social environment during development affects the survival and fungal resistance of ant brood of known maternal origin. We manipulated one component of the social environment, the worker-to-brood ratio, of brood originating from single queens of *Formica selysi*. We monitored the survival of brood and measured the head size and ability to resist the entomopathogenic fungus *Beauveria bassiana* of the resulting callow workers. The worker-to-brood ratio and origin of eggs affected the survival and maturation time of the brood and the size of the resulting callow workers. The survival of the callow workers varied greatly according to their origin, both in controls and when challenged with *B. bassiana*. However, there was no interaction between the fungal challenge and either the worker-to-brood ratio or origin of eggs, suggesting that these factors did not affect parasite resistance in the conditions tested. Overall, the social conditions during brood rearing and the origin of eggs had a strong impact on brood traits that are important for fitness. We detected a surprisingly large amount of variation among queens in the survival of their brood reared in standard queenless conditions, which calls for further studies on genetic, maternal and social effects influencing brood development in the social insects.

INTRODUCTION

The relative influence of genotype, maternal effects and rearing environment on the phenotypes of offspring has been the topic of heated debate in humans (e.g. Meaney 2001), and is the subject of ongoing research in a wide range of species (Russell & Lummaa 2009; Schwander *et al.* 2010). Social insects provide an interesting opportunity to investigate these influences. They live in mother-daughter associations within well-defined colonies, which allows for complex social interactions. Egg laying and brood rearing are generally performed by different colony members: the queen and nurse workers, respectively. Thus, the offspring's phenotype is shaped by a combination of factors including the genotype inherited from the parents, maternal effects, as well as indirect genetic effects and environmental effects due to the interactions with its social partners, especially the workers that provide daily care (Brian & Carr 1960; Linksvayer 2006; Kapheim *et al.* 2011).

Many studies have investigated the mechanisms of caste determination in insect societies. Recent findings suggest that the control of caste differentiation during offspring development can range from predominantly genetic to primarily environmental, with most of the investigated species falling at intermediate points along the continuum (reviewed in Schwander et al. 2010). Within castes, it seems that the genotype, maternal effects and care received during development may have some influence on the number of individuals produced (e.g. Linksvayer

2008), their development time (e.g. Howard & Jeanne 2004) and their size (e.g. Schwander, Rosset & Chapuisat 2005; Fournier *et al.* 2008; Kovacs *et al.* 2010). The relationships between these inputs remain unclear, and experimental studies manipulating the social environment are needed to better evaluate the respective influence of each factor (Linksvayer & Wade 2005).

The ratio of workers to brood is one element of the social environment that may differ greatly across colonies of different ages and social structures, and that may influence the survival and quality of the brood. In general, most studies have found that a larger worker-to-brood ratio increases the survival and the size of new workers in a diverse range of ant subfamilies (e.g. Brian 1957; Gray 1971; Wilson 1983; Porter & Tschinkel 1985; Tschinkel 1988), although this benefit may diminish at larger group sizes (Brian 1953). Moreover, workers seem to be unable to properly care for the brood when there are fewer workers than brood (Evesham 1985; Cassill & Tschinkel 1999; Cassill 2002; Hartmann *et al.* 2003). Little is known about how the worker-to-brood ratio affects other aspects of offspring quality, such as maturation time and resistance to infection. Recent studies of parasite resistance in social insects indicate that gene by environment interactions are probably important in worker susceptibility (e.g. Reber et al. 2008). Both genetic and environmental factors are known to affect disease resistance in other organisms, including birds (Saino, Calza & Moller 1997), mammals (e.g. Lubach, Coe & Ershler 1995; Prager *et al.* 2010), and insects (e.g.

Cotter, Kruuk & Wilson 2004; Moreno-García, Lanz Mendoza & Córdoba-Aguilar 2010).

In this study, we examined the effect of one component of the social environment, the worker-to-brood ratio, on the survival and fungal resistance of ant brood of controlled origin. We monitored how brood laid by each queen performed when reared by different numbers of nestmate workers in the ant *Formica selysi*. In this species, the number of queens varies among colonies (Chapuisat et al. 2004). Each queen usually mates with one male, rarely with two (Chapuisat et al. 2004; Schwander et al. 2005). The number of workers per colony is highly variable, with approximately 10 times more workers in colonies headed by multiple queens than in colonies headed by a single queen (Rosset & Chapuisat 2007). Workers from single-queen (= monogyne) colonies are also significantly larger than workers from multiple-queen (= polygyne) colonies (Schwander et al. 2005) and have a slightly higher activity in one component of the immune system causing bacterial growth inhibition, even when controlling for body size (Castella, Christe & Chapuisat 2010).

Surprisingly, *F. selysi* workers originating from polygyne colonies showed a lower survival rate than workers from monogyne colonies when they were experimentally challenged with an entomopathogenic fungus, despite the fact that experimental groups with higher diversity had higher resistance (Reber et al. 2008). This result suggests that, while diversity *per se* improves the resistance of

the group, some other aspect of the polygyne lifestyle reduces the ability of workers to resist infections. The observed difference in parasite resistance might result from variation in immune response (Castella *et al.* 2010; Vitikainen & Sundström 2011) and/or body size (Schwander et al. 2005). In turn, both factors may be influenced by genetics, maternal effects, and/or social environment experienced during development.

We hypothesize that the ratio of workers to brood might affect the survival and maturation time of brood, as well as the size and parasite resistance of the resulting workers. The direction of the effect is somewhat difficult to predict, and we do not know how the worker-to-brood ratio varies among field colonies. We do, however, expect the number of workers caring for brood to influence the nutrition, feeding rate, and hygiene of the larvae. In turn, larval nutrition has been shown to strongly influence the immune response of insects (Suwanchaichinda & Paskewitz 1998; Valtonen, Roff & Rantala 2011). Therefore, we expect that a higher worker-to-brood ratio might result in higher brood survival, with the resulting workers being larger and more resistant to parasites. We simultaneously assessed if brood originating from different mothers varied in survival, maturation time, size and parasite resistance.

METHODS

Brood rearing experiment

In March-April, 2010, we collected 22 ovipositing queens and many workers from a total of 16 polygyne colonies in a large *Formica selysi* population located along the Rhône river between Sierre and Susten in Valais, Switzerland (7°36'30"E, 46°18'30"N, altitude 565 m). In most cases, we collected or at least observed multiple queens in each of these colonies. In the few ambiguous cases, we verified that the colonies were polygynous by genotyping eight workers per colony at eight microsatellite loci (Chapuisat et al. 2004).

In the lab, we placed each queen and about 50 workers from her field colony (but no pre-existing brood) in an individual plastic box (15 x 13 x 6 cm) lined with Fluon GP1 (Whitford Plastics, Diez, Germany) to prevent ants from escaping. We supplied water in a glass tube with moist cotton, and covered this tube with a piece of aluminium foil to provide a dark, humid nest. We also provided *ad libitum* access to standard ant food (Meunier & Chapuisat 2009). The ants were kept at 24 ± 2 °C with 50% humidity under a 12 : 12 h light : dark cycle. For four weeks (late April through early May), we monitored these boxes regularly and removed newly laid eggs for use in the experiment. At the end of this egg collection period, we measured the heads of the queens using a Leica MZ12.5 microscope (Leica Application Suite 2.8.1) after they were cooled to 10°C for

about 30 minutes to minimize movement. Head size is a good indicator of overall body size (Schwander et al. 2005).

We placed the freshly collected eggs on a small Petri dish, where we observed and counted them under a dissecting microscope. We then divided all of the intact eggs into groups of 20 and placed them on separate Petri dishes. At the same time, we divided the workers from the same field colonies as the eggs into groups of 10, 20, 100, and 200 workers (rearing groups). We placed these groups in large boxes (38 x 22 x 14 cm) with a covered glass water tube, *ad libitum* access to standard ant food, and fluon lining to prevent escape. In cases when queens had produced enough eggs for each of the four worker-to-brood ratio treatments (at least 80 eggs), we haphazardly assigned the groups of 20 eggs to each treatment. A total of nine queens produced at least 80 eggs, and 20 queens produced at least 20 eggs. When queens failed to produce enough eggs for a full treatment block, we allocated the groups of 20 eggs in priority to the rearing groups of 20 and 100 workers. With these eggs, we created 16 10:20 worker-to-brood ratio rearing groups with the eggs of 13 queens, 20 20:20 worker-to-brood ratio rearing groups with the eggs of 14 queens, 21 100:20 worker-to-brood ratio rearing groups with the eggs of 16 queens, and 16 200:20 worker-to-brood ratio rearing groups with the eggs of 13 queens.

Brood survival and development

To start the rearing experiment, we placed the open Petri dishes containing eggs near the entrance to the glass tubes in the boxes with the rearing workers immediately after the eggs were collected and counted. After 48 hours, all of the eggs had been collected and moved to the water tube by workers in 66 out of 73 rearing groups. In the remaining groups, a median of one and a maximum of three eggs were left on the Petri dish, and these groups proceeded with fewer brood. We monitored the number of brood visible, the instar of the brood, and the mortality of workers at least once per week until pupation.

After pupation, we monitored the boxes daily to ensure that we could identify newly emerged callow workers by their light colour. We immediately removed callow workers and placed them in a small plastic box containing *ad libitum* food and water with two nurses from the same colony, each of which was marked with a small dot of paint on her abdomen. During this period, the mortality rate of callow workers was 6%. For each rearing group, we measured brood survival, calculated as the number of live callow workers that emerged out of 20 eggs, as well as the minimum maturation time of brood for each group that successfully reared new workers, calculated as the number of days between the hatching of the first egg and the emergence of the first callow worker.

Callow survival and fungal challenge

When the callow workers were five days old and fully sclerotized, we exposed them to either the entomopathogenic fungus *Beauveria bassiana* (10^8 conidia/mL suspended in 0.05% Tween 20 buffer) or to a control (0.05% Tween 20 buffer) by depositing a 2 μL droplet directly on the thorax. Previous experiments on *F. selysi* found that, for the specific strain of *B. bassiana* that we collected from our field site, this concentration resulted in intermediate mortality rates (Reber and Chapuisat unpubl. data). A similarly high concentration of conidia has also been used in other studies on the response of different insects to *B. bassiana* (e.g. Conteiro Castilho *et al.* 2010; Mukawa, Tooyama & Ikegami 2011). The first callow worker from each rearing group was randomly assigned to either the fungal exposure or the control treatment. Thereafter, we alternated subsequent callow workers emerging from the same rearing group with the fungal exposure and control treatment. In this way, we ensured that callow workers from the same rearing group were evenly represented in both the exposure and the control treatments. We isolated the individuals in small Petri dishes (3.5 cm diameter) containing food and water and monitored their survival daily for 14 days. We removed dead individuals from the Petri dishes, measured their heads, surface sterilized the corpses, and placed them in tubes with wet cotton wool for 30 days to record which corpses produced *B. bassiana* conidia. At the end of the experiment, we measured the heads of the remaining live workers. Two workers

escaped late in the observation period, and five others were mixed up during head measurement. These individuals were excluded from the head size analysis.

Statistical analysis

We constructed generalized linear models (GLMs) to separate the effect of the worker-to-brood ratio from the impact of the origin of brood on the survival, maturation time and size of the resulting workers, respectively. In each model, we used the brood measurement as the response variable and the mother identity, worker-to-brood ratio treatment, as well as the interaction between the two as fixed effects. For the model of callow head size, we used the size of individuals as the response variable, and we nested their rearing group within mother identity to account for any variation among rearing groups. For all of the comparisons in which maternal identity influenced our response variable, we additionally investigated whether specific queen traits influenced brood performance. In these GLMs, the response variables were the brood measurements and the fixed effects were maternal (= queen) head size, maternal fecundity, as well as the interaction between the two. For the comparisons wherein the response variable was influenced by worker-to-brood ratio treatment, we also performed pairwise post-hoc tests to determine which worker-to-brood ratio treatments differed significantly from one another. For these tests, we used the brood measurement as the response variable, the worker-to-brood ratio treatment as a fixed effect, and the maternal identity as a random factor. We reported the analysis of deviance

and the GLM family (linear for normally distributed data, poisson for count data, and binomial for survival data) that we used for each comparison. Finally, we used Pearson correlation tests to investigate whether our measures were related to one another.

We investigated how the rearing environment and origin of the brood each influenced the ability of the resulting workers to resist an immune challenge using a parametric survival analysis (the survreg function in R 2.10). In this model, the response variable was the number of days that each individual survived after the infection or control solution was administered. This variable was censored when workers survived beyond our observation period, and for the two individuals that escaped during the observation period. The fixed effects included the worker-to-brood ratio treatment, the maternal identity, the fungal challenge versus control and the interaction terms. We used the Weibull distribution, which produced the minimum error deviance. All statistical analyses were carried out in R 2.10 (R Development Core Team, 2009).

RESULTS

Brood survival and development

The survival of the brood from the egg stage to the emergence of callow workers was influenced by both the worker-to-brood ratio during development and the

origin of the eggs (Table 1). The relationship between brood survival and worker-to-brood ratio was non-linear: the groups of 20 workers provided with 20 eggs exhibited the highest mean number of brood emerging (Fig. 1a) and reared significantly more offspring than the groups of 200 workers (post-hoc comparison, $p = 0.0052$), but the other pairwise comparisons were not significant after Bonferroni correction ($p = 0.023$, 0.38 and 0.20 when comparing the worker-to-brood ratio 10:20 to 20:20, 100:20 and 200:20, respectively; $p = 0.029$ and 0.038 when comparing the worker-to-brood ratio 100:20 to 20:20 and 200:20, respectively). The maternal origin of eggs greatly influenced brood survival, which ranged from 0 to 29%, with a mean of 6%. This variation in brood survival was not, however, related to the maternal head size or fecundity (Table 2). Queen fecundity varied from 0 to more than 200 eggs produced over the four weeks during which we collected their eggs, but this was not correlated with queen size ($r = 0.30$, $p = 0.23$).

Response variable	Model factors			GLM family
	Maternal identity	Worker-to-brood ratio	Interaction	
Brood survival	$\chi^2 = 110.3$, d.f. = 15, $p < 0.0001$	$\chi^2 = 27.6$, d.f. = 3, $p < 0.0001$	$\chi^2 = 42.9$, d.f. =33, $p = 0.12$	poisson
Maturation time	$F_{13, 16} = 12.6$, $p = 0.013$	$F_{3, 13} = 1.07$, $p = 0.45$	$F_{9, 4} = 6.06$, $p = 0.049$	linear
Reduced maturation time (without 200:20)	$F_{13,13} = 11.4$, $p = 0.015$	$F_{2,11} = 1.16$, $p = 0.40$	$F_{7,4} = 7.77$, $p = 0.033$	linear
Callow head size	$F_{12, 72} = 1.98$, $p = 0.017$	$F_{3, 69} = 4.84$, $p = 0.005$	$F_{9, 60} = 1.57$, $p = 0.15$	linear
Reduced head size (without 200:20)	$F_{12, 68} = 2.06$, $p = 0.014$	$F_{2, 66} = 6.86$, $p = 0.002$	$F_{7, 59} = 1.66$, $p = 0.14$	linear

Table 1 Influence of worker-to-brood ratio and origin of eggs on brood survival, maturation time, and head size of the resulting callow workers. For the callow head size comparisons, rearing group identity is nested within maternal identity.

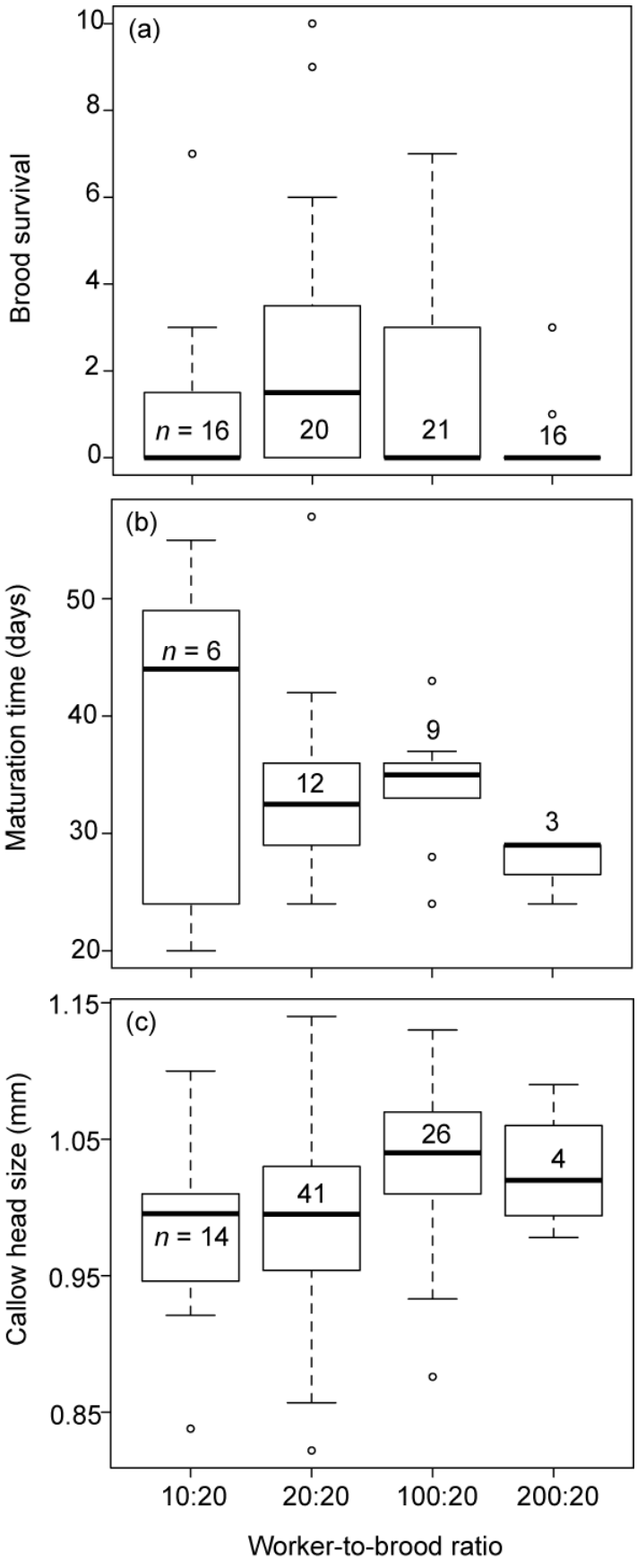

(a)
Brood survival
10
8
6
4
2
0
n = 16
20
21
16
(b)
Maturation time (days)
50
40
30
20
n = 6
12
9
3
(c)
Callow head size (mm)
1.15
1.05
0.95
0.85
n = 14
41
26
4
10:20
20:20
100:20
200:20
Worker-to-brood ratio

Fig.1 Effect of the worker-to-brood ratio on (a) brood survival, expressed as the number of callow workers emerging out of 20 eggs, (b) maturation time, expressed as the shortest time from egg hatching to callow emergence in each nest, and (c) head size of the resulting callow workers. The boxplots show the median values and the upper and lower quartiles. The whiskers encompass 1.5 times the interquartile range, and the circles represent outliers. The samples sizes for each category (the number of rearing groups in a and b, and the number of newly emerged workers in c) are shown in each box.

The maturation time of brood was influenced by the origin of eggs, as well as the interaction between the origin of eggs and the worker-to-brood ratio (Table 1). The shortest time from hatching to callow emergence (maturation time) varied greatly among rearing groups, ranging from 20 to 55 days, and eggs from rearing groups with greater worker-to-brood ratios generally developed faster (Fig. 1b). The variation in maturation time of brood was also linked to an interaction between maternal size and fecundity (Table 2). We found a negative correlation between the number of brood produced and the maturation time (r = -0.40, p = 0.031).

The worker-to-brood ratio and egg origin also had a significant impact on the head size of the resulting workers (Table 1). Brood reared by a greater number of workers were generally larger (Fig. 1c), with groups of 100 workers producing significantly larger workers than groups of 20 workers (post-hoc comparison, p = 0.0067) or 10 workers (p = 0.0004) and no differences between rearing groups

with 10 and 20 workers (p = 0.51). Head size was also weakly heritable from mother to daughter, with maternal head size explaining 5% of the variance in callow head size (Table 2).

	Model factors			
Response variable	Maternal head size	Maternal fecundity	Interaction	GLM family
Brood survival	$\chi^2 = 0.55$, d.f. = 1, $p = 0.46$	$\chi^2 = 0.87$, d.f. = 1 $p = 0.35$	$\chi^2 = 0.60$, d.f. = 1, $p = 0.44$	binomial
Maturation time	$F_{1,26} = 0.91$, $p = 0.35$	$F_{1,25} = 0.34$, $p = 0.57$	$F_{1,24} = 7.54$, $p = 0.011$	linear
Callow head size	$F_{1,81} = 4.27$, $p = 0.042$	$F_{1,80} = 0.77$, $p = 0.38$	$F_{1,79} = 1.16$, $p = 0.29$	linear

Table 2 Influence of maternal characteristics on brood survival and maturation time, as well as head size and survival rate of the resulting callow workers.

Callow survival and fungal challenge

The survival of callow workers was influenced by exposure to *B. bassiana* (χ^2 = 6.64, d.f. = 1, p = 0.010) and by the origin of the eggs (χ^2 = 27.26, d.f. = 12, p = 0.0071), but not by the worker-to-brood ratio (χ^2 = 3.21, d.f. = 2, p = 0.20) or any of the interactions (p > 0.4; full model χ^2 = 65.64, d.f. = 87, p = 0.11). Sequential removal of interaction terms did not influence the qualitative results of this analysis; the best fit model contained only the effects of queen and infection status

(reduced model $\chi^2 = 36.04$, d.f. = 13, $p = 0.0006$). Exposure to the fungal parasite had a negative impact on the survival of callow workers (Fig. 2), and survival was not correlated with the head size of callow workers in our experiment ($r = 0.22$, $p = 0.16$). In the groups exposed to the parasite, 60% of the ant corpses yielded *B. bassiana* conidia, but no conidia were observed on corpses from the control. The worker-to-brood ratio had no significant effect on the survival of callow workers in the absence of infection, nor on their ability to survive the fungal challenge (Fig. 2). In contrast, the offspring of some mothers had higher survival than others, both when exposed to the fungal parasite and in controls. Hence, the origin of eggs affected the overall survival of callow workers, but did not influence their relative ability to resist the pathogen, as indicated by the lack of interaction between the factors fungal challenge and origin. The overall survival of brood throughout development and during fungal infection thus depended upon worker-to-brood treatment, maternal origin, and infection status (Electronic supplementary materials, Appendix 1).

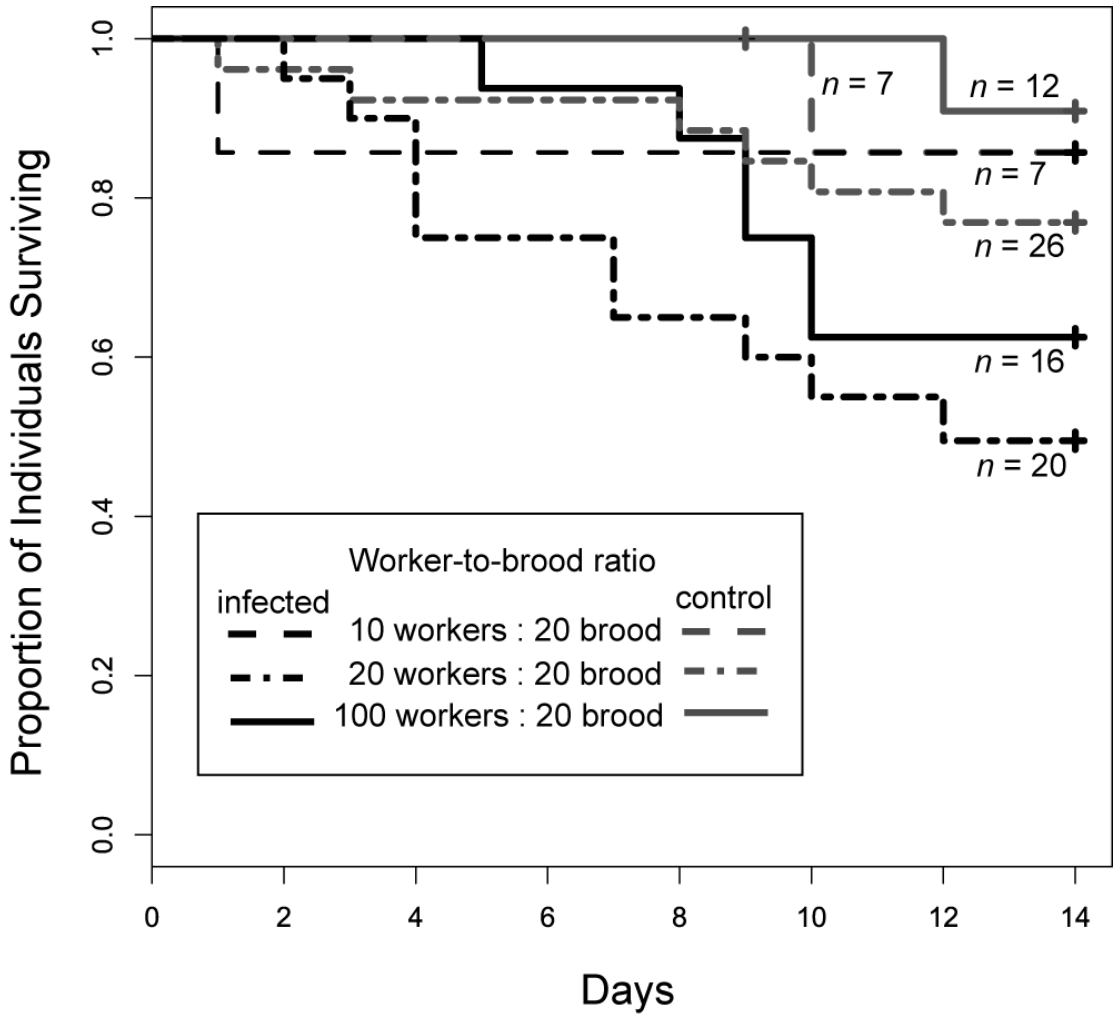


Fig. 2 The proportion of newly emerged workers surviving in controls (gray lines) and when challenged with the entomopathogenic fungus *B. bassiana* (black lines). The 200:20 worker-to-brood ratio treatment was omitted from the analysis due to small sample size. The number of individuals from each worker-to-brood ratio treatment is shown for each category.

DISCUSSION

In animals with cooperative brood care, a lot of the phenotypic variability may result not only from genetic variation, but also from the social conditions during development. Here, we examined the impact of one important component of the

social environment, the availability of worker nurses, on the development, survival and parasite resistance of brood of known maternal origin. We distributed eggs laid by single ant queens among experimental rearing groups having four worker-to-brood ratios. In line with previous studies of social insects (e.g. Schwander et al. 2010), we found that the relationship between environmental and parental inputs is complex, with both the social conditions and origin of eggs influencing major traits of the brood. The worker-to-brood ratio influenced the survival and adult size of the brood. Queens varied greatly in their fecundity during our observation period, as well as in the viability of their brood reared in standard queenless conditions. The callow workers originating from different queens also varied significantly in their post emergence survival. We did not find an impact of either the worker-to-brood treatment or the maternal origin of eggs on the ability of callow workers to resist the fungal infection.

At the outset, we expected that having more workers available to care for the brood would improve the quality of care. Instead, we found that the worker-to-brood ratio generally had non-linear effects on the survival and maturation time of brood, as well as on the head size of resulting callow workers. This suggests that there may be developmental trade-offs associated with variation in the worker-to-brood ratio. For example, when one worker was available to care for each egg (20:20 treatment), the largest number of brood survived, but this brood matured more slowly than the brood reared by a larger number of workers. Some

previous studies found a similar trade-off, with larger colony size being associated with a decline in the efficiency of brood care and an increase in the size of workers (reviewed by Tschinkel 1988).

In most of the rearing groups, the workers raised only a small proportion of the eggs until adulthood (Appendix 1). Other experimental studies also had very low brood survival under similar laboratory conditions (e.g. Abril, Oliveras & Gómez 2010). There are several potential causes for this high rate of attrition. First, the presence of queens in colonies has been found to have a major effect on worker investment in brood in other ant species (e.g. Brian & Carr 1960; Vienne, Errard & Lenoir 1998). Second, it is possible that larger groups performed poorly under our laboratory conditions. We housed the colonies in large boxes with *ad libitum* access to food and water, so we don't believe that crowding *per se* could have caused this pattern. One source of stress on the brood could have been the higher traffic of adults in and out of the nest tube, where the brood is attended.

Eggs laid by different queens varied greatly with respect to many of the metrics that we measured on the resulting brood, even though the queens were not present in the rearing groups. This variation may have multiple causes. First, the broods differ in their maternal and paternal genotype. Second, they might have been influenced by maternal effects and by environmental or social factors associated with the colony of origin of the queens, including indirect genetic effects through the nestmate workers caring for the brood (Wolf et al. 1998; Linksvayer 2007).

The high variation among queens in both the viability of their immature brood and the resilience of their callow workers is somewhat surprising, as selection should favour queens with high productivity, thereby decreasing genetic variance in reproductive output. A high variation among queens in brood viability has been documented in other polygyne species (e.g. Holzer et al. 2006). Moreover, previous studies have found that queens from polygyne colonies varied more in their fecundity than queens from monogyne colonies (e.g. Keller 1988). It is thus possible that selection is somewhat relaxed in colonies with multiple queens. Alternatively, this variation may have no genetic basis. For example, competition among queens might have reduced the reproductive output of some queens (e.g. Keller & Reeve 1994) or there may be differences based on queen age. Finally, queens may also differ in their allocation of reproductive effort toward producing sexual *versus* worker offspring. It is interesting to note that the same queens whose brood performed well during this experiment also continued to produce a larger number of callow workers in the lab throughout the season (Pearson $r = 0.65$, $p = 0.015$). Thus it appears that some queens and colonies were consistently more productive than others, at least under laboratory conditions. To better understand the causes of this variation among queens, it would be interesting to quantify the degree of selection on queen fecundity, brood viability and reproductive success in different social contexts (e.g. number of queens present, queen age).

The factors influencing parasite resistance also deserve further investigation. Although both the worker-to-brood ratio and egg origin influenced some aspects of brood development and offspring quality, we have not yet detected specific factors that contribute to variation in resistance to the fungal parasite *B. bassiana*. Indeed, we found no interaction between the fungal challenge and either the worker-to-brood ratio or origin of eggs, suggesting that these factors did not affect parasite resistance in the conditions tested. It is however likely that there is genetic and social variation for disease resistance (e.g. Hughes & Boomsma 2004; Cremer *et al.* 2007) and given our sample size, small effects of maternal origin or worker-to-brood ratio might have remained undetected. There are also many more ways by which social interactions, including grooming or sharing of antibiotics both before and/or during infection, may influence brood and callow immunity (Calleri *et al.* 2006; Cremer *et al.* 2007; Chapuisat *et al.* 2007; Hamilton *et al.* 2011; Reber *et al.* 2011). An interesting next step would be to manipulate other aspects of the social or abiotic environment during brood development, and to compare the relative effects of egg origin and social environment on resistance to a variety of naturally-occurring pathogens.

Overall, the number of workers available to care for brood is likely to change over the course of the colony life-span, and in response to the number of queens in the colony (e.g. Heinze). Our results suggest that the worker-to-brood ratio plays an important role in the ontogeny of colony characteristics, such as colony

growth rate and worker size, which will in turn influence colony success (e.g. Billick 2001; Nielsen, Agrawal & Hajek 2010). Further research is required to identify which factors influence the ability of individual workers to resist fungal infections, and also to determine whether colonies can adjust the worker-to-brood ratio or brood production strategy in response to the short-term worker requirements of the group.

Acknowledgments

We wish to thank Alain Reymond and Dominique Bays for their help with data collection. Thanks also to Alan Brelsford and three anonymous reviewers for comments on the manuscript. This study was funded by a Swiss National Science Foundation grant (31003A_125306) to MC.

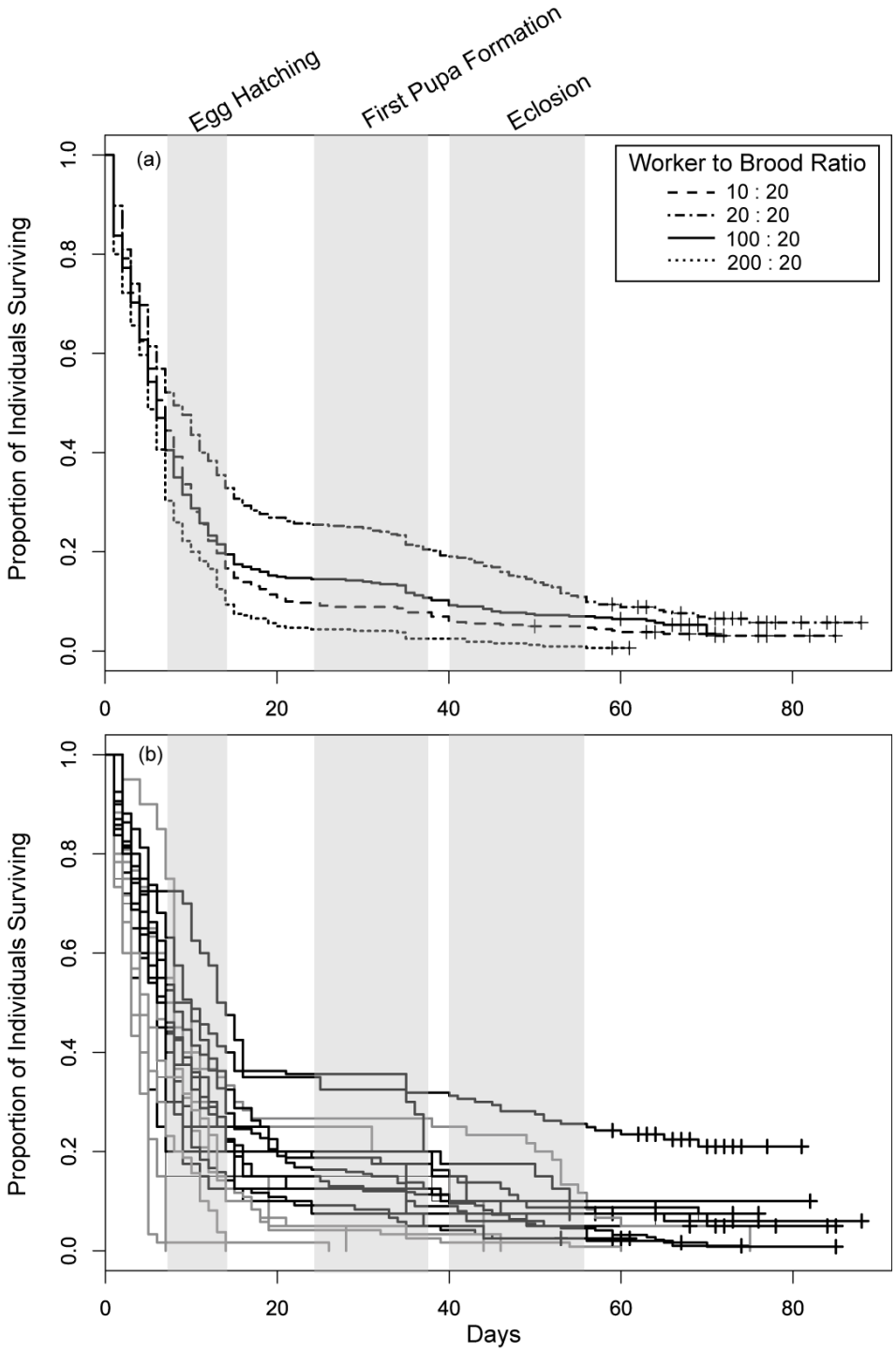
Appendix 1

Here, we further analyse the survival of brood during the entire experimental period from the egg stage until adulthood. Interestingly, most of the observed mortality in brood occurred early in development, at the egg or early larval stage (Appendix Fig. 1). We find an effect of maternal origin, rearing group, and infection treatment, plus an interaction between maternal origin and rearing group, on the survival trajectory of brood (Full parametric survival model: $\chi^2 = 1168$, d.f. = 316, $p < 0.0001$; maternal origin: $\chi^2 = 323.2$, d.f. = 19, $p < 0.0001$; rearing group: $\chi^2 = 110.6$, d.f. = 3, $p < 0.0001$; infection treatment: $\chi^2 = 538.5$, d.f. =

3, $p < 0.0001$; maternal origin * rearing group: $\chi^2 = 150.0$, d.f. = 57, $p < 0.0001$). Among the rearing group treatments (Appendix Fig. 1a), we find that brood from the 20:20 worker-to-brood ratio groups perform best until adulthood, when the mortality rate increases during the infection experiment (Fig. 2). We also find that the offspring of nearly half of the queens do not survive until adulthood (grey lines, Appendix Fig. 1b), and there is still substantial variation in survival rates among the queens that do produce surviving offspring (black lines).

Appendix Methods:

We performed routine counts of the number and approximate age of the developing brood throughout their maturation period. Prior to the first pupation event, observations were done once or twice a week. During this time, we smoothed the mortality curves by averaging mortality across the days intervening our observations. After pupation, the rearing groups were checked daily. In our analysis, all survival lines begin on the first day that the brood was placed with the rearing group (Appendix Fig. 1). We performed a parametric survival analysis using the Weibull distribution, which produced the minimum error deviance for this model.



Appendix Fig. 1 The survival of brood from the time that we placed eggs into rearing groups until the end of the fungal challenge experiment, based on their rearing group treatment (a) and their maternal origin (b). Grey boxes show the mean (+/- standard deviation) timing of major phenological events, including egg hatching, pupation, and eclosion. Maternal origin lines are distinguished between those that had surviving offspring after the complete

experiment (black lines) *versus* those that did not (grey lines). Much of the mortality that we observed in all conditions occurred very early in development, during the egg and early larval stages. Tick marks show individuals that were censored in our survival analysis.

Chapter 4: Wood ants protect their brood with tree resin

Timothée Brütsch & Michel Chapuisat

Brütsch, T., & Chapuisat, M. (2014). Wood ants protect their brood with tree resin. *Animal Behaviour*, *93*, 157–161.

ABSTRACT

Social insects use multiple lines of collective defences to combat pathogens. One example of a behavioural group defence is the use of anti-microbial plant compounds to disinfect the nest. Indeed, wood ants collect coniferous tree resin, and the presence of resin in their nest protects them against fungal and bacterial pathogens. Many questions remain on the mechanisms of resin use, including which factors elicit resin collection and placement within nests. Here, we investigate whether the presence of brood induces *Formica paralugubris* workers to collect more resin, and whether the workers preferentially place resin near the brood. We also test if the collection and placement of resin depends on the presence of the fungal entomopathogen *Beauveria bassiana*. We show that workers bring more resin to their nest when brood is present, and preferentially place the resin near the brood. In contrast, workers do not increase resin collection in response to exposure to *B. bassiana*, nor do they place resin closer to contaminated brood or contaminated areas of the nest. This lack of response may be explained by a limited effect of resin against the germination and growth of *B. bassiana in vitro*. Overall, our main result is that woods ants actively position resin near the brood, which probably confers prophylactic protection against other detrimental microorganisms.

INTRODUCTION

The use of plant compounds to combat parasites has been documented in various animal taxa, including insects, birds and mammals (Clayton & Wolfe 1993; Chapuisat *et al.* 2007; Simone, Evans & Spivak 2009; Lefèvre *et al.* 2010). The mechanisms are also varied, from direct ingestion and topical application to nest fumigation (Huffman 2003; Gwinner & Berger 2006; Villalba, Provenza & Shaw 2006). Plant use may be prophylactic or curative, and may benefit the individual or its offspring (de Roode, Lefèvre & Hunter 2013). For example, monarch butterflies preferentially lay eggs on toxic plants when they are infected by protozoan parasites, which reduces the growth of the parasite in their offspring (Lefèvre et al. 2010; 2012). However, in many cases the mechanisms governing the use of medicinal substances by animals are still poorly known, and it is notably difficult to demonstrate that the contact with the substance is deliberate and primarily aimed at fighting parasites (Clayton & Wolfe 1993; Gwinner & Berger 2005; Manson, Otterstatter & Thomson 2010; Suárez-Rodríguez, López-Rull & Garcia 2013).

In social insects, many defences are collective and contribute to diminish the parasite pressure at the colony level (Cremer *et al.* 2007; Wilson-Rich *et al.* 2009; de Roode & Lefèvre 2012). The use of medicinal plant substances has been primarily documented in wood ants and bees (Christe *et al.* 2003; Chapuisat *et al.* 2007; Simone-Finstrom & Spivak 2010). Indeed, wood ants, honeybees and

stingless bees collect and incorporate plant resin into their nests (Christe *et al.* 2003; Duangphakdee *et al.* 2009; Simone *et al.* 2009). Due to its anti-fungal and anti-bacterial properties, this resin may protect the colony against multiple pathogens (Banskota, Tezuka & Kadota 2001; Christe *et al.* 2003; Chapuisat *et al.* 2007; Simone-Finstrom & Spivak 2010). For example, in the wood ant *Formica paralugubris*, the presence of resin increased the survival of adult workers and larvae exposed to the bacteria *Pseudomonas fluorescens*, as well as the survival of larvae exposed to the fungal pathogen *Metarhizium anisopliae* (Chapuisat et al. 2007). Moreover, in wood ants and honeybees, the presence of resin reduced the microbial load and allowed individuals to downregulate some components of their immune system (Christe et al. 2003; Castella et al. 2008a; Simone et al. 2009).

The mechanisms governing the use of resin by wood ants remain little known. Field experiments revealed that workers foraging on trails prefer to collect resin over other kinds of nest material, such as twigs and small stones (Castella et al. 2008b). The preference for resin was higher in spring and summer than in autumn, raising the hypothesis that resin collection might primarily serve to protect the brood, which is produced at this time of the year (Castella et al. 2008b).

Laboratory experiments also suggested that the use of resin was prophylactic and constitutive rather than curative and infection-dependent, as the workers did not increase resin collection when their colonies were exposed to *M. anisopliae*

(Castella et al. 2008b). However, the behavioural response may depend on the parasite. For example, honeybee colonies tended to increase resin collection after being challenged with the fungal pathogen *Ascophaera apis* (Simone-Finstrom & Spivak 2012). Hence, more fungal pathogens should be tested in wood ants. Moreover, the hypothesis that workers preferentially place the resin close to contaminated brood or contaminated nest areas, as compared to non-contaminated ones, has not been tested so far.

Here, we investigate whether the presence of brood and/or of the virulent fungal entomopathogen *Beauveria bassiana* influences the rate of resin collection by wood ant workers, as well as the spatial distribution of resin in the nests. If *F. paralugubris* workers use resin to protect brood, we expect that they will collect more resin when brood is present in their nest, and that they will place the resin close to the brood. If the ants use resin in response to the fungal contamination rather than as a constitutive prophylaxis, we expect workers to increase resin collection after exposure to *B. bassiana*, and to preferentially place resin near contaminated brood or contaminated nest areas.

MATERIAL AND METHODS

In our experiment, workers collected resin from foraging arenas and placed it in experimental nests (Figure 1). We sampled *F. paralugubris* workers, brood and nest material from 20 field nests in the "Chalet à Roch" population. The study

population is located in sparse spruce forest (*Asplenio-Piceetum*) at an altitude between 1320 and 1420 m in the Swiss Jura mountains (Cherix 1980). It consists of hundreds of large and highly polygynous nests interconnected by trails and forming a supercolony (Holzer et al. 2006). The sampled brood consisted of pupae, which are abundant and easy to sample in the upper parts of mounds. Because of their soft cuticles, ant pupae tend to be highly susceptible to fungal entomopathogens (Tragust et al. 2013b).

In the laboratory, we removed the resin already present in the nest material. We split the samples from each field nest into four experimental nests, assigned to four treatments, in full factorial design: presence or absence of pupae and presence or absence of *Beauveria bassiana*, a virulent generalist fungal entomopathogen (Uma Devi et al. 2008). *B. bassiana* has been reported to successfully infect and kill adult ant workers (Purcell *et al.* 2012; Reber & Chapuisat 2012a) and brood (Broome, Sikorowski & Norment 1976; Patterson & Briano 1993).

To monitor the spatial distribution of resin, each experimental nest had two internal compartments, one that received nest material with pupae and/or parasite contamination, while the other received only nest material (Fig. 1). The nest consisted of a dark plastic box (13.8 x 18.3 x 6.2 cm) filled with resin-free nest material up to a height of 1.5 to 2 cm. The two equal-sized internal compartments were separated by a thin wall of plastic 3.5 cm high that divided nest material but

did not reach the top of the box, so that the ants could easily move between compartments inside the nest. Each compartment had a small entrance hole giving way to a foraging arena consisting of a plastic tray (22 x 35 x 15 cm) lined with Fluon to prevent ants from escaping. Each experimental nest received 200 workers.

In the foraging arena, the workers had access to 2.5 g of coniferous tree resin, in the form of approximately 300 grains of resin of various sizes that were previously removed from the nest material. Workers also had ad libitum access to water and standard jelly food (Reber & Chapuisat 2012b).

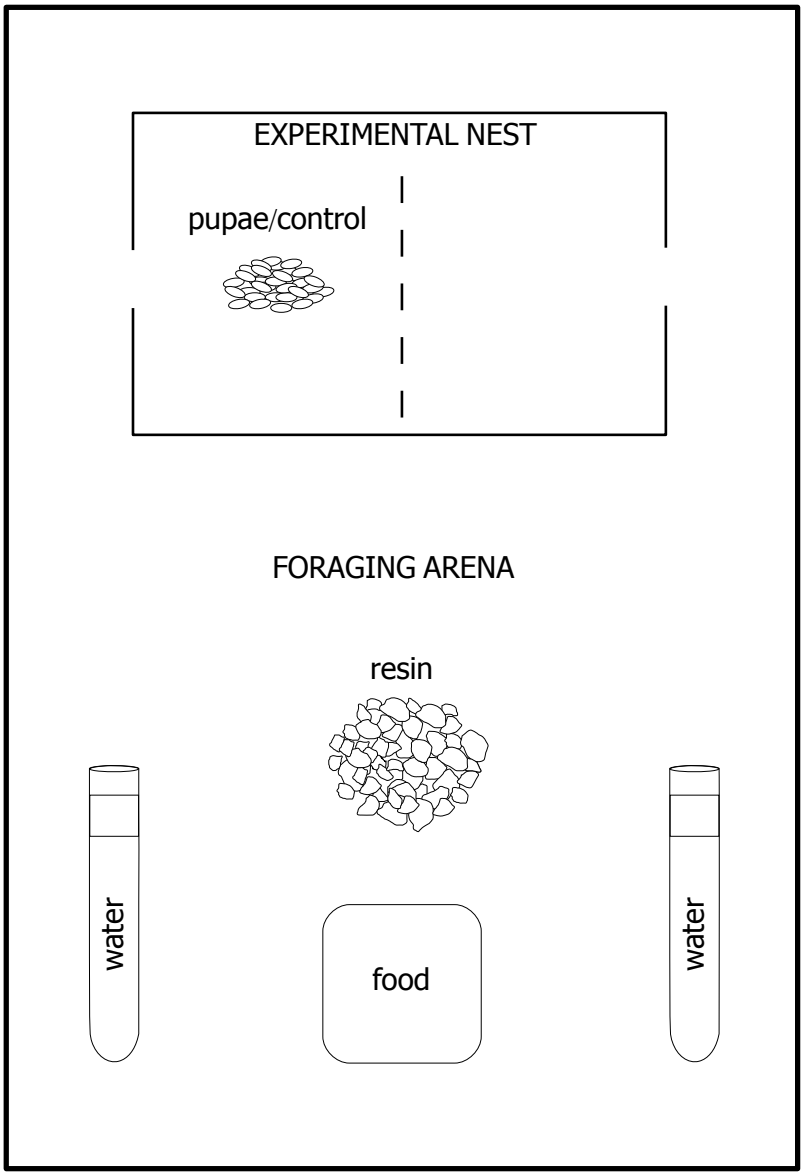


Figure 1. Outline of the experimental set up. The dashed line indicates the internal separation of the nest material in two compartments. See material and methods for details.

For each of the 20 field nests, one of the four experimental nests received one of the four following experimental treatments:

1. Presence of non-contaminated brood (Brood +, Pathogen -). We placed 100 pupae originating from the same field nest as the workers in one randomly chosen compartment of the nest. The group of pupae had been sprayed with approximately 220 µl of control solution (0.05 % sterile Tween 20).

2. Presence of non-contaminated control items (Brood -, Pathogen -). One of the nest compartments received 100 small plastic pieces similar to pupae in size and shape (approximately 4 mm long and 3 mm wide). These pieces had been sprayed with control solution.

3. Presence of brood contaminated by the fungal pathogen (Brood +, Pathogen +). One of the nest compartments received 100 pupae that had been sprayed with approximately 220 µl of *B. bassiana* spore solution (4.6×10^7 conidia/ ml).

4. Presence of control items contaminated by the fungal pathogen (Brood -, Pathogen +). One of the nest compartments received 100 small plastic pieces that had been sprayed with *B. bassiana* spore solution.

We checked the content of the experimental nests on a daily basis, recording the position of brood or control plastic pieces. In three cases, the workers transferred all the brood to the opposite compartment towards the end of the experiment. We conservatively kept these nests and their original brood compartment in the

analyses. However, we checked that excluding these three nests or redefining their brood compartments did not affect the outcomes of the statistical tests. After one week, we weighted the total amount of resin that the ants had placed in each compartment of each nest. At the end of the experiment, the workers were euthanized in a -20°C freezer.

In follow-up experiments aiming at further distinguishing between a constitutive and therapeutic use of resin, we assessed if the resin inhibited the germination and growth of *B. bassiana* in vitro. We performed two types of growth inhibition assays. First, we spread 100 µl of a spore solution (10^7 spores per ml in 0.05 % Tween 20) on 9 cm diameter petri dishes containing malt extract agar (MEA). We placed four pieces of resin on each petri dish (e.g. Chapuisat et al. 2007). Second, we performed well-diffusion assays, adapted from Mandeel et al. (2005). In petri dishes, we mixed spores with MEA, using three final concentrations: 2 x 10^6, 6 x 10^5 and 10^4 spores per ml, respectively. In each plate, we cut four 4 mm diameter holes. Two of these holes were filled with resin dissolved in ethanol (100 % mass/volume). One of the remaining two holes was filled with ethanol, as a negative control while the other was filled with 14% bleach, a potent, large spectrum anti-fungal substance, as a positive control. We incubated the plates at 25°C for four days.

Statistical Analyses

To test whether the presence of pupae, the exposure to the pathogen or an interaction between the two factors influenced the total amount of resin that workers brought to the nest, we constructed a mixed-effect model with pupae presence and exposure to the pathogen as fixed factors, and the field nest as a random factor, using the lmer function (Bates et al. 2014) in R version 3.0.2 (R Development Core Team 2013). We obtained *P* values from likelihood ratio tests comparing models with and without the variable of interest.

To examine if the workers preferentially deposited the resin near the brood and/or the fungal pathogen, we compared the amount of resin in the compartment containing pupae and/or pathogen to the one in the compartment containing only nest material. We used paired sample Welch *t*-tests, as the compartments are paired within each experimental nest.

RESULTS

Workers brought significantly more resin to the nest in presence of pupae than in control, broodless conditions (Fig. 2a ; $\chi^2_1 = 12.2$, P = 0.0005). In contrast, workers did not change their rate of resin collection when the pupae or control plastic pieces were contaminated with pathogenic *B. bassiana* spores ($\chi^2_1 = 0.36$, P = 0.55). There was no significant interaction between the presence of pupae and

the exposure to the pathogen, which indicates that the impact of brood presence on resin collection is independent of fungal contamination ($\chi^2_1 = 0.18$, P = 0.67).

Within experimental nests, workers preferentially placed the resin near pupae, independently of the fungal contamination. Indeed, the mass of resin was significantly higher in the compartments containing non-contaminated pupae (paired samples Welch t-test: $t_{19} = 3.3$, P = 0.003) or in the compartments containing *Beauveria* contaminated pupae ($t_{19} = 2.6$, P = 0.02) than in the corresponding broodless compartments of the same experimental nests (Fig. 2b). In contrast, workers did not place more resin in compartments with control plastic pieces ($t_{19} = 1.1$, P = 0.28) or in compartments with *Beauveria* contaminated plastic pieces ($t_{19} = -0.22$, P = 0.83) than in the broodless compartments of the same experimental nests (Fig. 2b). Usually, the resin tended to be distributed evenly in the compartment containing the brood. However, in one uncontaminated nest, the resin was clearly placed around the pupae.

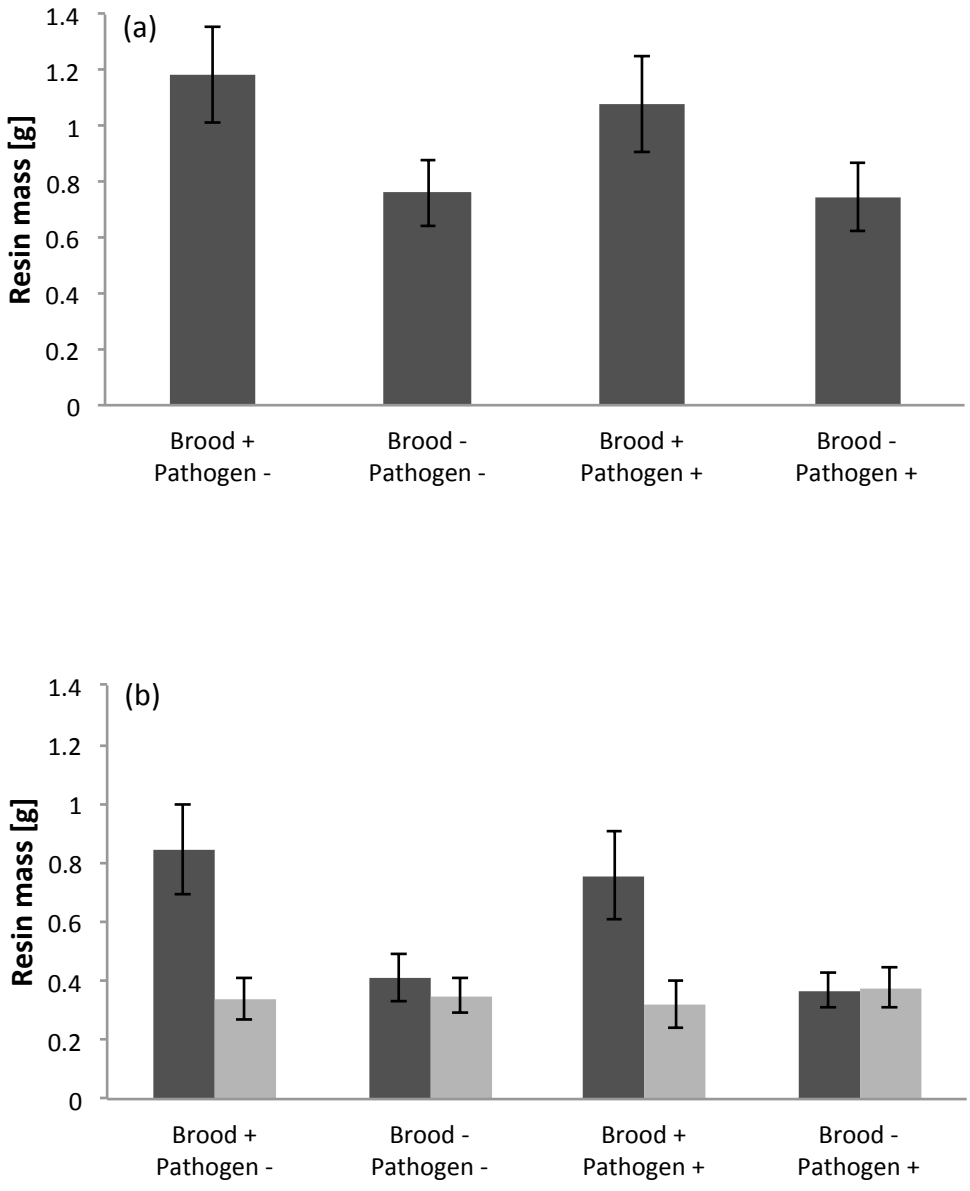


Figure 2. (a) Average total amount of resin that wood ant workers brought to the experimental nests and (b) average amount of resin that wood ant workers brought in each nest compartment, in function of brood presence and exposure to the fungal pathogen *B. bassiana*. Each nest had two compartments, of which one randomly chosen received the treatment. In (b), black and grey bars show the average amount of resin placed in treated compartments and in compartments that

contained only nest material, respectively. Brood + / - indicates pupae presence / absence, and pathogen + / - pathogen presence / absence, respectively. Sample size was 20 experimental nests per treatment. Error bars indicate standard error of the mean quantity of resin collected.

In the follow-up experiments testing the effect of resin against *B. bassiana* in vitro, we did not observe distinctive inhibition halos around the pieces of resin, nor around the wells filled with resin dissolved in ethanol. We observed large fungus-free halos around the wells containing bleach, but not around the ones containing only ethanol. This suggests that the resin has little effect against the germination and growth of *B. bassiana*, at least in the conditions tested.

DISCUSSION

Wood ant workers collected significantly more tree resin when brood was present in their nests. Specifically, the presence of pupae in experimental nests led workers to bring 50% more resin from the foraging arenas to the nests, as compared to the broodless situation. Moreover, within the nests, workers preferentially placed the resin near pupae. On average, 71% of the resin collected by workers was placed in the nest compartment containing brood; the rest was deposited in the compartment containing only nest material.

The experimental findings that workers retrieve more resin when pupae are present in their nest and that they preferentially place the resin near pupae

strongly support the hypothesis that workers use resin to protect their brood from pathogens. This is in line with earlier findings showing that the presence of resin decreases the prevalence of bacteria and fungi in nest material (Christe et al. 2003), and protects the larvae against some specific virulent bacterial and fungal pathogens (Chapuisat et al. 2007). A higher rate of resin collection when brood is present is also fully consistent with the field observation that workers collect proportionally more resin in spring and summer, when brood is produced, than in autumn (Castella et al. 2008b).

In the social insects, brood is of crucial importance for the future of the colony, and often receives extra protection (Ayasse & Paxton 2002; Cremer *et al.* 2007). The brood is likely to be particularly sensitive to pathogens, because larvae and pupae do not have a fully sclerotized and melanised cuticle, which facilitates the penetration of fungal spores (Ortiz-Urquiza & Keyhani 2013). The brood also lacks some of the antibiotic-producing glands of adult ants, like metapleural glands (Stow & Beattie 2008).

Many behavioural defences seem to be targeted at brood protection against pathogens. Some ants preventively self-groom before entering the brood chamber (Morelos-Juárez et al. 2010), while others stay away from brood when they are contaminated (Ugelvig & Cremer 2007). Some ants even place venom on fungus-exposed brood to disinfect them (Tragust et al. 2013a). The maintenance of a strict nest hygiene, removal of diseased individuals, and allo-grooming of all

individuals returning to the colony are also efficient but less specific ways to prevent the spread of diseases (Hart & Ratnieks 2002; Wilson-Rich *et al.* 2009; Reber *et al.* 2011; Tragust *et al.* 2013b). Given the strong and broad-spectrum antimicrobial activity of resin (Banskota et al. 2001; Christe et al. 2003; Chapuisat et al. 2007), depositing resin near brood appears to be another powerful measure to reduce the risk of infection in brood.

In our experiment, workers did not increase resin collection when the brood had been exposed to *B. bassiana*, nor did they place resin closer to contaminated brood or contaminated areas of the nest. There are several possible explanations for this lack of response. First, resin may have little effect against *B. bassiana*. In line with this hypothesis, in our follow-up assay in vitro the resin did not inhibit the germination and growth of the pathogen. Second, *B. bassiana* may have only limited impact on ant pupae in natural conditions, for example due to cocoon presence or systematic allo-grooming (Reber et al. 2011; Tragust et al. 2013b). Third, workers may not be able to detect the presence of spores or of infected pupae. In our experiment, we did not detect any removal of contaminated or dead pupae from the nests.

Overall, our experiment suggests that resin collection is constitutive and prophylactic, as it does not depend on the presence of specific pathogens. These results are similar to the ones obtained when challenging these ants with another generalist fungal entomopathogen, *M. anisopliae*, which, in contrast to *B.*

bassiana, was inhibited by resin and detrimental to the ants (Chapuisat et al. 2007; Castella et al. 2008b). Prophylactic defences are often perceived as fixed, whereas therapeutic defences are seen as plastic, varying with the risk and predictability of infection (de Roode & Lefèvre 2012). Here, we show that resin collection and placement is both prophylactic and plastic, as it depends on the presence of brood in the nest.

Although a prophylactic, multi-target use of resin is probably common, a therapeutic response might still occur to fight more specific pathogens, as has been documented in the honeybee (Simone-Finstrom & Spivak 2012). Conditional, adaptive responses to endoparasite infections have also been reported in monarch butterflies, which lay eggs on toxic plants (Lefèvre et al. 2010; 2012), and fruit fly larvae, which increase ethanol consumption (Milan, Kacsoh & Schlenke 2012).

In conclusion, wood ants brought more resin to their nests when brood was present, and they deposited the resin near the brood, independently of the presence of a fungal pathogen. When combined with our previous findings on the protective effects of resin (Christe et al. 2003; Chapuisat et al. 2007), these new results indicate that wood ants actively place resin near the brood in order to prophylactically protect these vulnerable and precious colony members from detrimental microorganisms.

Acknowledgements:

We thank Antoine Felden for help in the field and the lab and Jessica Purcell, Raphaël Jeanson and three anonymous reviewers for helpful comments on the manuscript. The study was supported by grant 31003A_125306 from the Swiss National Science Foundation to MC.

Chapter 5: Wood ants produce a potent antimicrobial agent by applying formic acid on tree-collected resin

Timothée Brütsch*, Geoffrey Jaffuel*, Armelle Vallat-Michel, Ted C. J. Turlings and Michel Chapuisat

*These authors contributed equally to the work

Brütsch, T., Jaffuel, G., Vallat-Michel, A., Turlings, T. & Chapuisat, M. (2017). Wood ants produce a potent antimicrobial agent by applying formic acid on tree-collected resin. Accepted for publication in *Ecology and Evolution*

ABSTRACT

Wood ants fight pathogens by incorporating tree resin with antimicrobial properties into their nests. They also produce large quantities of formic acid in their venom gland, which they readily spray to defend or disinfect their nest. Mixing chemicals to produce powerful antibiotics is common practice in human medicine, yet evidence for the use of such "defensive cocktails" by animals remains scant. Here, we test the hypothesis that wood ants enhance the antifungal activity of tree resin by treating it with formic acid. In a series of experiments, we document that (i) tree resin had much higher inhibitory activity against the common entomopathogenic fungus *Metarhizium brunneum* after having been in contact with ants, while no such effect was detected for other nest materials; (ii) wood ants applied significant amounts of endogenous formic and succinic acid on resin and other nest materials; and (iii) the application of synthetic formic acid greatly increased the antifungal activity of resin, but had no such effect when applied to inert glass material. Together, these results demonstrate that wood ants obtain an effective protection against a detrimental micro-organism by mixing endogenous and plant-acquired chemical defences. In conclusion, the ability to synergistically combine antimicrobial substances of diverse origins is not restricted to humans and may play an important role in insect societies.

INTRODUCTION

Animals living in large social groups are exposed to a high risk of epidemics. In response to this threat, social animals have evolved sophisticated individual and collective means to control disease, which combine immunological, behavioural and organizational defences (Naug & Smith 2007; Cremer *et al.* 2007; Wilson-Rich *et al.* 2009). Collective defences include ways to keep the environment hygienic, for example by removing or neutralizing infectious particles (Morelos-Juárez *et al.* 2010; Tragust *et al.* 2013a).

An original way to fight enemies is to exploit the defensive chemicals produced by other organisms (de Roode *et al.* 2013). Humans use a myriad of chemicals from multiple sources, alone or in synergistic combinations, to medicate themselves, clean their environment, or control pests (Mason & Singer 2015). Animals also harness chemicals produced by other species for their own defence (de Roode *et al.* 2013; Mason & Singer 2015). For example, many insect herbivores sequester plant secondary metabolites to gain protection against predators or parasites (Nishida 2002; Lefèvre *et al.* 2010). It has been proposed that animals may combine multiple acquired chemicals to benefit from synergistic effects (Mason & Singer 2015). However, evidence for the use of such "defensive cocktails" by animals remains scant (Mason & Singer 2015).

Wood ants and honeybees incorporate tree resin with antimicrobial properties into their nest (Christe *et al.* 2003; Simone-Finstrom & Spivak 2010). In the wood

ant *Formica paralugubris*, workers actively collect large amounts of resin from coniferous trees, which they bring back to their nest and place near their brood (Castella *et al.* 2008b; Brütsch & Chapuisat 2014). Coniferous resin is rich in secondary metabolites with antimicrobial properties (Phillips & Croteau 1999). The presence of resin decreases the overall microbial load in wood ant nests and protects the ants against bacterial and fungal pathogens (Christe *et al.* 2003; Chapuisat *et al.* 2007).

Wood ants are also chemical factories. They produce large quantities of formic acid in their venom gland, which they spray at prey and enemies (Blum 1992; Morgan 2008). In other ant species, formic acid is also present in the trophallactic fluid following oral uptake from the venom gland (Tragust *et al.* 2013a), and other acids have been found in the metapleural glands (Vieira *et al.* 2012). Formic acid has well known antimicrobial properties. It is widely used by humans, as cleaning agent and as preservative additive in livestock food (Iba & Berchieri 2007). Moreover, formic acid is effective against *Metarhizium*, a common fungal pathogen of ants (Graystock & Hughes 2011), and is used by *Lasius neglectus* ants to disinfect their brood (Tragust *et al.* 2013a). This suggests that wood ants may combine endogenous acids with tree resin.

Here we test the hypothesis that wood ants apply self-produced acids on tree-collected resin to produce a more potent antimicrobial agent. Specifically, we examined if i) ants enhance the antifungal activity of resin, ii) ants add

endogenous acids to resin, and iii) these acids increase the antifungal activity of resin.

MATERIALS AND METHODS

Effect of wood ants on the antifungal activity of resin

In a first experiment, we tested if spruce tree resin that had been in contact with wood ants had increased inhibitory activity against the generalist entomopathogenic fungus *Metarhizium brunneum,* compared to resin that had not been contacted by ants. As controls, we used twigs and small stones. Twigs are major constituents of wood ant nests and small stones are commonly found in some of the nests (Castella *et al.* 2008b).

We established experimental groups of workers from field colonies of *Formica paralugubris* (Chapuisat *et al.* 1997; Christe *et al.* 2003). We collected pieces of fresh resin from spruce tree, as well as twigs and stones of similar size, in areas away from ant colonies. The pieces of resin, twigs and stones were disinfected under UV light (30 mn under a UV lamp radiating at 254 nm in a Biosafety Cabinet BSC - 700II, HMC Europe).

Each tested material (pieces of resin, twigs and stones) was kept with and without ant workers for two weeks. In ant-exposed treatments, four pieces of the tested material were kept with 40 workers in a small plastic box (13.5 x 15 x 5 cm; n =

25 replicates for each material). In ant-free controls, four pieces of the tested material were kept in a box without workers (n = 25 replicates for each material). The edges of the boxes were treated with Fluon to prevent ant escape. The workers were free to interact with the pieces of resin, twigs and stones. They had *ad libitum* access to water and jelly food consisting of chicken eggs, honey, water and agar (Reber & Chapuisat 2012b).

After this two-week period of exposure to ants or ant-free control conditions, we measured the inhibitory activity of resin, twigs and stones against the fungus *M. brunneum*. We used a strain that had been isolated from Valais, Switzerland, and showed high virulence against *Formica selysi* (Reber & Chapuisat 2012a). *M. brunneum* was described in 2009, and was previously known as *M. anisopliae anisopliae* (Bischoff *et al.* 2009). A strain of the latter species complex caused high mortality to *F. paralugubris* (Chapuisat *et al.* 2007). *M. brunneum* is used here as a model fungal pathogen, while other pathogens might be important in the field. Indeed, the resin affects a broad spectrum of fungi and bacteria that are potential pathogens of *F. paralugubris* (Christe *et al.* 2003; Chapuisat *et al.* 2007).

Inhibitory activity was measured on Malt extract agar nutritive medium in 8.5 cm diameter petri dishes, inoculated by plating 100 µl of 0.05% Tween 20 solution containing 7×10^5 asexual spores (= conidia) of *M. brunneum*. The four pieces of each material (resin, twigs or stone) coming from the same experimental box were placed together in a petri dish. The petri dishes (n = 25 per material and treatment)

were incubated at 25°C for six days. We then photographed each petri dish and measured the spore-free areas around the tested material with the *ImageJ* software (Schneider, Rasband & Eliceiri 2012). Spore-free areas were either void of both spores and mycelium, or consisted of white and mostly sterile mycelium known as sectors (Ryan *et al.* 2002).

For the statistical analysis, we used one estimate of inhibitory activity per experimental box (= replicate). We therefore measured the overall spore-free area in each petri dish, and divided it by four. This is a conservative estimate of the average inhibition halo around each item, because large halos were overlapping. We constructed a model with the spore-free area as response variable, and the material (resin, twigs or stone) and previous contact with workers (presence or absence of workers in the box) as explanatory variables. The response variable was square root transformed to achieve homogeneity of variances and normal distribution of residuals, as required for an ANOVA. We carried out post-hoc comparisons with Tukey's HSD tests.

Transfer of endogenous acids to resin and other types of nest material

In a second experiment, we examined if ants applied endogenous acids to pieces of resin, twigs or stones. We placed four pieces of the tested material (resin, twigs or stone) in boxes with and without ants as described above, except that there were 50 workers per box in the treatment with ants (n = 10 replicates for each

material and treatment type, with or without ants). As organic acids are very soluble in water, we sampled the acids from each material (resin, twigs or stone) by immersing the four items from the same experimental box in 1 ml of MilliQ water for 30 seconds. The samples were stored at -20°C until HPLC analysis (see below).

We also tested if the retention and subsequent detection of formic acid varied with the type of material (resin, twigs and stone). For this, 1µl of 60 % synthetic formic acid (CAS number 64-18-6) was deposited on each type of material (10 replicates per material and treatment). After 24 hours, each item was immersed in 500 µl of MilliQ water for 30 seconds. The samples were stored at -20°C until HPLC analysis.

To identify the origin of the acids detected on nest materials, we extracted the content of the venom gland, trophallaxis fluid and metapleural glands from ten workers anaesthetised with CO_2. For venom and trophallaxis fluid, we gently pressed their gaster and collected the liquid with a micro capillary. For the metapleural glands, we introduced the tip of a micro capillary in the gland opening and extracted the liquid by capillarity. We diluted these extracts in 500 µl of MilliQ water. The samples were stored at -20°C until HPLC analysis.

To measure the organic acids in the samples, we analysed them by High Performance Liquid Chromatography (HPLC), using an Agilent HP1100 HPLC system equipped with a diode array detector (DAD), with UV detection

wavelength set at 210 ± 2 nm. To remove small particles, the samples were centrifuged (3 min at 1400 rpm) and the supernatant was transferred to 2 ml glass vials (Interchim, Swiss Labs, Mulhouse, F). We injected 40 µl of the samples onto a 300 mm x 7.8 mm BP-100 H carbohydrate column (Benson Polymeric, USA). The temperature of the column was maintained at 40°C and MilliQ water was used as a solvent with 20 mM of sulfuric acid (analysis grade 95-97%, Honeywell, Germany) at a flow rate of 0.4 ml/min. Succinic (CAS number 150-90-3, Acros organics, USA) and formic (CAS number 141-53-7, Sigma Aldrich, USA) acids were quantified in the samples by external calibration. The linearity of the method was established using 6 standard solutions at concentration levels from 5 µg/mL to 1.3 mg/mL.

We constructed a model with acid quantity as response variable and the material (resin, twigs or stone) and previous contact with workers (presence or absence of workers in the box) as explanatory variables. We analysed the data of each acid separately.

Effects of acids on the antifungal activity of resin

In a third experiment, we tested if combinations of synthetic acids corresponding to the composition of ant endogenous acids enhanced the antifungal activity of the resin. We mixed commercially available acids with MilliQ water to obtain a formic acid solution (formic acid 58.5 %), a venom-like mix (formic acid 58.5%,

succinic acid 1.2%) and a trophallaxis-like mix (succinic acid 3.6%, formic acid 0.06%) corresponding to the proportions of the main acids found in the venom and trophallactic fluid, respectively (see results). MilliQ water was used as control.

Pieces of spruce resin and pieces of safety glass were dipped in water, 58.5% formic acid, venom-like or trophallaxis-like mixes of synthetic acids, respectively. Safety glass was chosen as control because it is chemically inert. The amount of acid retained by pieces of glass and resin (after being dipped in acid) was not significantly different (0.011 ± 0.003 vs 0.013 ± 0.006 g, respectively; t = -1.56, d.f. = 42.83, p = 0.13; N = 30 pieces of each material). Inhibitory activity against *M. brunneum* was estimated by measuring the spore-free area around each item. We used the procedure described above, except that we plated 250 µl of solution containing 4.5 x 10^6 spores of *M. brunneum* on a nutritive medium of sabouraud glucose agar complemented with the antibiotics dodine, cycloheximide and chloramphenicol, in 13.5 cm diameter petri dishes, which allowed for better fungal growth. For each material, we placed one item subjected to each of the three acid treatments (dipped in 58.5% formic acid, venom-like and trophallaxis-like mixes of synthetic acids) and to control conditions (dipped in water) on the same petri dish (n = 25 replicates per material).

For each material, we calculated the increase in antifungal activity due to exposure to acids. Specifically, we subtracted the spore-free area produced by the

control from the area produced by the acid-exposed material in the same petri dish. We used Wilcoxon rank sum tests to examine if the acid-induced changes in antifungal activity differed between resin and inert glass material. All statistical analyses were performed in R version 3.3.0 (R Core Team 2016).

RESULTS

Effect of ants on the antifungal activity of resin

Pieces of resin that had been kept with wood ant workers showed a significantly higher inhibitory activity against *M. brunneum* than pieces of resin that had not been in contact with ants. In contrast, the presence of workers had no effect on the antifungal activity of twigs and stones (Fig. 1; ANOVA, interaction between material and contact with workers: d.f. = 2, $F = 3.9$, $p = 0.022$; Tukey's HSD post-hoc tests: resin vs resin that had been in contact with workers: $p < 0.0001$; twigs vs twigs that had been in contact with workers: $p = 0.99$; stones vs stones that had been in contact with workers: $p = 0.99$). Overall, resin had higher antifungal activity than twigs or stones (Fig. 1; ANOVA: d.f. = 2, $F = 57.6$, $p < 0.0001$).

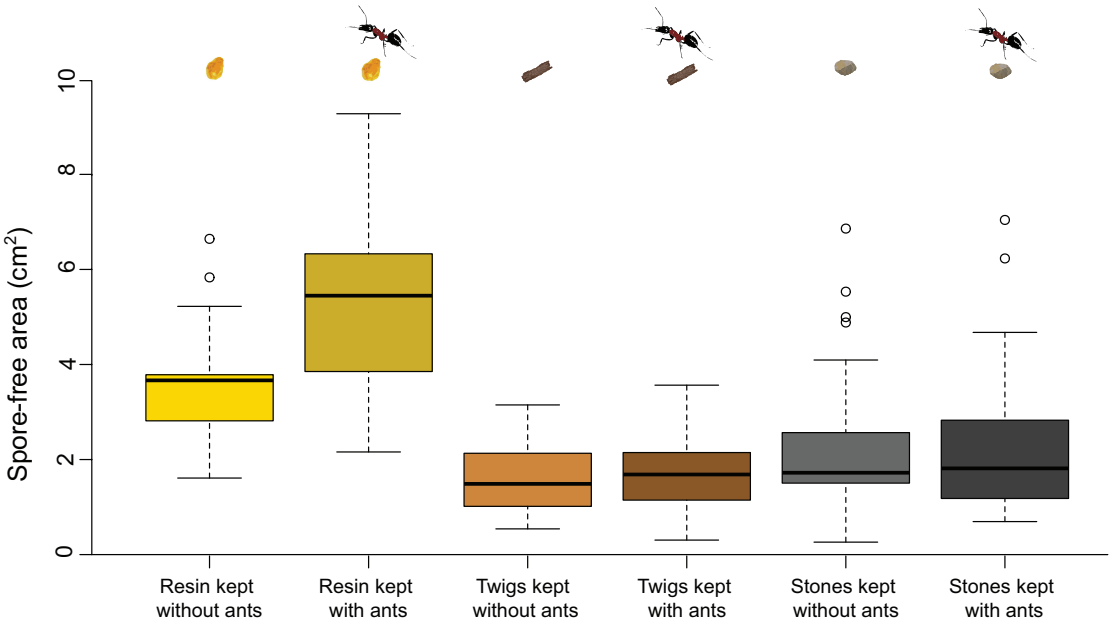


Figure 1. Antifungal activity of pieces of resin, twigs and stones that had been kept without or with ants, respectively. The boxplots show the median values of spore-free areas around the tested items, as well as the upper and lower quartiles. The whiskers encompass 1.5 times the interquartile range. Outliers are indicated by circles.

Transfer of endogenous acids to resin and other types of nest material

Both formic and succinic acids were found on resin, twigs and stones that had been in contact with workers (Table 1). In contrast, we did not detect these two acids on resin that had not been in contact with workers. We detected some succinic acid on twigs and formic acid on stones that had not been in contact with workers, but in much smaller quantities than on similar materials that had been in contact with workers (Table 1). Overall, we detected significantly more acids on materials that had been kept with ants (ANOVA, main effect of contact with

workers: formic acid, $F = 34.8$, d.f. = 1, $p < 0.0001$; succinic acid, $F = 28.1$, d.f. = 1, $p < 0.0001$).

	Resin		Twigs		Stones	
	Kept without ants	Kept with ants	Kept without ants	Kept with ants	Kept without ants	Kept with ants
Formic acid	0 (0)	0.022 μl ± 0.029 (10)	0 (0)	0.031 μl ± 0.015 (10)	0.058 μl ± 0.047 (9)	4.6 μl ± 2.46 (10)
Succinic acid	0 (0)	0.13 μl ± 0.13 (10)	0.004 μl ± 0.0094 (2)	0.049 μl ± 0.031 (10)	0 (0)	0.097 μl ± 0.083 (10)

Table 1. Mean quantity of acids detected on resin, twigs and stones that had been kept without ants or with ants, expressed as volume of acid in µl ± SD. The number of samples in which the acid was detected is given in parentheses (out of 10 samples).

For formic acid, there was a significant interaction between material and contact with ants (Table 1; $F = 33.6$, d.f. = 2, $p < 0.0001$). The high amount of formic acid detected on stones that had been kept with ants can be explained by large differences among the three materials in their ability to sequester and release formic acid. Indeed, when we experimentally deposited a controlled amount of 1µl of 60% formic acid on each type of material, we detected much more acid on stones than on resin and twigs, respectively (mean in µl ± SD: stones, 0.41 ± 0.11;

resin, 0.00044 ± 0.0014; twigs, 0.013 ± 0.0043; Kruskal-Wallis rank sum test: $\chi 2 = 26.5$, d.f. = 2, $p < 0.0001$).

Worker ants produced large quantities of formic acid and comparatively small amounts of succinic acid. The venom gland extracts contained on average 58.5 % of formic acid (detected in all 10 samples) and 1.2 % of succinic acid (5 samples). The trophallactic fluid contained 3.6 % of succinic acid (9 samples) and 0.06 % of formic acid (1 sample). Fumaric acid was detected in trace quantities in the venom and trophallactic fluid. We did not detect any acid in the metapleural gland extracts.

Effects of acids on the antifungal activity of resin

The treatment with synthetic formic acid at a concentration corresponding to the one of venom increased the inhibitory activity of resin against *M. brunneum* (Fig. 2). Formic acid had a significantly stronger impact on the antifungal activity of resin than of inert glass material, which is indicative of a synergistic interaction (Fig. 2; Wilcoxon rank sum test: $W = 323$, $p < 0.0001$). The treatment with the venom-like mix (formic + succinic acids) also increased the antifungal activity of resin, but not more than formic acid alone, at the same concentration (Fig. 2). The increase in antifungal activity due to the venom-like mix was also stronger for resin than for glass ($W = 312$, $p < 0.0001$). The treatment with the trophallaxis-like mix, which contains more succinic acid and only traces of formic acid,

slightly decreased the antifungal activity of resin and had no effect on the antifungal activity of glass (Fig. 2; $W = 70$, $p = 0.0009$).

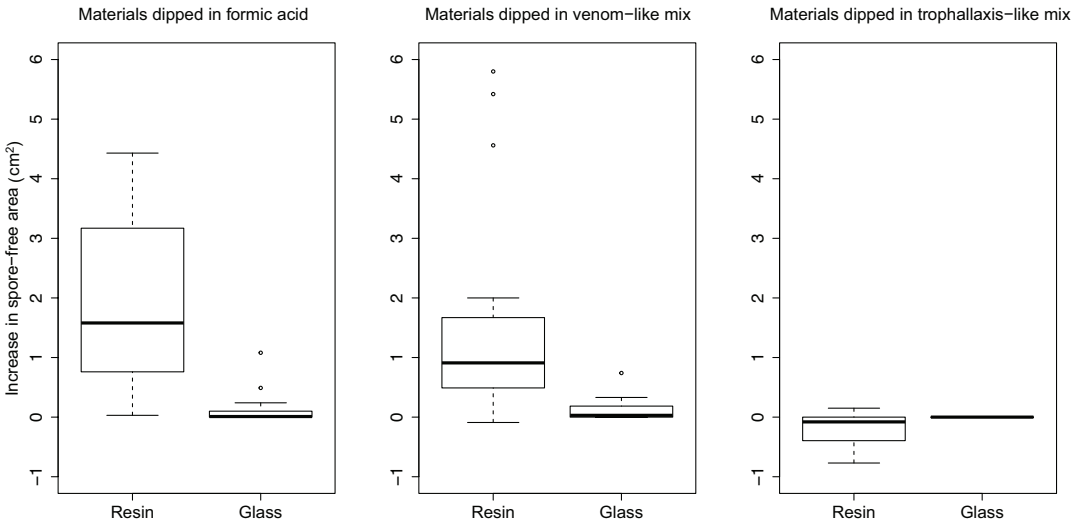


Figure 2. Increase in the antifungal activity of resin and glass dipped in 58.5% formic acid, venom-like and trophallaxis-like mixes of synthetic acids, respectively, relative to controls (same materials dipped in water).

DISCUSSION

Wood ants are known to incorporate plant resin with antiseptic properties into their nests (Christe *et al.* 2003; Simone-Finstrom & Spivak 2010). Here, we show that wood ants enhance the antifungal activity of tree-collected resin by supplementing it with endogenous formic acid. Three lines of experimental evidence support this claim. First, tree resin showed significantly higher inhibitory activity against the fungal pathogen *M. brunneum* after having been in

contact with wood ants. In sharp contrast, the contact with ants did not affect the antifungal activity of control materials commonly found in wood ant nests, namely twigs and small stones.

Second, the ants applied significant amounts of endogenous formic and succinic acid on resin and other types of nest material. The proportion of formic acid and succinic acid varied with substrate, which likely reflects differences among substrates in their ability to take up and release formic acid (Al-Hosney *et al.* 2005). Large quantities of formic acid and small amounts of succinic acid were found in the venom of wood ants.

Third, the treatment of resin with synthetic formic acid greatly increased the antifungal activity of the resin, but had no such effect on pieces of glass. This interaction between formic acid and substrate reveals a synergistic effect. Indeed, the combination of formic acid and resin produced a higher antifungal activity than the additive effect of each compound. The application of formic acid on resin was sufficient to obtain this synergistic effect, and succinic acid did not appear to contribute to the antifungal activity of resin. Together, these results provide strong evidence that wood ants apply formic acid produced in their venom gland on tree resin, which results in a synergistic increase in the antifungal activity of resin.

Documented cases of "defensive mixology", whereby animals actively combine antimicrobial substances of diverse sources to obtain a synergistic protection, are

extremely rare (Mason & Singer 2015). Honeybee workers manipulate tree resin with their mandibles, but there is no evidence that this process modifies the chemical composition of the resin (Simone-Finstrom & Spivak 2010). Stingless bees collect resin from several plant genera. Although these resins vary in their antibacterial properties, mixing them had no synergistic effect against a fungus and various bacteria (Drescher *et al.* 2014). Synergistic defences may also occur in herbivores or nectar-feeding animals (Mason & Singer 2015). For example, a dietary treatment with a mix of thymol and nicotine tended to reduce the load of a protozoan parasite in bumblebees (Biller *et al.* 2015).

Like humans, social insects have extraordinary capacities to exploit and modify their environment (Wilson 1971), and they rely on sophisticated means to keep pathogens at bay (Cremer *et al.* 2007). Here, we provide evidence that wood ants do not only collect tree resin with antimicrobial properties, they also supplement it with formic acid. Thus, wood ants combine their own chemical defences with the ones of plants to produce a more potent antimicrobial agent that contributes to nest hygiene (Christe *et al.* 2003; Castella *et al.* 2008b; Brütsch & Chapuisat 2014) and protects the ants against detrimental micro-organisms (Chapuisat *et al.* 2007). Together, these findings demonstrate that the ability to synergistically combine antimicrobial substances of diverse origins is not restricted to humans and may play an important role in insect societies.

Acknowledgements

We thank Matthias Held, Gregory Röder, Patrick Joye and Morgan Geuning for help in the laboratory. We thank the “Centre de Conservation de la faune et de la nature du Canton de Vaud” for the authorisation to collect ants (Authorisation 1660). Funding was provided by the Swiss National Science Foundation grants 31003A_125306 and 31003A-146641 to MC.

General discussion

In the first part of this thesis, I investigated the defenses against pathogens during colony founding and brood rearing. In **chapter 1**, we examined if young founding queens were able detect and avoid contaminated nest sites. We found that young queens were able to detect the entomopathogenic fungi *M. brunneum* and *B. bassiana*, but were surprisingly attracted to them instead of being repelled by them. Since the publication of our results, others have reported similar findings in another context (Pontieri et al. 2014). Pharaoh ants, *Monomorium pharaonis,* when relocating to a new nest site, preferred nest sites infected with *M. brunneum* sporulating cadavers of nestmates than nest sites containing uninfected cadavers (Pontieri et al. 2014). In previous studies, collembolans were attracted to *B. bassiana*, *Beauveria brongniartii* and *Metarhizium anisopliae* (Dromph & Vestergaard 2002) and mosquitoes were attracted to *B. bassiana* and *M. anisopliae* (George et al. 2013). Although the list of studies showing attraction by generalist fungal entomopathogens is growing, none of them can so far convincingly explain these observations. Several of the proposed hypotheses, such as fungal manipulation or insects seeking vaccination and trans-generational immune priming by contact with fungi imply that the fungi are pathogens. However, we observed a similar attraction with one of the two non-entomopathogenic fungi tested in our study, *Fusarium graminearum* (Brütsch et

al. 2014). Moreover, deliberately getting into contact with the pathogen implies that infection risk is lower than the potential benefits derived from contact. This strategy seems risky as these fungi are often lethal to the host species tested, and in the case of ants, even a single cadaver has been shown to make small colonies crash (Loreto & Hughes 2016). It is also not clear why these particular insect species are attracted to the pathogens, while some others, like termites, clearly avoid them (Mburu et al. 2009; Rath 2010; Yanagawa et al. 2012). Finally, it is interesting to note that non-pathogenic fungi sometimes repelled insects, like one of the two non-entomopathogens tested in our study, *Petromyces alliaceus* (Brütsch *et al.* 2014) or *Penicillium spp.* in the study with mosquitoes (George *et al.* 2013).

Although the results from these studies still lack convincing explanations, the observed patterns are very strong, and further studies with a variety of entomopathogenic and non-entomopathogenic fungi will be needed to understand this intriguing behavior. For instance, to test if contact with a pathogen provides benefits in term of vaccination or immune priming, ant queens (or other insects) would have to be infected with a non-lethal dose of the pathogen, to see if they (or their offspring) resist better to subsequent lethal doses of the pathogen, compared to individuals that were not previously exposed.

In the second part of **chapter 1** and in **chapter 2**, we tested the hypothesis that queens associate during colony founding to benefit from social immunity. In

chapter 1, we found that the presence of a pathogen in a potential nest site did not incite *F. selysi* queens to associate during colony founding. In line with these results, we found in **chapter 2** that neither queens of *L. niger* founding in association nor their workers benefitted from social immunity. On the contrary, queens tended to invest more in some components of internal individual immunity and in self-grooming. These results, with the ones of others (Pull et al. 2013), provide strong evidence that social immunity is not an advantage of pleometrosis, at least in the species and conditions tested. Studying the next stage of colony development, we found in **chapter 3** that in *F. selysi*, neither the worker brood ratio nor the origin of workers (their mother queen) influenced resistance to the fungal pathogen *B. bassiana*. In contrast, worker brood ratio influenced other traits, such as the survival and adult size of the brood.

The aim of the second part of this thesis was to investigate the use of antimicrobial resin by the wood ant *Formica paralugubris*. In **chapter 4**, we found that *F. paralugubris* workers retrieve more resin when brood is present in the nest, and that they place resin near the brood. This showed that although resin collection is prophylactic (Castella *et al.* 2008b) its use is also plastic, as it depends on the presence of brood in the nest.

In **chapter 5**, we tested if wood ants treat the resin to increase its antifungal potential. We found that workers create a potent antimicrobial agent by depositing self-produced formic acid on pieces of resin. This constitutes a rare

demonstration of an antibiotic "defensive mixology" (Mason & Singer 2015). In future studies, it would be interesting to test if ants produce antimicrobial cocktails in other conditions. Workers may for example mix several self-produced antibiotics such as the ones found in the metapleural glands (Veal, Trimble & Beattie 1992; Fernández-Marín *et al.* 2006; Yek & Mueller 2011), the venom gland (Graystock & Hughes 2011; Tragust *et al.* 2013a), or the trophallaxis regurgitates (de Souza *et al.* 2008; Hamilton *et al.* 2011). In genetically diverse nests, workers from different genetical origins may also mix their self-produced chemicals to create more potent or larger spectrum antimicrobials.

Concerning the resin itself, future studies may investigate the potential costs of its use, as novel terminology describing the use of antimicrobial substances (self-medication, prophylactic medication, compensatory diet choice, prophylactic consumption) depends on the potential detrimental effect of the substance on the host in absence of infection (Singer, Mace & Bernays 2009; Abbott 2014). In previous studies, resin did not have a negative effect on the survival of workers or larvae (Chapuisat *et al.* 2007). However, resin may have sub-lethal detrimental effects. For example, propolis (resin used by honeybees in their hive) induced narcosis and reduced the metabolic rate of the mite *Varroa destructor* (Garedew *et al.* 2002), provoking a lower resistance to thermal stress (Garedew, Schmolz & Lamprecht 2003). Similarly, propolis reduced the metabolic rate and shorted

the pupal duration of the greater wax moth *Galleria mellonella* larvae (Garedew, Schmolz & Lamprecht 2004). Resin may also have detrimental effects on more fragile life stages, like the eggs.

In conclusion, this thesis illustrates the interest of testing multiple lines of antipathogen defenses in social insect colonies, as some of the results do not match with seemingly obvious predictions. This also calls for future studies investigating in further detail the antipathogen defenses of particular life stages, like colony founding or during brood rearing. The second part of this thesis provides important insights on how social insects use antibiotic substances such as resin.

Bibliography

Abbott, J. (2014) Self-medication in insects: current evidence and future perspectives. *Ecological Entomology*, **39**, 273–280.

Abril, S., Oliveras, J. & Gómez, C. (2010) Effect of temperature on the development and survival of the Argentine ant, *Linepithema humile*. *Journal of Insect Science*, **10**, 97–13.

Adams, E.S. & Tschinkel, W.R. (1995) Effects of foundress number on brood raids and queen survival in the fire ant *Solenopsis invicta*. *Behavioral Ecology and Sociobiology*, **37**, 233–242.

Al-Hosney, H.A., Carlos-Cuellar, S., Baltrusaitis, J. & Grassian, V.H. (2005) Heterogeneous uptake and reactivity of formic acid on calcium carbonate particles: a Knudsen cell reactor, FTIR and SEM study. *Physical Chemistry Chemical Physics*, **7**, 3587–3595.

Anderson, R.M. & May, R.M. (1985) Vaccination and herd immunity to infectious diseases. *Nature*, **318**, 323–329.

Aron, S., Steinhauer, N. & Fournier, D. (2009) Influence of queen phenotype, investment and maternity apportionment on the outcome of fights in cooperative foundations of the ant *Lasius niger*. *Animal Behaviour*, **77**, 1067–1074.

Ayasse, M. & Paxton, R.J. (2002) Brood protection in social insects. *Chemoecology of insect eggs and egg deposition*, Blackwell (eds M. Hilker & T. Meiners), pp. 117–148. Blackwell Publishing Ltd, Berlin.

Ayre, G.L. (1967) The relationships between food and digestive enzymes in five species of ants (Hymenoptera: Formicidae). *The Canadian Entomologist*, **99**, 408–411.

Baer, B., Armitage, S.A.O. & Boomsma, J.J. (2006) Sperm storage induces an immunity cost in ants. *Nature*, **441**, 872–875.

Banskota, A.H., Tezuka, Y. & Kadota, S. (2001) Recent progress in pharmacological research of propolis. *Phytotherapy Research*, **15**, 561–571.

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2014) lme4: Linear mixed-effects models using Eigen and S4.

Baverstock, J., Roy, H.E. & Pell, J.K. (2010) Entomopathogenic fungi and insect behaviour: from unsuspecting hosts to targeted vectors. *BioControl*, **55**, 89–102.

Bernasconi, G. & Strassmann, J. (1999) Cooperation among unrelated individuals: the ant foundress case. *Trends in Ecology & Evolution*, **14**, 477–482.

Biller, O.M., Adler, L.S., Irwin, R.E., McAllister, C. & Palmer-Young, E.C. (2015) Possible synergistic effects of thymol and nicotine against *Crithidia bombi* parasitism in bumble bees. *PLoS ONE*, **10**, e0144668.

Billick, I. (2001) Density dependence and colony growth in the ant species *Formica neorufibarbis*. *Journal of Animal Ecology*, **70**, 895–905.

Bischoff, J.F., Rehner, S.A. & Humber, R.A. (2009) A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia*, **101**, 512–530.

Blum, M.S. (1992) Ant venoms: Chemical and pharmacological properties. *Toxin Reviews*, **11**, 115–164.

Boos, S., Meunier, J., Pichon, S. & Kölliker, M. (2014) Maternal care provides antifungal protection to eggs in the European earwig. *Behavioral Ecology*, **25**, 754–761.

Brian, M.V. (1953) Brood-rearing in relation to worker number in the ant *Myrmica*. *Physiological Zoology*, **26**, 355–366.

Brian, M.V. (1957) The growth and development of colonies of the ant *Myrmica*. *Insectes Sociaux*, **4**, 177–190.

Brian, M.V. & Carr, C.A.H. (1960) The influence of the queen on brood rearing in ants of the genus *Myrmica*. *Journal of Insect Physiology*, **5**, 81–94.

Broome, J.R., Sikorowski, P.P. & Norment, B.R. (1976) A mechanism of pathogenicity of *Beauveria bassiana* on larvae of the imported fire ant, *Solenopsis richteri*. *Journal of Invertebrate Pathology*, **28**, 87–91.

Brütsch, T. & Chapuisat, M. (2014) Wood ants protect their brood with tree resin. *Animal Behaviour*, **93**, 157–161.

Brütsch, T., Felden, A., Reber, A. & Chapuisat, M. (2014) Ant queens (Hymenoptera: Formicidae) are attracted to fungal pathogens during the initial stage of colony founding. *Myrmecological News*, **20**, 71–76.

Calleri, D.V., Reid, E.M., Vargo, E.L., Traniello, J.F.A. & Rosengaus, R.B. (2006) Inbreeding and disease resistance in a social insect: effects of heterozygosity on immunocompetence in the termite *Zootermopsis angusticollis*. *Proceedings of the Royal Society of London, Series B*, **273**, 2633–2640.

Camargo, R.S., Forti, L.C., Fujihara, R.T. & Roces, F. (2011) Digging effort in leaf-cutting ant queens (*Atta sexdens rubropilosa*) and its effects on survival and colony growth during the claustral phase. *Insectes Sociaux*, **58**, 17–22.

Cannon, C.A. & Fell, R.D. (2002) Patterns of macronutrient collection in the black carpenter ant, *Camponotus pennsylvanicus* (De Geer) (Hymenoptera: Formicidae). *Environmental Entomology*, **31**, 977–981.

Cassill, D.L. (2002) Brood care strategies by newly mated monogyne *Solenopsis invicta* (Hymenoptera: Formicidae) queens during colony founding. *Annals of the Entomological Society of America*, **95**, 208–212.

Cassill, D.L. & Tschinkel, W.R. (1999) Effects of colony-level attributes on larval feeding in the fire ant, *Solenopsis invicta*. *Insectes Sociaux*, **46**, 261–266.

Castella, G., Chapuisat, M., Moret, Y. & Christe, P. (2008a) The presence of conifer resin decreases the use of the immune system in wood ants. *Ecological Entomology*, **33**, 408–412.

Castella, G., Christe, P. & Chapuisat, M. (2008b) Prophylaxis with resin in wood ants. *Animal Behaviour*, **75**, 1591–1596.

Castella, G., Christe, P. & Chapuisat, M. (2009) Mating triggers dynamic immune regulations in wood ant queens. *Journal of Evolutionary Biology*, **22**, 564–570.

Castella, G., Christe, P. & Chapuisat, M. (2010) Covariation between colony social structure and immune defences of workers in the ant *Formica selysi*. *Insectes Sociaux*, **57**, 233–238.

Cerenius, L. & Söderhäll, K. (2004) The prophenoloxidase-activating system in invertebrates. *Immunological Reviews*, **198**, 116–126.

Chapuisat, M. & Keller, L. (1999) Extended family structure in the ant *Formica paralugubris*: the role of the breeding system. **46**, 405–412.

Chapuisat, M., Bocherens, S. & Rosset, H. (2004) Variable queen number in ant colonies: no impact on queen turnover, inbreeding, and population genetic differentiation in the ant *Formica selysi*. *Evolution*, **58**, 1064–1072.

Chapuisat, M., Goudet, J. & Keller, L. (1997) Microsatellites reveal high population viscosity and limited dispersal in the ant *Formica paralugubris*. *Evolution*, **51**, 475–482.

Chapuisat, M., Oppliger, A., Magliano, P. & Christe, P. (2007) Wood ants use resin to protect themselves against pathogens. *Proceedings of the Royal Society B*, **274**, 2013–2017.

Cherix, D. (1980) Note préliminaire sur la structure, la phénologie et le régime alimentaire d'une super-colonie de *Formica lugubris* zett. *Insectes Sociaux*, **27**, 226–236.

Christe, P., Oppliger, A., Bancala, F., Castella, G. & Chapuisat, M. (2003) Evidence for collective medication in ants. *Ecology Letters*, **6**, 19–22.

Clayton, D.H. & Wolfe, N.D. (1993) The adaptive significance of self-medication. *Trends in Ecology & Evolution*, **8**, 60–63.

Conteiro Castilho, A.M., Fraga, M.E., Aguiar-Menezes, E. de L. & da Rocha Rosa, C.A. (2010) Selection of *Metarhizium anisopliae* and *Beauveria bassiana* isolates pathogenic to *Atta bisphaerica* and *Atta sexdens rubropilosa* soldiers under laboratory conditions. *Ciencia Rural*, **40**, 1243–1249.

Cotter, S.C. & Kilner, R.M. (2010) Personnal immunity versus social immunity. *Behavioral Ecology*, **21**, 663–668.

Cotter, S.C., Kruuk, L.E.B. & Wilson, K. (2004) Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *Journal of Evolutionary Biology*, **17**, 421–429.

Cremer, S., Armitage, S.A.O. & Schmid-Hempel, P. (2007) Social immunity. *Current*

Biology, **17**, R693–702.

de Roode, J.C. & Lefèvre, T. (2012) Behavioral immunity in insects. *Insects*, **3**, 789–820.

de Roode, J.C., Lefèvre, T. & Hunter, M.D. (2013) Self-medication in animals. *Science*, **340**, 150–151.

de Souza, D.J., Van Vlaenderen, J., Moret, Y. & Lenoir, A. (2008) Immune response affects ant trophallactic behaviour. *Journal of Insect Physiology*, **54**, 828–832.

Drescher, N., Wallace, H.M., Katouli, M., Massaro, C.F. & Leonhardt, S.D. (2014) Diversity matters: how bees benefit from different resin sources. *Oecologia*, **176**, 943–953.

Dromph, K.M. & Vestergaard, S. (2002) Pathogenicity and attractiveness of entomopathogenic hyphomycete fungi to collembolans. *Applied Soil Ecology*, **21**, 197–210.

Duangphakdee, O., Koeniger, N., Deowanish, S., Hepburn, H.R. & Wongsiri, S. (2009) Ant repellent resins of honeybees and stingless bees. *Insectes Sociaux*, **56**, 333–339.

Ebert, D. & Hamilton, W.D. (1996) Sex against virulence: the coevolution of parasitic diseases. *Trends in Ecology & Evolution*, **11**, 79–82.

Ebert, D. & Weisser, W.W. (1997) Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites. *Proceedings of the Royal Society of London, Series B*, **264**, 985–991.

Elliot, S.L. & Hart, A.G. (2010) Density-dependent prophylactic immunity reconsidered in the light of host group living and social behavior. *Ecology*, **91**, 65–72.

Evesham, E.J.M. (1985) The interaction of food distribution and the caste composition of an ant colony (*Myrmica rubra* L.). *Journal of Zoology*, **207**, 241–250.

Fernández-Marín, H., Zimmerman, J.K., Rehner, S.A. & Wcislo, W.T. (2006) Active use of the metapleural glands by ants in controlling fungal infection. *Proceedings of the Royal Society of London, Series B*, **273**, 1689–1695.

Fjerdingstad, E.J. & Keller, L. (2004) Relationships between phenotype, mating behavior, and fitness of queens in the ant *Lasius niger*. *Evolution*, **58**, 1056–1063.

Fournier, D., Battaille, G., Timmermans, I. & Aron, S. (2008) Genetic diversity, worker size polymorphism and division of labour in the polyandrous ant *Cataglyphis cursor*. *Animal Behaviour*, **75**, 151–158.

Garedew, A., Lamprecht, I., Schmolz, E. & Schricker, B. (2002) The varroacidal action of propolis: a laboratory assay. *Apidologie*, **33**, 41–50.

Garedew, A., Schmolz, E. & Lamprecht, I. (2003) Microcalorimetric and respirometric investigation of the effect of temperature on the antivarroa action of the natural bee product-propolis. *Thermochimica Acta*, **399**, 171–180.

Garedew, A., Schmolz, E. & Lamprecht, I. (2004) Effect of the bee glue (propolis) on the

calorimetrically measured metabolic rate and metamorphosis of the greater wax moth *Galleria mellonella*. *Thermochimica Acta*, **413**, 63–72.

Gálvez, D. & Chapuisat, M. (2014) Immune priming and pathogen resistance in ant queens. *Ecology and Evolution*, **4**, 1761–1767.

George, J., Jenkins, N.E., Blanford, S., Thomas, M.B. & Baker, T.C. (2013) Malaria mosquitoes attracted by fatal fungus. *PLoS ONE*, **8**, e62632.

Gillespie, J.P., Bailey, A.M., Cobb, B. & Vilcinskas, A. (2000) Fungi as elicitors of insect immune responses. *Archives of Insect Biochemistry and Physiology*, **44**, 49–68.

Gillespie, J.P., Kanost, M.R. & Trenczek, T. (1997) Biological mediators of insect immunity. *Annual Review of Entomology*, **42**, 611–643.

Godfrey, S.S. (2013) Networks and the ecology of parasite transmission: A framework for wildlife parasitology. *International Journal for Parasitology: Parasites and Wildlife*, **2**, 235–245.

Gray, B. (1971) A morphometric study of the ant species, *Myrmecia dispar* (Clark) (Hymenoptera: Formicidæ). *Insectes Sociaux*, **18**, 95–109.

Graystock, P. & Hughes, W.O.H. (2011) Disease resistance in a weaver ant, *Polyrhachis dives*, and the role of antibiotic-producing glands. *Behavioral Ecology and Sociobiology*, **66**, 2319–2327.

Gwinner, H. & Berger, S. (2005) European starlings: nestling condition, parasites and green nest material during the breeding season. *Journal of Ornithology*, **146**, 365–371.

Gwinner, H. & Berger, S. (2006) Parasite defence in birds: the role of volatiles. *Acta Zoologica Sinica*, **52**, 280–283.

Hajek, A.E. & St Leger, R.J. (1994) Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology*, **39**, 293–322.

Hamilton, C., Lejeune, B.T. & Rosengaus, R.B. (2011) Trophallaxis and prophylaxis: social immunity in the carpenter ant *Camponotus pennsylvanicus*. *Biology Letters*, **7**, 89–92.

Hart, A.G. & Ratnieks, F.L.W. (2002) Waste management in the leaf-cutting ant *Atta colombica*. *Behavioral Ecology*, **13**, 224–231.

Hartmann, A., Wantia, J., Torres, J.A. & Heinze, J. (2003) Worker policing without genetic conflicts in a clonal ant. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 12836–12840.

Heinze, J. Social plasticity: ecology, genetics, and the structure of ant societies. *Ecology of social evolution* (eds K. J & J. Heinze), pp. 129–150. Berlin.

Holzer, B., Chapuisat, M., Kremer, N., Finet, C. & Keller, L. (2006) Unicoloniality, recognition and genetic differentiation in a native *Formica* ant. *Journal of Evolutionary Biology*, **19**, 2031–2039.

Howard, K.J. & Jeanne, R.L. (2004) Rates of brood development in a social wasp: effects of colony size and parasite infection. *Insectes Sociaux*, **51**, 179–185.

Huffman, M.A. (2003) Animal self-medication and ethno-medicine: exploration and exploitation of the medicinal properties of plants. *Proceedings of the Nutrition Society*, **62**, 371–381.

Hughes, W.O.H. & Boomsma, J.J. (2004) Genetic diversity and disease resistance in leaf-cutting ant societies. *Evolution*, **58**, 1251–1260.

Hughes, W.O.H., Eilenberg, J. & Boomsma, J.J. (2002) Trade-offs in group living: transmission and disease resistance in leaf-cutting ants. *Proceedings of the Royal Society of London, Series B*, **269**, 1811–1819.

Iba, A.M. & Berchieri, A., Jr. (2007) Studies on the use of a formic acid-propionic acid mixture (Bio-add™) to control experimental *Salmonella* infection in broiler chickens. *Avian Pathology*, **24**, 303–311.

Johnson, R.A. (2004) Colony founding by pleometrosis in the semiclaustral seed-harvester ant *Pogonomyrmex californicus* (Hymenoptera: Formicidae). *Animal Behaviour*, **68**, 1189–1200.

Kapheim, K.M., Bernal, S.P., Smith, A.R., Nonacs, P. & Wcislo, W.T. (2011) Support for maternal manipulation of developmental nutrition in a facultatively eusocial bee, *Megalopta genalis* (Halictidae). *Behavioral Ecology and Sociobiology*, **65**, 1179–1190.

Keller, L. (1988) Evolutionary implications of polygyny in the Argentine ant, *Iridomyrmex humilis* (mayr) (Hymenoptera: Formicidae): an experimental study. *Animal Behaviour*, **36**, 159–165.

Keller, L. (1991) Queen number, mode of colony founding, and queen reproductive success in ants (hymenoptera, formicidae). *Ethology Ecology & Evolution*, **3**, 307–316.

Keller, L. & Reeve, H.K. (1994) Genetic variability, queen number, and polyandry in social hymenoptera. *Evolution*, **48**, 694–704.

Klinger, E., Groden, E. & Drummond, F. (2006) *Beauveria bassiana* horizontal infection between cadavers and adults of the colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Environmental Entomology*, **35**, 992–1000.

Kohlmeier, P., Dreyer, H. & Meunier, J. (2015) PO-CALC: A novel tool to correct common inconsistencies in the measurement of phenoloxidase activity. *Journal of Insect Physiology*, **75**, 80–84.

Konrad, M., Vyleta, M.L., Theis, F.J., Stock, M., Tragust, S., Klatt, M., Drescher, V., Marr, C., Ugelvig, L.V. & Cremer, S. (2012) Social transfer of pathogenic fungus promotes active immunisation in ant colonies. *PLoS Biology*, **10**, e1001300.

Kovacs, J.L., Hoffman, E.A., Marriner, S.M., Rekau, J.A. & Goodisman, M.A.D. (2010) Environmental and genetic influences on queen and worker body size in the social wasp *Vespula maculifrons*. *Insectes Sociaux*, **57**, 53–65.

Lefèvre, T., Chiang, A., Kelavkar, M., Li, H., Li, J., de Castillejo, C.L.F., Oliver, L., Potini, Y., Hunter, M.D. & de Roode, J.C. (2012) Behavioural resistance against a protozoan parasite in the monarch butterfly. *Journal of Animal Ecology*, **81**, 70–79.

Lefèvre, T., Oliver, L., Hunter, M.D. & de Roode, J.C. (2010) Evidence for trans-generational medication in nature. *Ecology Letters*, **13**, 1485–1493.

Linksvayer, T.A. (2006) Direct, maternal, and sibsocial genetic effects on individual and colony traits in an ant. *Evolution*, **60**, 2552–2561.

Linksvayer, T.A. (2007) Ant species differences determined by epistasis between brood and worker genomes. *PLoS ONE*, **2**, e994.

Linksvayer, T.A. (2008) Queen-worker-brood coadaptation rather than conflict may drive colony resource allocation in the ant *Temnothorax curvispinosus*. *Behavioral Ecology and Sociobiology*, **62**, 647–657.

Linksvayer, T.A. & Wade, M.J. (2005) The evolutionary origin and elaboration of sociality in the aculeate Hymenoptera: maternal effects, sib-social effects, and heterochrony. *The Quarterly Review of Biology*, **80**, 317–336.

Little, T.J. & Kraaijeveld, A.R. (2004) Ecological and evolutionary implications of immunological priming in invertebrates. *Trends in Ecology & Evolution*, **19**, 58–60.

Little, T.J., O'Connor, B., Colegrave, N., Watt, K. & Read, A.F. (2003) Maternal transfer of strain-specific immunity in an invertebrate. **13**, 489–492.

Lord, J.C. (2001) Response of the wasp *Cephalonomia tarsalis* (Hymenoptera: Bethylidae) to *Beauveria bassiana* (Hyphomycetes: Moniliales) as free conidia or infection in its host, the sawtoothed grain beetle, *Oryzaephilus surinamensis* (Coleoptera: Silvanidae). *Biological Control*, **21**, 300–304.

Loreto, R.G. & Hughes, D.P. (2016) Disease in the society: infectious cadavers result in collapse of ant sub-colonies. *PLoS ONE*, **11**, e0160820.

Lowe, S., Browne, M., Boudjelas, S. & De Poorter, M. (2000) *100 of the World's Worst Invasive Alien Species. a Selection From the Global Invasive Species Database*. The Invasive Species Specialist Group (ISSG) a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN).

López, J.H., Schuehly, W., Crailsheim, K. & Riessberger-Gallé, U. (2014) Trans-generational immune priming in honeybees. *Proceedings of the Royal Society B*, **281**, 20140454–20140454.

Lubach, G.R., Coe, C.L. & Ershler, W.B. (1995) Effects of early rearing environment on immune responses of infant rhesus monkeys. *Brain, Behavior, and Immunity*, **9**, 31–46.

Mandeel, Q. & Taha, A. (2005) Assessment of in vitro antifungal activities of various extracts of indigenous Bahraini medicinal plants. *Pharmaceutical Biology*, **43**, 164–172.

Mankowski, M.E. & Morrell, J.J. (2011) Role of relative humidity in colony founding and queen survivorship in two carpenter ant species. *Journal of Economic Entomology*, **104**,

740–744.

Manson, J.S., Otterstatter, M.C. & Thomson, J.D. (2010) Consumption of a nectar alkaloid reduces pathogen load in bumble bees. *Oecologia*, **162**, 81–89.

Mason, P.A. & Singer, M.S. (2015) Defensive mixology: combining acquired chemicals towards defence. *Functional Ecology*, **29**, 441–450.

Mburu, D.M., Ochola, L., Maniania, N.K., Njagi, P.G.N., Gitonga, L.M., Ndung'u, M.W., Wanjoya, A.K. & Hassanali, A. (2009) Relationship between virulence and repellency of entomopathogenic isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to the termite *Macrotermes michaelseni*. *Journal of Insect Physiology*, **55**, 774–780.

Meaney, M.J. (2001) Nature, nurture, and the disunity of knowledge. *Annals of the New York Academy of Sciences*, **935**, 50–61.

Mersch, D.P., Crespi, A. & Keller, L. (2013) Tracking individuals shows spatial fidelity is a key regulator of ant social organization. *Science*, **340**, 1090–1093.

Meunier, J. (2015) Social immunity and the evolution of group living in insects. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **370**, 20140102–20140102.

Meunier, J. & Chapuisat, M. (2009) The determinants of queen size in a socially polymorphic ant. *Journal of Evolutionary Biology*, **22**, 1906–1913.

Meyling, N.V. & Eilenberg, J. (2007) Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. *Biological Control*, **43**, 145–155.

Meyling, N.V. & Pell, J.K. (2006) Detection and avoidance of an entomopathogenic fungus by a generalist insect predator. *Ecological Entomology*, **31**, 162–171.

Milan, N.F., Kacsoh, B.Z. & Schlenke, T.A. (2012) Alcohol consumption as self-medication against blood-borne parasites in the fruit fly. *Current Biology*, **22**, 1–6.

Morelos-Juárez, C., Walker, T.N., Lopes, J.F.S. & Hughes, W.O.H. (2010) Ant farmers practice proactive personal hygiene to protect their fungus crop. *Current Biology*, **20**, R553–4.

Moreno-García, M., Lanz Mendoza, H. & Córdoba-Aguilar, A. (2010) Genetic Variance and Genotype-by-Environment Interaction of Immune Response in *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology*, **47**, 111–120.

Moret, Y. & Schmid-Hempel, P. (2001) Immune defence in bumble-bee offspring. *Nature*, **414**, 506.

Morgan, E.D. (2008) Chemical sorcery for sociality: exocrine secretions of ants (Hymenoptera: Formicidae). *Myrmecological News*, **11**, 79–90.

Mukawa, S., Tooyama, H. & Ikegami, T. (2011) Influence of humidity on the infection of western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae), by

Beauveria bassiana. *Applied Entomology and Zoology*, **46**, 255–264.

Myers, J.H. & Rothman, L.E. (1995) Virulence and transmission of infectious diseases in humans and insects: evolutionary and demographic patterns. *Trends in Ecology & Evolution*, **10**, 194–198.

Naug, D. & Smith, B. (2007) Experimentally induced change in infectious period affects transmission dynamics in a social group. *Proceedings of the Royal Society B*, **274**, 61–65.

Nickerson, J.C., Whitcomb, W.H., Bhatkar, A.P. & Naves, M.A. (1975) Predation on founding queens of *Solenopsis invicta* by workers of *Conomyrma insana*. *Florida Entomologist*, **58**, 75–82.

Nielsen, C., Agrawal, A.A. & Hajek, A.E. (2010) Ants defend aphids against lethal disease. *Biology Letters*, **6**, 205–208.

Nishida, R. (2002) Sequestration of defensive substances from plants by Lepidoptera. *Annual Review of Entomology*, **47**, 57–92.

Ortiz-Urquiza, A. & Keyhani, N.O. (2013) Action on the surface: Entomopathogenic fungi versus the insect cuticle. *Insects*, **4**, 357–374.

Patterson, R.S. & Briano, J.A. (1993) Potential of three biological control agents for suppression of *Solenopsis invicta*, the red imported fire ant., pp. 35–43. Exeter, UK.

Phillips, M.A. & Croteau, R.B. (1999) Resin-based defenses in conifers. *Trends in Plant Science*, **4**, 184–190.

Pontieri, L., Vojvodic, S., Graham, R., Pedersen, J.S. & Linksvayer, T.A. (2014) Ant colonies prefer infected over uninfected nest sites. *PLoS ONE*, **9**, e111961.

Porter, S.D. & Tschinkel, W.R. (1985) Fire ant polymorphism (Hymenoptera Formicidae): Factors affecting worker size. *Annals of the Entomological Society of America*, **78**, 381–386.

Poulin, R. & Morand, S. (2000) The Diversity of Parasites. *The Quarterly Review of Biology*, **75**, 277–293.

Poulin, R., Brodeur, J. & Moore, J. (1994) Parasite manipulation of host behaviour: Should hosts always lose? *Oikos*, 479–484.

Prager, G., Stefanski, V., Hudson, R. & Roedel, H.G. (2010) Family matters: Maternal and litter-size effects on immune parameters in young laboratory rats. *Brain, Behavior, and Immunity*, **24**, 1371–1378.

Pull, C.D., Hughes, W.O.H. & Brown, M.J.F. (2013) Tolerating an infection: an indirect benefit of co-founding queen associations in the ant *Lasius niger*. *Naturwissenschaften*, **100**, 1125–1136.

Purcell, J., Brelsford, A., Wurm, Y., Perrin, N. & Chapuisat, M. (2014) Convergent genetic architecture underlies social organization in ants. *Current Biology*, **24**, 2728–2732.

Purcell, J., Brütsch, T. & Chapuisat, M. (2012) Effects of the social environment on the survival and fungal resistance of ant brood. *Behavioral Ecology and Sociobiology*, **66**, 467–474.

R Core Team. (2016) *R: a Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rath, A.C. (2010) The use of entomopathogenic fungi for control of termites. *Biocontrol Science and Technology*, **10**, 563–581.

Reber, A. & Chapuisat, M. (2012a) Diversity, prevalence and virulence of fungal entomopathogens in colonies of the ant *Formica selysi*. *Insectes Sociaux*, **59**, 231–239.

Reber, A. & Chapuisat, M. (2012b) No evidence for immune priming in ants exposed to a fungal pathogen. *PLoS ONE*, **7**, e35372.

Reber, A., Castella, G., Christe, P. & Chapuisat, M. (2008) Experimentally increased group diversity improves disease resistance in an ant species. *Ecology Letters*, **11**, 682–689.

Reber, A., Meunier, J. & Chapuisat, M. (2010) Flexible colony-founding strategies in a socially polymorphic ant. *Animal Behaviour*, **79**, 467–472.

Reber, A., Purcell, J., Buechel, S.D., Buri, P. & Chapuisat, M. (2011) The expression and impact of antifungal grooming in ants. *Journal of Evolutionary Biology*, **24**, 954–964.

Rosengaus, R.B., Malak, T. & MacKintosh, C. (2013) Immune-priming in ant larvae: social immunity does not undermine individual immunity. *Biology Letters*, **9**, 20130563.

Rosset, H. & Chapuisat, M. (2007) Alternative life-histories in a socially polymorphic ant. *Evolutionary Ecology*, **21**, 577–588.

Roth, O., Joop, G., Eggert, H., Hilbert, J., Daniel, J., Schmid-Hempel, P. & Kurtz, J. (2010) Paternally derived immune priming for offspring in the red flour beetle, *Tribolium castaneum*. *Journal of Animal Ecology*, **79**, 403–413.

Ruiz-González, M.X., Moret, Y. & Brown, M.J.F. (2009) Rapid induction of immune density-dependent prophylaxis in adult social insects. *Biology Letters*, **5**, 781–783.

Russell, A.F. & Lummaa, V. (2009) Maternal effects in cooperative breeders: from hymenopterans to humans. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **364**, 1143–1167.

Ryan, M.J., Bridge, P.D., Smith, D. & Jeffries, P. (2002) Phenotypic degeneration occurs during sector formation in *Metarhizium anisopliae*. *Journal of Applied Microbiology*, **93**, 163–168.

Sadd, B.M., Kleinlogel, Y., Schmid-Hempel, R. & Schmid-Hempel, P. (2005) Trans-generational immune priming in a social insect. *Biology Letters*, **1**, 386–388.

Saino, N., Calza, S. & Moller, A.P. (1997) Immunocompetence of nestling barn swallows in relation to brood size and parental effort. *Journal of Animal Ecology*, **66**, 827–836.

Sanders, C.J. (1964) The biology of carpenter ants in New Brunswick. *The Canadian Entomologist*, **104**, 894–909.

Sanders, D. & van Veen, F.J.F. (2011) Ecosystem engineering and predation: the multi-trophic impact of two ant species. *Journal of Animal Ecology*, **80**, 569–576.

Schmid-Hempel, P. & Ebert, D. (2003) On the evolutionary ecology of specific immune defence. *Trends in Ecology & Evolution*, **18**, 27–32.

Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature methods*, **9**, 671–675.

Schulenburg, H., Kurtz, J., Moret, Y. & Siva-Jothy, M.T. (2009) Introduction. Ecological immunology. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **364**, 3–14.

Schultz, T.R. (2000) In search of ant ancestors. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 14028–14029.

Schwander, T., Lo, N., Beekman, M., Oldroyd, B.P. & Keller, L. (2010) Nature versus nurture in social insect caste differentiation. *Trends in Ecology & Evolution*, **25**, 275–282.

Schwander, T., Rosset, H. & Chapuisat, M. (2005) Division of labour and worker size polymorphism in ant colonies: the impact of social and genetic factors. *Behavioral Ecology and Sociobiology*, **59**, 215–221.

Shykoff, J.A. & Schmid-Hempel, P. (1991) Parasites and the advantage of genetic variability within social insect colonies. *Proceedings of the Royal Society of London, Series B*, **243**, 55–58.

Simone, M., Evans, J.D. & Spivak, M. (2009) Resin collection and social immunity in honey bees. *Evolution*, **63**, 3016–3022.

Simone-Finstrom, M.D. & Spivak, M. (2010) Propolis and bee health: the natural history and significance of resin use by honey bees. *Apidologie*, **41**, 295–311.

Simone-Finstrom, M.D. & Spivak, M. (2012) Increased resin collection after parasite challenge: a case of self-medication in honey bees? *PLoS ONE*, **7**, e34601.

Singer, M.S., Mace, K.C. & Bernays, E.A. (2009) Self-medication as adaptive plasticity: increased ingestion of plant toxins by parasitized caterpillars (ed RC May). *PLoS ONE*, **4**, e4796.

Siva-Jothy, M.T. & Thompson, J.J.W. (2002) Short-term nutrient deprivation affects immune function. *Physiological Entomology*, **27**, 206–212.

Siva-Jothy, M.T., Moret, Y. & Rolff, J. (2005) Insect immunity: an evolutionary ecology perspective. *Advances in Insect Physiology*, **32**, 1–48.

Sommer, K. & Hölldobler, B. (1995) Colony founding by queen association and determinants of reduction in queen number in the ant *Lasius niger*. *Animal Behaviour*, **50**, 287–294.

Söderhäll, K. & Cerenius, L. (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. *Current opinion in immunology*, **10**, 23–28.

Spatafora, J.W., Sung, G.-H., Sung, J.-M., Hywel-Jones, N.L. & White, J.F. (2007) Phylogenetic evidence for an animal pathogen origin of ergot and the grass endophytes. *Molecular Ecology*, **16**, 1701–1711.

Stow, A.J. & Beattie, A. (2008) Chemical and genetic defenses against disease in insect societies. *Brain, Behavior, and Immunity*, **22**, 1009–1013.

Suárez-Rodríguez, M., López-Rull, I. & Garcia, C.M. (2013) Incorporation of cigarette butts into nests reduces nest. *Biology Letters*, **9**, 20120931.

Suwanchaichinda, C. & Paskewitz, S.M. (1998) Effects of larval nutrition, adult body size, and adult temperature on the ability of *Anopheles gambiae* (Diptera: Culicidae) to melanize sephadex beads. *Journal of Medical Entomology*, **35**, 157–161.

Szathmáry, E. & Smith, J.M. (1995) The major evolutionary transitions. *Nature*, **374**, 227–232.

Therneau, T.M. (2015) Mixed effects cox models [R package coxme version 2.2-5].

Thomas, M.B. & Read, A.F. (2007) Can fungal biopesticides control malaria? *Nature reviews. Microbiology*, **5**, 377–383.

Thompson, S.R. & Brandenburg, R.L. (2005) Tunneling responses of Mole Crickets (Orthoptera: Gryllotalpidae) to the entomopathogenic fungus, *Beauveria bassiana*. *Environmental Entomology*, **34**, 140–147.

Tidbury, H.J., Pedersen, A.B. & Boots, M. (2011) Within and transgenerational immune priming in an insect to a DNA virus. *Proceedings of the Royal Society B*, **278**, 871–876.

Tragust, S., Mitteregger, B., Barone, V., Konrad, M., Ugelvig, L.V. & Cremer, S. (2013a) Ants disinfect fungus-exposed brood by oral uptake and spread of their poison. *Current Biology*, **23**, 76–82.

Tragust, S., Ugelvig, L.V., Chapuisat, M., Heinze, J. & Cremer, S. (2013b) Pupal cocoons affect sanitary brood care and limit fungal infections in ant colonies. *BMC Evolutionary Biology*, **13**, 225.

Traniello, J.F.A., Rosengaus, R.B. & Savoie, K. (2002) The development of immunity in a social insect: Evidence for the group facilitation of disease resistance. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 6838–6842.

Tranter, C. & Hughes, W.O.H. (2015) Acid, silk and grooming: alternative strategies in social immunity in ants? *Behavioral Ecology and Sociobiology*, 1–13.

Tranter, C., Graystock, P., Shaw, C., Lopes, J.F.S. & Hughes, W.O.H. (2013) Sanitizing the fortress: protection of ant brood and nest material by worker antibiotics. *Behavioral Ecology and Sociobiology*, 1–9.

Tschinkel, W.R. (1988) Colony growth and the ontogeny of worker polymorphism in the fire

ant, *Solenopsis invicta*. *Behavioral Ecology and Sociobiology*, **22**, 103–115.

Tschinkel, W.R. (1998) An experimental study of pleometrotic colony founding in the fire ant, *Solenopsis invicta*: what is the basis for association? *Behavioral Ecology and Sociobiology*, **43**, 247–257.

Turnbull, C., Hoggard, S., Gillings, M., Palmer, C., Stow, A.J., Beattie, D., Briscoe, D., Smith, S., Wilson, P. & Beattie, A. (2011) Antimicrobial strength increases with group size: implications for social evolution. *Biology Letters*, **7**, 249–252.

Ugelvig, L.V. & Cremer, S. (2007) Social prophylaxis: group interaction promotes collective immunity in ant colonies. *Current Biology*, **17**, 1967–1971.

Ugelvig, L.V., Kronauer, D.J.C., Schrempf, A., Heinze, J. & Cremer, S. (2010) Rapid anti-pathogen response in ant societies relies on high genetic diversity. *Proceedings of the Royal Society B*, **277**, 2821–2828.

Uma Devi, K., Padmavathi, J., Uma Maheswara Rao, C., Khan, A.A.P. & Mohan, M.C. (2008) A study of host specificity in the entomopathogenic fungus B*eauveria bassiana* (Hypocreales, Clavicipitaceae). *Biocontrol Science and Technology*, **18**, 975–989.

Valtonen, T.M., Roff, D.A. & Rantala, M.J. (2011) Analysis of the effects of early nutritional environment on inbreeding depression in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, **24**, 196–205.

Van Loon, A.J., Boomsma, J.J. & Andrásfalvy, A. (1990) A new polygynous *Lasius* species (Hymenoptera; Formicidae) from central Europe. *Insectes Sociaux*, **37**, 348–362.

Veal, D.A., Trimble, J.E. & Beattie, A.J. (1992) Antimicrobial properties of secretions from the metapleural glands of *Myrmecia gulosa* (the Australian bull ant). *Journal of Applied Bacteriology*, **72**, 188–194.

Vieira, A.S., Morgan, E.D., Drijfhout, F.P. & Camargo-Mathias, M.I. (2012) Chemical composition of metapleural gland secretions of fungus-growing and non-fungus-growing ants. *Journal of Chemical Ecology*, **38**, 1289–1297.

Vienne, C., Errard, C. & Lenoir, A. (1998) Influence of the queen on worker behaviour and queen recognition behaviour in ants. *Ethology*, **104**, 431–446.

Villalba, J.J., Provenza, F.D. & Shaw, R. (2006) Sheep self-medicate when challenged with illness-inducing foods. *Animal Behaviour*, **71**, 1131–1139.

Villani, M.G., Allee, L.L., Preston-Wilsey, L., Consolie, N., Xia, Y. & Brandenburg, R.L. (2002) Use of radiography and tunnel castings for observing mole cricket (Orthoptera: Gryllotalpidae) behavior in soil. *American Entomologist*, **48**, 42–50.

Villani, M.G., Krueger, S.R., Schroeder, P.C., Consolie, F., Consolie, N.H., Preston-Wilsey, L.M. & Roberts, D.W. (1994) Soil application effects of *Metarhizium anisopliae* on Japanese beetle (Coleoptera: Scarabaeidae) behavior and survival in turfgrass microcosms. *Environmental Entomology*, **23**, 1–12.

Vitikainen, E. & Sundström, L. (2011) Inbreeding and caste-specific variation in immune

defence in the ant *Formica exsecta*. *Behavioral Ecology and Sociobiology*, **65**, 899–907.

Walker, T.N. & Hughes, W.O.H. (2009) Adaptive social immunity in leaf-cutting ants. *Journal of Invertebrate Pathology*, **97**, 165–170.

Ward, P.S. (2014) The phylogeny and evolution of ants. *Annual Review of Ecology*, **45**, 23–43.

Wilson, E.O. (1971) *The Insect Societies*. Harvard University Press, Cambridge.

Wilson, E.O. (1983) Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: Atta). *Behavioral Ecology and Sociobiology*, **14**, 47–54.

Wilson, E.O. (1987) Causes of ecological success: The case of the ants. *The Journal of Animal Ecology*, **56**, 1.

Wilson, K. & Cotter, S.C. (2008) Density-dependent prophylaxis in insects. *Phenotypic Plasticity of Insects: Mechanisms and Consequences* (eds T. Ananthakrishnan & D. Whitman), pp. 381–420. Phenotypic plasticity of insects, Plymouth, UK.

Wilson, K. & Reeson, A.F. (1998) Density-dependent prophylaxis: evidence from Lepidoptera–baculovirus interactions? *Ecological Entomology*, **23**, 100–101.

Wilson, K., Thomas, M.B., Blanford, S., Doggett, M., Simpson, S.J. & Moore, S.L. (2002) Coping with crowds: density-dependent disease resistance in desert locusts. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 5471–5475.

Wilson-Rich, N., Spivak, M., Fefferman, N.H. & Starks, P.T. (2009) Genetic, individual, and group facilitation of disease resistance in insect societies. *Annual Review of Entomology*, **54**, 405–423.

Wolf, J.B., Brodie, E.D., III, Cheverud, J.M., Moore, A.J. & Wade, M.J. (1998) Evolutionary consequences of indirect genetic effects. *Trends in Ecology & Evolution*, **13**, 64–69.

Yanagawa, A., Fujiwara-Tsujii, N., Akino, T., Yoshimura, T., Yanagawa, T. & Shimizu, S. (2012) Odor aversion and pathogen-removal efficiency in grooming behavior of the termite *Coptotermes formosanus*. *PLoS ONE*, **7**, e47412.

Yek, S.H. & Mueller, U.G. (2011) The metapleural gland of ants. *Biological reviews of the Cambridge Philosophical Society*, **86**, 774–791.

Yek, S.H., Boomsma, J.J. & Schiøtt, M. (2013) Differential gene expression in *Acromyrmex* leaf-cutting ants after challenges with two fungal pathogens. *Molecular Ecology*, **22**, 2173–2187.